MOLECULAR ANALYSIS OF THE ROLE OF FcγRIIb, SHIP AND PI 3-KINASE IN MACROPHAGE Fcγ RECEPTOR FUNCTION

DISSERTATION

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

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2007

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ABSTRACT

Fcγ Receptors expressed on macrophages are engaged and activated by immune-complexes and tumor cells opsonized with antibodies. This activation leads to elimination of immune-complexes via the process of phagocytosis and the destruction of antibody-coated tumor cells through a process called antibody-dependent cell-mediated cytotoxicity (ADCC). Both these events are accompanied by the release of inflammatory mediators. Although essential for the elimination of invading microbes or tumor cells, the release of inflammatory mediators needs to be regulated to prevent collateral tissue damage. FcγR-mediated responses are regulated by simultaneous engagement of activating and inhibitory Fcγ receptors and by the activation of various kinases and phosphatases. In this study we have examined the molecular details of regulation of FcγR functions by the inhibitory FcγRIIb and the intracellular lipid phosphatases and lipid kinases. Specifically we have investigated the role of FcγRIIb, the inositol phosphatase SHIP-1 and phosphatidylinositol 3-kinase (PI 3-Kinase).

In the first part of this dissertation, we tested the functional differences between the two isoforms of human FcγRIIb, b1 and b2, in macrophages. The results of these experiments revealed that both FcγRIIb1 and FcγRIIb2 are equivalently surface

expressed, undergo tyrosine phosphorylation upon activation and induce the phosphorylation of SHIP-1. Both FcγRIIb1 and FcγRIIb2 downregulate phagocytosis and TNFα production to a similar extent. Together these findings demonstrate that hFcγRIIb1 and b2 are both functional inhibitory receptors in the phagocytic process.

In the second part, we examined the influence of inositol phosphatase SHIP-1 on phagocytosis-associated events and show for the first time that SHIP-1 negatively regulates FcγR-induced production of inflammatory cytokines IL-1β and IL-6 as well as superoxide generation in macrophages. Analysis of the molecular mechanisms of regulation established that SHIP-1 regulates the activation of PI 3-kinase and Ras/Erk pathway upstream of IL-1β and IL-6 production and the activation of Rac upstream of superoxide production. Interestingly, the modulation of the PI 3-kinase pathway by SHIP-1 is dependent on its catalytic activity whereas the regulation of the Ras/Erk pathway is independent of SHIP-1's catalytic activity.

In the last part of this dissertation, we investigated the molecular mechanisms involved in the process of macrophage-mediated ADCC. We show for the first time that activation of the PI 3-kinase/Akt pathway plays a critical role in macrophage-mediated ADCC against B cell lymphoma cells. Our results further show that in addition to the release of lytic mediators such as nitric oxide, the process of conjugate formation between macrophages and antibody-coated tumor cells is essential for cytolysis of tumor cells. We then demonstrate that the PI 3-kinase/Akt pathway regulates macrophage-mediated ADCC through its influence on cytoskeletal remodeling required for conjugate formation event.

Taken together these findings provide novel insights into the regulation of the phagocytic process by FcγRIIb and inositol phosphatase SHIP-1 and regulation of macrophage-mediated ADCC by the PI 3-kinase/Akt pathway.

Dedicated to my family

ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Susheela Tridandapani, for her constant support and guidance through out my graduate training. Her commitment and dedication to the field of science has always been an inspiration for me. She not only taught me how to think independently but also inculcated a scientific discipline in me. I will always be grateful to her for providing a great learning atmosphere at the beginning of my scientific career.

I would like to thank my committee members Dr. Clay Marsh, Dr. Mark Wewers and Dr. Michael Ostrowski for their time, scientific input and for allowing me to share their lab resources.

I am also thankful to Dr. Donald Dean and Dr. Ross Dalbey, previous and current directors of the Ohio State Biochemistry Program for their support and encouragement. I am particularly grateful to Charlene Maxwell, program manager for the OSBP, for her help with administrative work during the first year.

I also want to thank Dr. Natarajan Muthusamy and Dr. John Byrd for their time, for offering valuable and insightful advice and for allowing me to work in the laboratory. I am grateful to Carolyn Cheney for her help with radioactivity assays and flow cytometry.

I want to thank all present and previous members of the lab, Latha Ganesan, Ruma Pengal, Huiqing Fang, Jing Ai, Kishore Parsa, Murugesan Rajaram, Jon Butchar, Ann-Sophie Wavreille and Donna Cain for their time, assistance and guidance. Special thanks go to Dr. Latha Ganesan for guiding and helping me with scientific and technical input during the initial phase of my research which was a great learning experience.

Most importantly, I want to thank my parents, my brother, sister-in-law and nephew for their unending love and encouragement. Their love and understanding have been my greatest strength. Without their support, the work contained herein would not have been possible.

Finally I would like to thank my fiancé Harsh who has always been there for me through the successes and the failures during my graduate school. I am grateful to him for his love, constant support and understanding throughout this research endeavor.

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PUBLICATIONS

Research Publications

1. **Joshi, T.**, Ganesan, L.P., Cao. X., Tridandapani, S. Molecular analysis of expression and function of hFcγRIIb1 and b2 isoforms in myeloid cells. Molecular Immunology, 2006; 43, 839 – 850.

2. *Ganesan, L.P., **Joshi, T.**, Fang, H., Kutala, V.K., Roda, J., Trotta, R., Lehman, A., Kuppusamy, P., Byrd, J.C., Carson, W.E., Caligiuri, M.A., Tridandapani, S. FcγR-induced production of superoxide and inflammatory cytokines is differentially regulated by SHIP through its influence on PI3K and/or Ras/Erk pathways. Blood, 2006; 108, 718 – 725.
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3. **Joshi, T.**, Butchar, J.P., Tridandapani, S. Fcγ receptor signaling in phagocytes. International Journal of Hematology, 2006; 84(3), 210 – 216.

4. Zhao, X.B., Lapalombella, R., **Joshi, T.**, Cheney, C., Gowda, A., Hayden-Ledbetter, M.S., Baum, P.R., Lin, T.L., Jarjoura, D., Lehman A., Kussewitt, D., Lee, R.J., Caligiuri, M.A., Tridandapani, S., Muthusamy, N., Byrd, J.C. Targeting $CD37^{+}$ lymphoid malignancies with a novel engineered small modular immunopharmaceutical. Blood, (Prepublished online, April 17, 2007).

FIELDS OF STUDY

Major Field: Ohio State Biochemistry Program

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ADCC	antibody-dependent cell-mediated cytotoxicity
BMM	bone marrow derived macrophages
BCR	B-cell receptor
CSF-1	colony-stimulating factor-1
DAG	diacylglycerol
DHE	dihydroethidium
FcγR	Fc gamma receptor
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor-bound protein 2
GST	glutathione S-transferase
IC	immune-complex
IFNγ	interferon gamma
IgG	Immunoglobulin G
IL-1β	interleukin 1-beta
IL-4	interleukin 4
IL-6	interleukin 6
IL-10	interleukin 10
IL-13	interleukin 13
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
LAT	linker for activation of T cells
MAPK	mitogen activated protein kinase

Myr-Akt	myristoylated Akt
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
PAMPs	pathogen-associated molecular patterns
PLCγ	phospholipase C gamma
PKCα	protein kinase C alpha
PKCε	protein kinase C epsilon
PRRs	pattern recognition receptors
PRD	proline-rich domains
PI 3-Kinase	Phosphatidylinositol 3-kinase
PtdIns3,4P_2	Phosphatidylinositol 3,4 bisphosphate
PtdIns3,4,5P_3	Phosphatidylinositol 3,4,5 trisphosphate
PTB	phosphotyrosine binding domains
PTEN	phosphatase and tensin homologue deleted on chromosome 10
ROS	reactive oxygen species
RNS	reactive nitrogen species
SH2	Src homology 2
SHIP-1	SH2 domain-containing inositol 5'-phosphatase-1
SHIP-2	SH2 domain-containing inositol 5'-phosphatase-2
SHP-1	SH2 domain-containing phosphatase-1
Sos	Son of sevenless
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha

CHAPTER 1

INTRODUCTION

The Immune System comprises of a complex network of cells and molecules that offer protection against diseases caused by invading pathogens. The vertebrate immune system is more evolved and offers protection through two layers of immune responses, namely innate immune responses and adaptive immune responses whereas invertebrates mount immune responses only through innate immunity. The term “innate” stands for “present from birth” and the innate immunity refers to the basic resistance mechanisms existent in organism to combat invading microorganism. Thus the innate immune system mounts an immediate and a non-specific response to an infection. On the other hand, adaptive immune response is more specialized and is developed only if the pathogen evades the innate immunity.

1.1 Macrophages and Innate Immunity

The innate immune response was initially discovered in insects and eventually recognized as evolutionarily conserved across the species. It carries out several processes including recognition of the invading pathogens and effector functions such as phagocytosis of microbes and activating the adaptive immune responses through antigen

presentation. The principal cell types involved in the innate immune responses include monocytes/macrophages, neutrophils, natural killer cells and dendritic cells.

In the bone marrow, committed myeloid precursor cells differentiate into monocytes which then enter into the blood. The circulating blood monocytes subsequently enter into tissue compartments of the body where they are differentiated to form resident tissue macrophages[1]. Resident macrophages in different organs are designated by different names, for e.g., alveolar macrophages (lung), Kupffer cells (liver) and microglia (central nervous system). The phenotypic characteristics of these macrophages differ substantially from each other and are governed by factors such as surface molecules and secretory proteins of surrounding cells and extra-cellular matrix[2].

The pleiotropic role of macrophages is further evident from the studies of macrophage-deficient mice. These mice, also called as *op/op* mice, have a mutation in the gene encoding colony-stimulating factor-1 (CSF-1) which is essential for differentiation of monocytes into macrophages. These mice exhibit infertility, sensory neuron dysfunction and severe defects in the development of mammary gland (reviewed in [1,3]).

Tissue resident macrophages perform a diverse array of functions depending on the mode of activation. Thus, if activated by T_H1 cytokines such as IFNγ (referred to as "classical activation"), macrophages actively clear invading microbes or host tumor cells; and in the process release several pro-inflammatory mediators such as TNFα, reactive oxygen species and reactive nitrogen species[2]. Although these pro-inflammatory responses bring about the clearance of pathogens, they also damage the surrounding host tissue. On the other hand, when macrophages are activated by T_H2 cytokines such as IL-4

and IL-13 (alternative activation), they show anti-inflammatory properties and have been proposed to be involved in functions such as wound healing and tissue repair[2].

The first step of immune response by macrophages involves the recognition and distinction of invading pathogens from self. This is achieved by a variety of receptors expressed by macrophages. These receptors identify and bind to conserved structural motifs of microbes. Such conserved structural motifs are termed pathogen-associated molecular patterns (PAMPs) and the receptors are called pattern recognition receptors (PRRs)[4,5]. A family of receptors called Toll-like receptors (TLRs) binds to numerous antigens such as bacterial lipopolysaccharide (TLR4), peptidoglycan (TLR2) and viral double stranded RNA (TLR3)[6]. Other than the TLRs, macrophages also express mannose receptors that bind to mannose and/or fucose residues of bacterial cell wall components and scavenger receptors that identify certain other structures of the microbial cell wall.

In addition to these PRRs, macrophages express two special types of receptors called complement receptors and Fc receptors. These receptors recognize microbial antigens that are opsonized with complement proteins (complement receptors) or antibodies (Fc receptors).

Antibodies are the most potent and antigen-specific opsonins. Through their $F(ab')_2$ end, antibodies bind to specific microbial antigens while their constant Fc region binds to Fc receptors on phagocytes. The recognition of antigen-antibody complex (immune-complex) by Fc receptors results in phagocytosis of immune-complex. The internalized antigen is degraded inside the cell and a part of the antigen is presented by antigen-presenting phagocytic cells to cells of adaptive immune system. Thus, Fc receptors serve as the link between innate and adaptive immunity.

The structure and function of Fc receptors, specifically Fcγ receptors, and the signaling pathways activated by Fcγ receptors is discussed below in detail.

1.2 Fcγ Receptors

Fcγ receptors bind to the Fc portion of IgG antibodies and play an important role in mediating inflammatory responses initiated by IgG immune-complexes and cytotoxic antibodies. It has been shown that Fcγ receptors are involved in mediating the effects of intravenous immunoglobulin (IVIG) therapy administered during certain autoimmune diseases[7]. The engagement of Fcγ receptors is also a predominant component of cytotoxic effects brought about by monoclonal antibody therapy of malignant disorders[8,9]. Furthermore, polymorphisms associated with FcγR have been linked to infectious and autoimmune diseases[10].

A variety of immune system cells including monocytes, macrophages, neutrophils, NK cells, B cells, mast cells and platelets express Fcγ receptors on their surface. Depending on the cell type, clustering of FcγR elicits a plethora of functions such as phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), inflammatory cytokines and reactive oxygen species production, degranulation, downregulation of antibody production and so on (reviewed in [11]).

There are two types of Fcγ receptors: activating receptors that facilitate the responses such as phagocytosis and ADCC, and inhibitory receptors that regulate the extent of the activating responses[11-13]. The activating Fc receptors contain a conserved motif called immunoreceptor tyrosine-based activation motif (ITAM) whereas the

inhibitory Fcγ receptor contains immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail.

Three classes of activating Fcγ receptors (FcγRI, FcγRIIa and FcγRIIIa) are expressed on human monocytes, macrophages and neutrophils[11,13]. Murine phagocytes do not express FcγRIIa. FcγRIIa contains the ITAM in its cytoplasmic tail, whereas FcγRI and FcγRIIIa associate with a dimer of small transmembrane protein subunits called the γ-chain that contains the ITAM (Figure 1.1). The ITAM motif within the γ chain and the cytoplasmic domain of FcγRIIa has two copies of the sequence Y-X-X-L[14-16]. Interestingly, the γ chain ITAM contains seven amino acid residues between the two YXXL motifs whereas the FcγRIIa ITAM contains twelve amino acids between the two YXXL sites[17]. The γ-chain not only contains the ITAM, but is also important for the surface expression FcγRI and IIIa[18]. Recently, the expression of a fourth activating Fcγ receptor, FcγRIV, has been described by Nimmerjahn et al on murine monocytes, macrophages and neutrophils[19]. FcγRIV also associates with the common γ-chain (that contains ITAM).

As opposed to the activating Fc receptors, the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) with a characteristic thirteen amino acid sequence AENTITYSLLKHP is a hallmark of the inhibitory Fcγ receptor FcγRIIb[11]. The ITIM is contained within the cytoplasmic tail of FcγRIIb. FcγRIIb is the only Fc receptor expressed by antibody-producing B cells. The co-clustering of FcγRIIb with B cell receptor (BCR) has been shown to mediate the inhibition of BCR-stimulated antibody

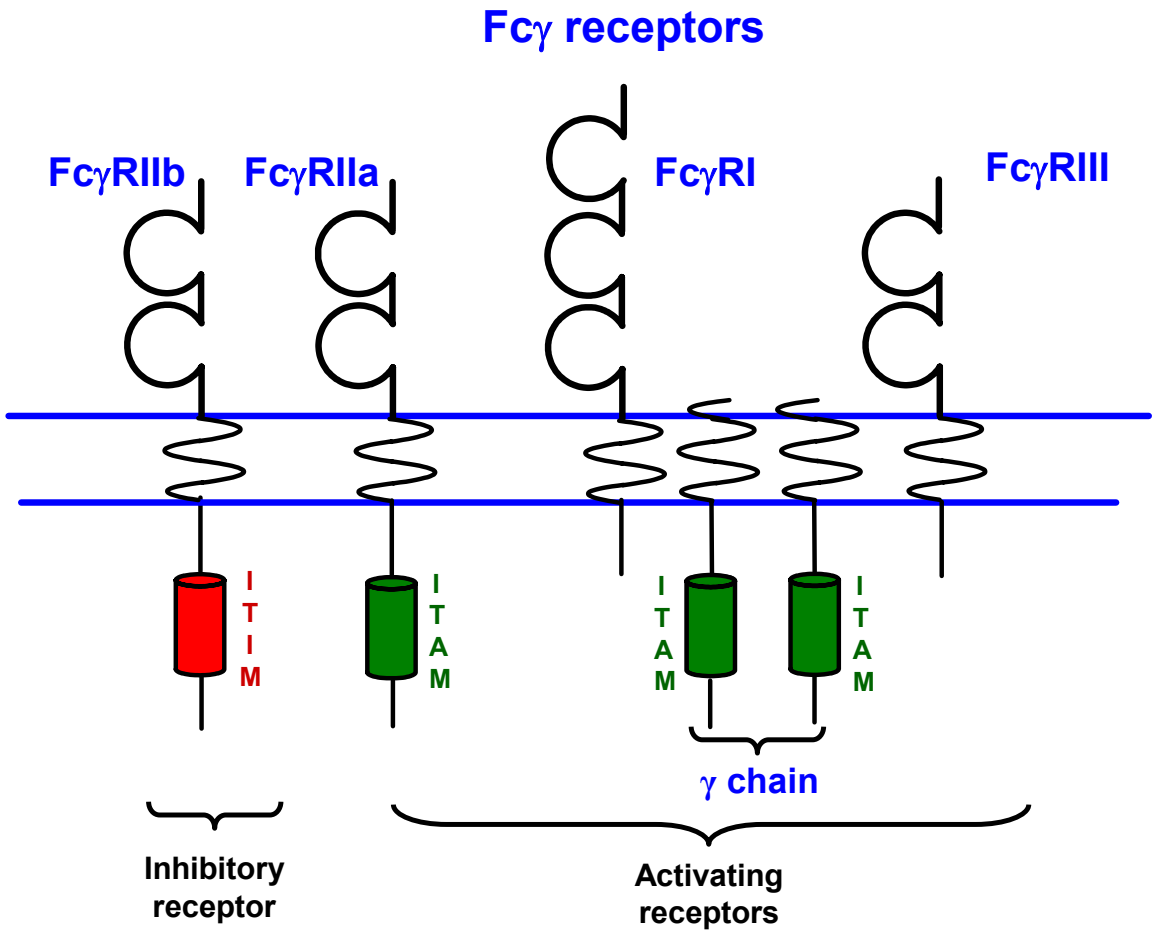


Figure 1.1: Fcγ receptors expressed on macrophages. Both human and mouse macrophages express FcγRI, FcγRIII and FcγRIIb, while mouse macrophages lack FcγRIIa. Recently mouse macrophages have been shown to express FcγRIV (not shown in this figure). Based on presence of ITAM or ITIM, these receptors are classified as activating or inhibitory.

production, calcium mobilization and cellular proliferation[20,21]. These studies are further confirmed by *in vivo* studies of FcγRIIb knock out mice which display elevated serum levels of IgG upon antigenic challenge[22]. FcγRIIb mRNA undergoes alternative splicing to generate two isoforms FcγRIIb1 and b2. The differences and functions of these two isoforms are discussed in detail in chapter 2.

The expression profile of Fcγ receptors on important phagocytic cells such as monocytes, macrophages and neutrophils is summarized in Table 1.

1.3 Overview of signaling pathways triggered by Fcγ receptors

The clustering of Fcγ receptors by IgG immune-complexes results in tyrosine phosphorylation of the ITAMs by membrane-associated Src-family tyrosine kinases[23,24]. The phosphorylated ITAMs serve as docking sites for SH2 domain-containing signaling molecules that in turn activate multiple signaling pathways. Tyrosine residues within the membrane-associated adapter LAT are also phosphorylated, providing additional docking sites for SH2-domain containing signaling molecules[25]. Thus proximal signaling events include the activation of Syk kinase, PI 3-kinase, MAP kinases and PLCγ.

The process of phagocytosis involves the internalization of IgG-coated particles and therefore requires extensive actin cytoskeletal rearrangements. Syk and PI 3-kinase play a critical role during actin polymerization and pseudopod formation. In addition to particle internalization, Fcγ receptor-mediated phagocytosis is accompanied by the release of various inflammatory mediators such as pro-inflammatory cytokines and ROS.

	Monocytes		Macrophages		Neutrophils	
	Mouse	Human	Mouse	Human	Mouse	Human
FcγRI	+	+	+	+	+	+
FcγRIIa	–	+	–	+	–	+
FcγRIIIa	+	+	+	+	+	+
FcγRIV	+	?	+	?	+	?
FcγRIIb	+	+	+	+	+	+

Table 1.1: Expression profile of activating and inhibitory FcγR on murine and human phagocytes.

The production of pro-inflammatory cytokines is dependent on the activation of MAP kinases and PI 3-kinase pathways. The generation of ROS, on the other hand, is mainly dependent upon the activation of PI 3-kinase pathway. Downstream of PI 3-kinase activation, there is an activation of Akt which then phosphorylates proteins involved in ROS generation while PI 3-kinase dependent Rac activation is also important for ROS production. A brief schematic of FcγR-mediated signaling is provided in Figure 1.2.

1.4 Src and Syk Kinases

The first step after co-clustering of activating and inhibitory FcγR is the phosphorylation of tyrosine residues in ITAM or ITIM. This step is catalyzed by Src-family tyrosine kinases[24]. The identification of the role of Src kinases during FcγR-mediated signaling emerged from the observation that, a tyrosine kinase inhibitor, herbimycin A, suppressed FcγR-induced phagocytosis in macrophages[26,27]. Moreover, Src kinases are shown to be constitutively associated with Fcγ receptors[24,28,29]. In a more recent study Cooney et al. demonstrated that the ITAM of FγRIIa is phosphorylated by Src kinases[23]. The requirement of Src kinases is evident from the significant reduction in the phagocytic ability of macrophages isolated from $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ mice[30]. Together these findings indicate that the activation of Src kinases is critical for FcγR-mediated phagocytosis.

The phosphorylated ITAMs then recruit SH2 domain-containing proteins such as Syk kinase[31,32]. Activation of Syk tyrosine kinase is critical for FcγR-mediated signaling

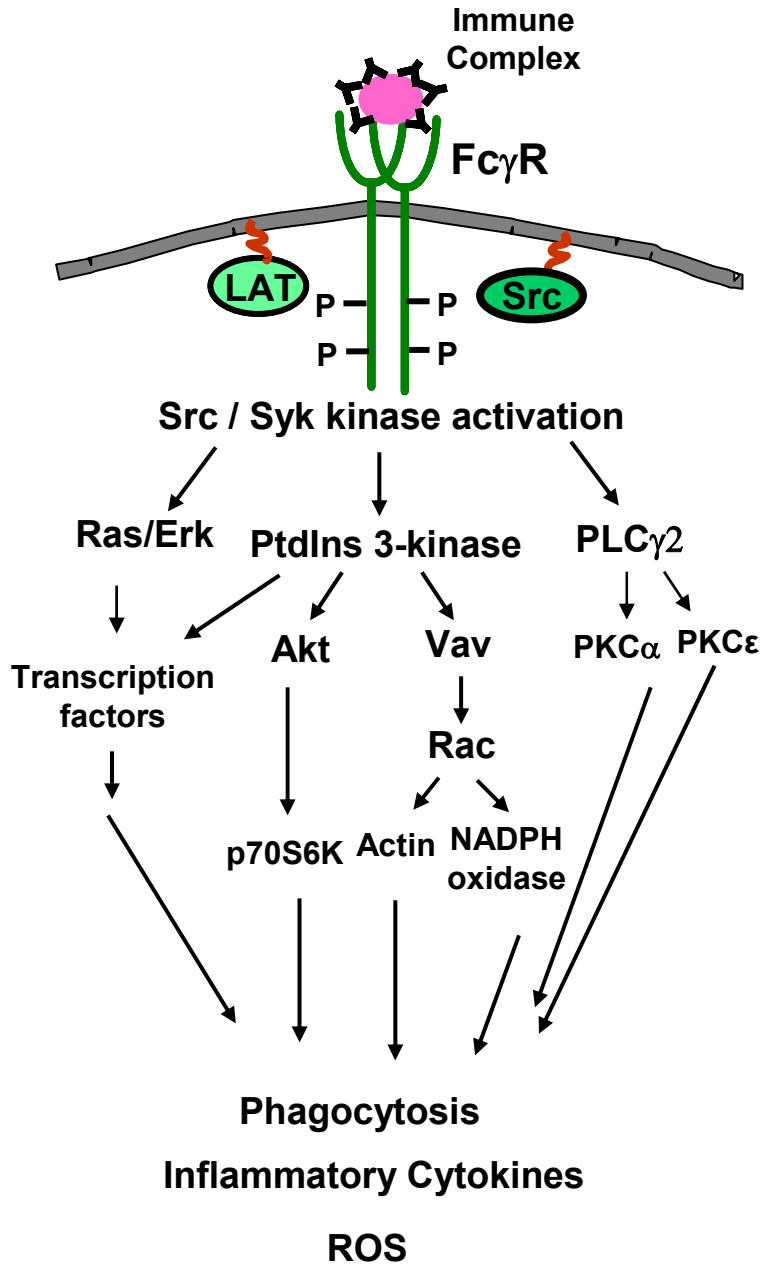


Figure 1.2: Schematic of signaling pathways activated by FcγR clustering. Upon activation, ITAMs of FcγR are phosphorylated by membrane-associated Src kinases resulting in activation of variety of signaling pathways.

in phagocytes. Syk-deficient macrophages exhibit a significant reduction in phagocytosis. Interestingly, Syk null neutrophils fail to produce ROS upon FcγR clustering[33]. In monocytes, suppression of Syk expression using siRNA led to decreased phagocytosis[34]. Conversely, increased phagocytic efficiency was seen in FcγR-expressing COS-1 fibroblasts co-transfected with Syk[35].

The precise mechanism by which Syk influences FcγR-mediated phagocytosis is not known. $Syk^{-/-}$ macrophages exhibit the formation of phagocytic cup; however they are incapable of ingesting IgG-coated particles[30]. This suggests that Syk may play an important role during actin cytoskeleton rearrangement. Interestingly, inhibition of PI 3-kinase using wortmannin or LY294002 also did not inhibit phagocytic cup formation; however closure of the phagosomes around the particles was blocked[36]. This suggests that Syk might be acting upstream of PI 3-kinase.

1.5 PI 3-Kinase

PI 3-kinase is activated by several different receptors, including FcγR. The family of PI 3-kinase is divided into four classes, I_A, I_B, II and III based on structural characteristics and substrate specificity. Class I_A PI 3-kinases are thought to be involved in FcγR-mediated signaling[37]. All members of PI 3-kinase serve to phosphorylate phosphatidylinositides at the 3' position[38]. Activated PI 3-kinase is found in the membrane within seconds of FcγR crosslinking[39]. Class I PI 3-kinases are heterodimers consisting of a catalytic p110 subunit and a regulatory p85 or p55 subunit. The p110 subunit has been shown to be activated and required for FcγR-mediated phagocytosis[40].

The SH2-domain-containing p85 subunit acts as a regulator by binding phosphotyrosine proteins at the cell membrane and inducing activation of the p110 subunit[41,42].

Activation of PI 3-kinase plays an important role in actin cytoskeleton remodeling that drives the process of phagocytosis. The initial phase of actin polymerization leading to phagocytic cup formation does not seem to be regulated by PI 3-kinase. However, PI 3-kinase is necessary for closure of the phagocytic cup[36,43]. Also, the requirement of PI 3-kinase activation is more pronounced for the engulfment of larger particles ($\geq$ 4.5 μm)[43]. This suggests that PI 3-kinase may play a role in regulation of membrane availability and thereby influences the overall phagocytic efficiency. In addition to IgG-induced phagocytosis, Allen et al. showed that PI 3-kinase regulated the actin cytoskeleton during the delayed phagocytosis of ulcerogenic *Heliobacter pylori*[44].

PI 3-kinase participates in cellular responses via attraction of pleckstrin-homology (PH) domain-containing proteins to the phosphatidylinositol 3,4,5 –trisphosphate (PtdIns3,4,5P_3) product. This includes proteins such as Akt, Btk and Vav, a guanine nucleotide exchange factor (GEF) for small GTPase Rac. The role of Rac during actin rearrangement and superoxide generation is extensively studied and is discussed below in brief. PtdIns3,4,5P_3 also activates GEF for the GTPase ARF6. ARF6 has been shown to be required for FcγR-mediated phagocytosis[45] as well as for focal exocytosis of membrane resulting in more membrane availability[46]. Recent work also shows that activation of Akt enhances FcγR-mediated phagocytosis via the mTOR/p70S6K pathway[47] ; thus uncovering another potential mechanism by which PI 3-kinase influences phagocytosis.

1.6 Rac

Rac GTPases play a crucial role during FcγR-mediated phagocytosis through their participation in actin rearrangement and ROS production by NADPH oxidase complex. In humans, three different isoforms of Rac have been described, namely Rac1, Rac2 and Rac3. Rac1 and Rac3 are ubiquitously expressed whereas Rac2 expression is restricted to hematopoietic cells[48,49]. Rac1 and Rac2 share 92% sequence homology. The main differences between Rac1 and Rac2 are found in the carboxy-terminal end of the molecules. Rac1 has six sequential basic amino acids (KKRKRK) in the C-terminal domain whereas Rac2 has only three basic amino acids interspersed with neutral amino acids (RQQKRA).

Upon activation of FcγRs, Rac is localized to the phagosome via its polybasic and/or prenylation regions[50]. Hoppe and Swanson demonstrated that there are two phases of GTPase activation: - in the first phase, GTPase activity colocalizes with actin in the extending pseudopods while in the second phase, GTPase activity peaks behind the actin-rich region during the closure of phagosome[51]. In this study, further examination of Rac1 and Rac2 in the two phases of activation revealed that Rac1 activity is observed around the phagocytic cup and is maximal during the closure. On the other hand, Rac2 activation is persistent during and after the closure of the phagocytic cup.

The activation of Rac is regulated by binding and hydrolysis of GTP. In resting condition, Rac is predominantly associated with GDP and is inactive. Upon stimulation, GDP is replaced by GTP with the help of proteins called guanine nucleotide exchange factors (GEF). Earlier studies indicated that Vav1 and Vav2 serve as GEFs for Rac activation during growth factor mediated signaling[52,53]. However, recent studies

demonstrate that Vav is dispensable for activation of Rac during Fcγ receptor mediated phagocytosis[54]. This finding is further supported by the observation that the Ras-GAP protein called CAPRI might serve as the adaptor protein for recruitment of Rac to phagocytic cup during Fcγ receptor mediated phagocytosis[55].

FcγR-mediated phagocytosis is accompanied by the production of superoxide. Superoxide generation is catalyzed by NADPH oxidase complex which is a multi-subunit enzyme. Rac is important in the assembly of functional NADPH oxidase. In the case of neutrophils, Rac2 isoform of Rac is present abundantly and has been shown to be necessary for NADPH oxidase activation (reviewed in [56,57]). Interestingly, studies by Zhao et al showed that human monocytes express more of Rac1 isoform than Rac2 and Rac1 participates in the assembly of functional NADPH oxidase complex[49].

1.7 Phospholipase Cγ (PLCγ)

PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG)[58]. DAG activates various isoforms of Protein Kinase C (PKC), while generation of IP3 leads to mobilization of Ca^{2+} from internal reserves[58]. There are two isoforms of PLCγ: PLCγ1 and PLCγ2. PLCγ2 is expressed exclusively in hematopoietic cells. Some studies indicate that both isoforms of PLCγ are activated when FcγR are clustered on monocytes and NK cells[59,60], while only PLCγ2 is activated in neutrophils upon Fc receptor clustering[61]. Further, it has been shown that inhibition of PLCγ2 in mouse macrophages results in diminished FcγR-mediated phagocytosis[62]. However, how PLCγ influences phagocytosis is not yet

completely understood. It is shown that the process of phagocytosis occurs normally in monocytes and macrophages in absence of Ca^{2+} [63,64]. However, Ca^{2+} plays an important role during degranulation of mast cells to release histamine or release of cytotoxic granules from NK cells or neutrophils[65]. Thus, mobilization of Ca^{2+} by PLCγ may not be important for phagocytic activity but activation of PLCγ may be more critical for neutrophil or NK cell FcγR-mediated functions such as ADCC.

1.8 Protein Kinase C (PKC)

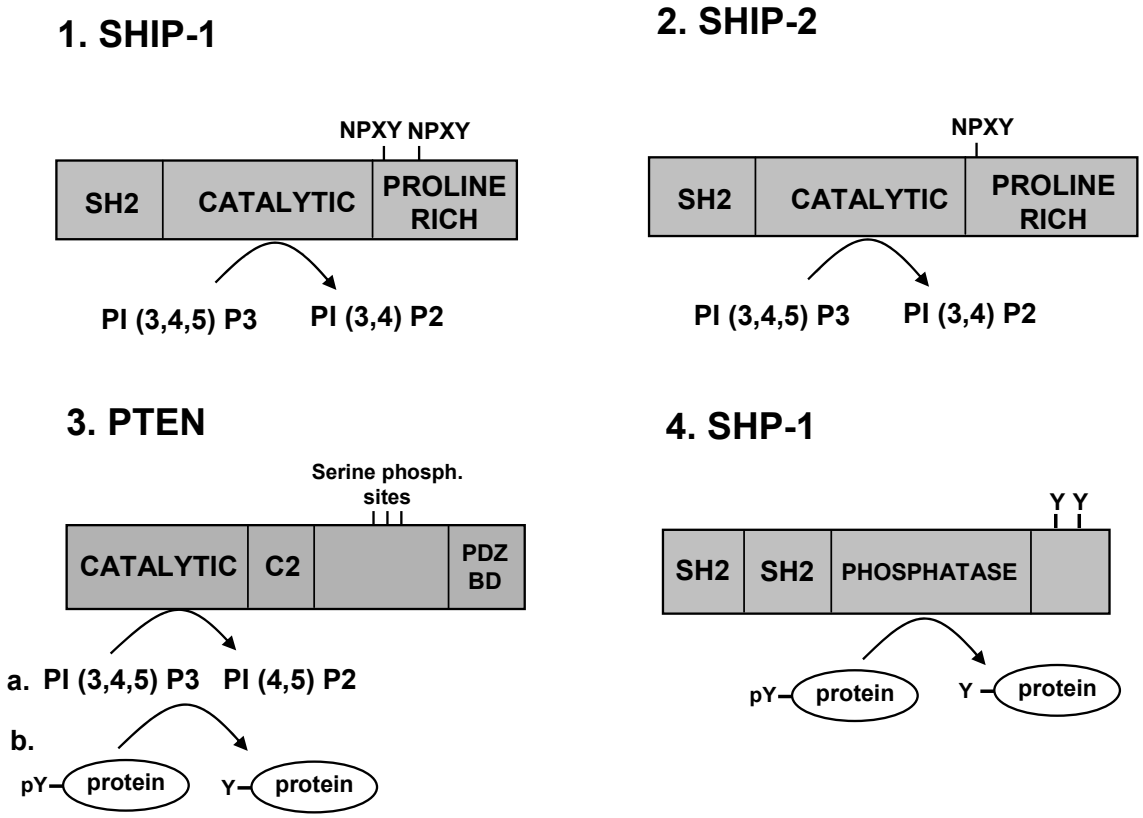
There are three classes of PKC enzyme. The classic isoforms include α, βI, βII and γ, which all require Ca^{2+}, DAG and phosphatidylserine (PS) for activity. The novel PKC isoforms δ, ε, η and θ are also activated by DAG and PS but do not require Ca^{2+} for activation. The atypical isoforms, PKC ζ and ι/λ, are activated only by PS and do not require Ca^{2+} nor DAG [66]. Measurements of PKC activity in phagosomes purified from monocytes stimulated with IgG-coated beads indicate that PKC is activated and is recruited to the phagocytic membrane[67]. A detailed understanding of differential involvement of PKC isoforms in IgG-stimulated phagocytosis and associated respiratory burst came from studies in the mouse macrophage cell line Raw 264.7. IgG-mediated phagocytic process required activation of novel PKCε, which is shown to localize to the phagosome[66,68]. On the other hand, there is an activation of the classic PKC isoform α during IgG-mediated respiratory burst[66]. Pharmacological inhibition of PKC using staurosporine or calphostin C results in reduced FcγR-mediated phagocytosis and ROS production[69].

1.9 Regulation of FcγR-mediated phagocytosis

The process of phagocytosis is central to the innate immune responses protecting the host from invading pathogens as well as clearing debris at the site of injury. However prolonged activation of such responses can cause significant damage to the host tissue and at times may result in the development of autoimmunity[70]. For example, in case of FcγR-mediated phagocytosis, inflammatory mediators such as pro-inflammatory cytokines and ROS are released that can cause collateral tissue damage. Therefore the process of phagocytosis needs to be controlled.

The phagocytic process is indeed regulated at several levels, including negative feedback mechanism, cytokine profile of the surrounding milieu, inhibitory FcγRIIb receptor and intracellular phosphatases such as SHIP-1, SHIP-2, PTEN and SHP-1 (Figure 1.3). A brief description of these negative regulators in phagocytosis is provided below.

Several studies demonstrated that IgG-mediated phagocytosis is downregulated by FcγRIIb. It has been shown that phagocytosis is enhanced in macrophages deficient in FcγRIIb expression[71]. In contrast, overexpression of FcγRIIb resulted in inhibition of phagocytosis[72]. These studies suggest that the phagocytosis of IgG-coated particles involves the simultaneous clustering of activating and inhibitory FcγR, and the ratio of the activating to inhibitory FcγR influence the magnitude of the phagocytic response. The ratio of activating to inhibiting FcγR is altered by cytokines present in surrounding milieu of monocytes and macrophages. For example, anti-inflammatory cytokines such as IL-4,



<u>Figure 1.3:</u> Domain structures of phosphatases. SHIP-1, SHIP-2, PTEN and SHP-1 act as negative regulators of FcγR-mediated signaling. Shown above is the schematic of domain structures and catalytic activities of phosphatases involved in FcγR signaling.

IL-13 or a combination of IL-4 with IL-10 can dramatically upregulate the expression of FcγRIIb, resulting in a dampened phagocytic response[73-75]. The inhibition by FcγRIIb is mediated by the recruitment of phosphatases, particularly SHIP-1 (SH2 domain-containing inositol 5' phosphatase-1) and SHIP-2 to the tyrosine phosphorylated ITIM within its cytoplasmic tail[76-78].

SH2 domain-containing inositol phosphatase, SHIP-1, is a multi-domain cytosolic protein. Its expression is restricted to hematopoietic cells. It contains an N-terminal SH2 domain, a central catalytic domain, two NPXY motifs and a C-terminal proline-rich domain (PRD). Each of these domains contributes to the inhibitory effects mediated by SHIP-1. The N-terminal SH2 domain binds with high affinity to phosphorylated ITIM of FcγRIIb[77,79,80] and with low affinity to phosphorylated ITAM[81,82]. The central catalytic domain hydrolyzes the phosphate group from the 5' position of both PtdIns3,4,5P_3 and 1,3,4,5-tetrakisphosphates (IP_4)[83,84]. The two NPXY motifs undergo tyrosine phosphorylation and bind to phosphotyrosine binding (PTB) domain-containing proteins such as Shc[84-86] and the RasGAP-binding protein Dok[87]. The C-terminal PRD domain has been shown to be constitutively associated with SH3 domains of Grb2[88]. Hydrolysis of PtdIns3,4,5P_3 by SHIP-1 leads to dampened activation of PH-domain containing enzymes such as Btk, Akt and Vav and subsequent downstream signaling[89-92].

In addition to its role during FcγR-signaling, SHIP-1 plays an important role during growth factor and cytokine-mediated signaling[93]. Moreover, generation of mice with targeted deletion of SHIP-1 gene revealed that SHIP-1 acts as an overall negative regulator of proliferation, survival and activation of hematopoietic cells. SHIP-$1^{-/-}$ mice have a shortened life span, show extensive proliferation of granulocytes and

macrophages, suffer from splenomegaly, lymphadenopathy and show elevated serum immunoglobulin levels[94-96]. These phenotypic characteristics underline the significance of the role of SHIP-1.

Under resting condition, SHIP-1 resides in the cytoplasm and translocates to the membrane upon cellular activation where it associates with the phosphorylated receptors through its SH2 domain. The inhibitory effect of SHIP-1 on phagocytosis was first demonstrated by studies using macrophages from SHIP-1 knockout mice. SHIP-$1^{-/-}$ macrophages showed enhanced phagocytic ability[97]. Further evaluation of the molecular details of SHIP-1 activation in macrophages demonstrated that SHIP-1 is recruited not only to phosphorylated ITIM motif but also to the phosphorylated ITAMs of the activating FcγR[81,82]. In B cells, SHIP-1 has been shown to inhibit Ras/Erk pathway through its interaction domains. Two models have been proposed to explain the mechanism of this inhibition. Tridandapani et al demonstrated that SHIP-1 competes with Grb2/Sos complex for the binding of Shc thereby preventing Grb2/Sos membrane translocation and activation of Ras[79]. In the second model, SHIP-1 was shown to associate with p62Dok, a RasGAP-binding protein which catalyzes the hydrolysis of GTP and inactivates Ras[87]. The role of SHIP-1 as a negative regulator of phagocytosis has been established. However, it was not known whether it plays any role in the regulation of phagocytosis-associated events such as the release of pro-inflammatory cytokines and ROS. In Chapter 3, I have presented and discussed our findings that illustrate that SHIP-1 downregulates phagocytosis-associated cytokines and ROS production through its influence on Ras/Erk and PI 3-kinase pathway.

Another inositol phosphatase, SHIP-2, was subsequently identified and was shown to be closely related to SHIP-1[98]. Unlike SHIP-1, SHIP-2 is expressed ubiquitously[99]. SHIP-2 displays a high sequence homology with SHIP-1, especially in the N-terminal SH2 domain and the catalytic domain[100,101]. The C-terminal PRD domain of SHIP-2 differs from that of SHIP-1 considerably. Also, SHIP-2 contains only one NPXY motif that binds to PTB domain-containing proteins upon phosphorylation[99]. SHIP-2 is phosphorylated upon FcγR clustering and negatively regulates phagocytosis through the inhibition of Rac activation[102,103].

Apart from the inositol phosphatases SHIP-1 and SHIP-2, two other phosphatases PTEN and SHP-1 have been shown to regulate Fc receptor-mediated activation events. PTEN (phosphatase and tensin homolog on chromosome 10) plays a pivotal role in the regulation of several biological functions. It possesses a unique phosphatase activity that can hydrolyze phospholipids at the 3' position as well as dephosphorylate tyrosine-phosphorylated proteins[104,105]. Evidence for the role of PTEN as a negative regulator comes from two studies. The first study showed that PTEN downregulates FcγR-mediated Rac activation and phagocytosis in transfected fibroblasts[106]. In the second study, PTEN-deficient murine macrophages were shown to exhibit enhanced phagocytosis and production of inflammatory cytokines. The latter study also demonstrated that PTEN negatively regulates FcγR-induced signaling events such as the activation of Akt and Erk[107]. However, the precise mechanism of PTEN activation following FcγR clustering is not yet known.

SHP-1 is a protein tyrosine phosphatase that is expressed mainly in hematopoietic cells. SHP-1 contains two N-terminal SH2 domains, a catalytic domain and two tyrosine

phosphorylation sites in the C-terminal domain[108,109]. The negative role of SHP-1 in FcγR signaling was first shown in J774A.1 macrophage-like cells. Transient over-expression of SHP-1 in these cells resulted in reduced phagocytosis of IgG-coated particles[110]. More recently, it was shown that SHP-1 associates specifically with the phosphorylated ITAM of FcγRIIa in human monocytic cells. In the same system, it was further demonstrated that SHP-1 downregulates FcγRIIa-induced NFκB-dependent gene transcription[111]. These observations suggest that SHP-1 is recruited during FcγR-mediated phagocytosis in macrophages probably in an FcγRIIb-independent manner.

It is evident from the above discussion that FcγR-mediated signaling events have been extensively studied. However, several questions and mechanisms still remain unanswered. During my dissertation research, we sought answers for some of these questions as discussed below in brief.

In Chapter 2, we examined the functional differences between the two isoforms of FcγRIIb. Previous studies in B cells have indicated that FcγRIIb1 and b2 are likely to serve some non-overlapping functions. However, none had compared the functional capacity of hFcγRIIb1 and b2 in macrophages. To examine the functional differences between b1 and b2 in the context of macrophages, we examined whether both FcγRIIb1 and b2 become tyrosine-phosphorylated and whether they both associate with SHIP-1 upon activation. We further tested whether both these isoforms are expressed on the cell surface equivalently and whether they are capable of downregulating FcγR-mediated functions.

The inhibitory effects of FcγRIIb are predominantly mediated by SH2 domain-containing inositol phosphatase, SHIP-1. Although it is known that SHIP-1 inhibits the uptake of IgG-coated particles in macrophages, its influence on phagocytosis-associated release of inflammatory mediators remained to be characterized. In Chapter 3, we describe our studies analyzing the influence of SHIP-1 on phagocytosis-associated events such as cytokine production and ROS generation.

Apart from their role in the clearance of immune-complexes, macrophages are also capable of eliminating tumor cells opsonized with antibodies by a process called antibody-dependent cell-mediated cytotoxicity (ADCC). The importance of FcγR activation in the clearance of antibody-coated tumor cells was convincingly demonstrated by Clynes et al[9]. However, molecular details of macrophage-mediated ADCC are not fully understood. Thus in Chapter 4, we investigated the mechanisms involved in macrophage-mediated ADCC against B cell lymphoma tumor targets coated with a monoclonal antibody.

CHAPTER 2

MOLECULAR ANALYSIS OF EXPRESSION AND FUNCTION OF HUMAN FcγRIIb1 AND b2 ISOFORMS IN MYELOID CELLS

2.1 Abstract

The inhibitory receptor FcγRIIb undergoes tyrosine phosphorylation and associates with the inositol phosphatase SHIP-1 to downregulate phagocytosis. The two splice variants of FcγRIIb, b1 and b2, are differentially expressed in hematopoetic cells. Both isoforms of FcγRIIb are expressed in human myeloid cells although FcγRIIb2 predominates. In murine B cells FcγRIIb2 associates with clathrin-coated pits and undergoes endocytosis, whereas FcγRIIb1 is excluded from the coated pits, indicating that the two isoforms serve partially differing functions. Studies examining the ability of two isoforms of human FcγRIIb to get phosphorylated have shown conflicting results and the functional capacities of these two isoforms are also not well understood. The aim of this study was to characterize the functional differences, if any, between the two isoforms of hFcγRIIb. Transfection experiments expressing human FcγRIIb1 or b2 in Raw 264.7

murine macrophage cells revealed that both isoforms are equivalently surface expressed. When co-clustered with activating receptors, both the isoforms undergo tyrosine phosphorylation and promote SHIP-1 phosphorylation. Finally, both b1 and b2 isoforms of FcγRIIb downregulate phagocytosis and TNFα production to a similar extent. Thus we conclude that hFcγRIIb1 and b2 are both functional inhibitory receptors in the phagocytic process.

2.2 Introduction

Fcγ receptor clustering on monocytes/macrophages initiates a series of signaling events that culminate in phagocytosis of IgG-coated particles, generation of inflammatory cytokines and the production of reactive oxygen and nitrogen radicals (reviewed in [112,113]). FcγRI, FcγRIIa and FcγRIIIa are all activating receptors, associate with immunoreceptor tyrosine-based activation motifs (ITAMs) and promote the phagocytic process. FcγRIIb, on the other hand, is an inhibitory receptor that serves to downregulate phagocytosis[114].

Muta et al demonstrated that the inhibitory potential of FcγRIIb is attributed to a tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail[21]. Mutation of the tyrosine to phenylalanine or deletion of the ITIM abolishes the inhibitory activity of FcγRIIb. When clustered with an ITAM-containing receptor, FcγRIIb ITIM is tyrosine phosphorylated and recruits the inositol phosphatase SHIP-1 via the SHIP-1 SH2 domain[76,77,79,115]. Association of SHIP-1 with the phosphorylated ITIM of FcγRIIb delivers SHIP-1 to the membrane where it can access and hydrolyze its substrate

PtdIns3,4,5P_3 to PtdIns3,4P_2, thereby downregulating the activation of downstream PtdIns3,4,5P_3-dependent enzymes. Deletion of SHIP-1 abrogates the inhibitory effect of FcγRIIb indicating that SHIP-1 is the effector molecule of FcγRIIb[77,78].

Both FcγRIIb and SHIP-1 negatively regulate FcγR-mediated phagocytosis. Thus, FcγRII$^{-/-}$ macrophages and SHIP-1$^{-/-}$ macrophages display enhanced phagocytic efficiency in comparison to their wild-type counterparts[71,81,97]. In human monocytes FcγRIIb expression is upregulated by IL-4, and results in diminished phagocytic efficiency[74,75]. Further studies from our laboratory indicate that in addition to IL-4, treatment of monocytes with a combination of IL-4 and IL-10 results in synergistic up-regulation of FcγRIIb protein as well as mRNA expression. The up-regulated FcγRIIb undergoes phosphorylation and monocytes show a concomitant decrease in the phagocytic ability.

FcγRIIb is expressed as two common isoforms, b1 and b2 (Figure 2.1). The two differ in their cytoplasmic tails. In humans, b1 isoform contains a 19 amino acid insert, encoded by exon C1, spliced out of the b2 isoform[116]. However, the downstream ITIM sequence remains the same in both the isoforms. In mouse, the insert is longer (46 amino acids) but the molecules are otherwise analogous[117-119]. Deglycosylated forms of the two proteins can be readily distinguished by size on SDS-PAGE [75,120,121]. Both isoforms are expressed in all cells expressing FcγRIIb although the b1 form predominates in B cells and mast cells whereas the b2 form predominates in cells of mononuclear phagocyte lineage[122,123]. Previous studies from our group demonstrate that both isoforms of FcγRIIb are up-regulated in monocytes when treated with IL-4 alone or IL-4 and IL-10 together.

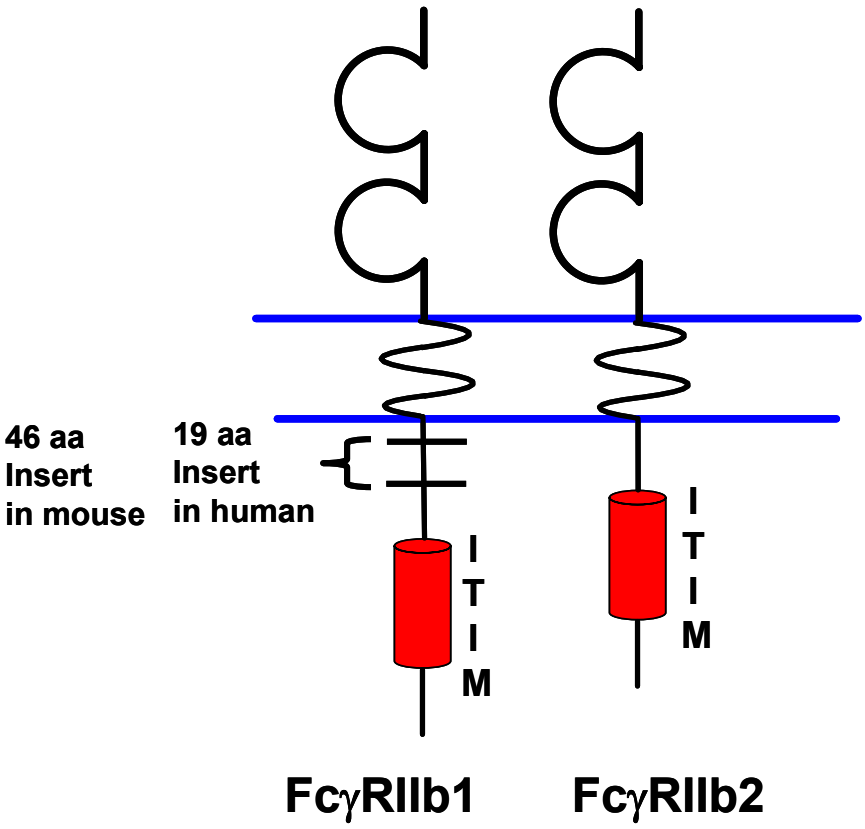


Figure 2.1: The two isoforms of FcγRIIb. In humans, the cytoplasmic domain of FcγRIIb1 contains 19 amino acids insert while in murine system, the insert is 46 amino acids long as compared to their respective FcγRIIb2 isoforms.

Previous studies have examined the functional differences between the two isoforms in B cells. Thus, it has been reported by several groups that both human FcγRIIb2 and murine FcγRIIb2 are capable of endocytosis[17,124,125], whereas FcγRIIb1 is not. In other studies, Budde et al observed that in B cells human FcγRIIb2 ITIM tyrosine is not phosphorylated although its ability to downregulate antigen receptor-induced calcium mobilization was comparable to that of FcγRIIb1, which is tyrosine phosphorylated[126]. Although the molecular details of this paradoxical observation are not fully understood, it is clear that FcγRIIb1 and b2 likely serve some non-overlapping functions in B cells. Of note, there are no studies to date comparing the functional capacity of hFcγRIIb1 and b2 in macrophages. Hunter et al used a COS-1 fibroblast model to study the inhibitory potential of hFcγRIIb. In this study, it was clearly demonstrated that hFcγRIIb1 can downregulate FcγR-mediated phagocytosis[72]. However, it is not clear whether hFcγRIIb2 can function in a comparable manner. Likewise, it is not known if both FcγRIIb1 and b2 are tyrosine phosphorylated in macrophages and function as inhibitory receptors during phagocytosis.

The purpose of this study was to examine the regulation of function of hFcγRIIb1 and b2 in myeloid cells. To test the functional characteristics of the two isoforms, we expressed hFcγRIIb1 and b2 in Raw 264.7 murine macrophage cells. We report that both isoforms of hFcγRIIb are equivalently surface expressed, suggesting that the presence or absence of the 19 amino acid insert has no influence on surface expression of these receptors. Interestingly, unlike in B cells, both isoforms are tyrosine phosphorylated upon

FcγR clustering. We extend these findings to show that both isoforms induce SHIP-1 activation, downregulate FcγR-mediated phagocytosis and TNFα production.

2.3 Materials and Methods

Cells, antibodies and reagents: Raw 264.7 murine macrophage cells were obtained from ATCC and maintained in RPMI with 5% fetal bovine serum. Phospho-FcγRIIb, phospho-Erk, phospho-p38 and phospho-SHIP antibodies were purchased from Cell Signaling Technology (Beverly, MA). Actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human FcγRIIb rabbit polyclonal antibody, Ab163, was a kind gift from Dr. Jean-Luc Teillaud, and was raised against a GST fusion protein of the cytoplasmic tail of FcγRIIb1. Rabbit anti-mouse FcγRIIb antibody was kindly obtained from Dr. John Cambier, Denver, Colorado. mAb FLI8.26 was purchased from BD Pharmingen (San Diego, CA). Anti-mouse CD16/32 (FcγRIII/II) was purchased from BD Pharmingen (Franklin Lakes, NJ). Mouse anti-rat antibody was from Jackson ImmunoResearch (West Grove, PA). Recombinant mouse CSF-1 and TNFα ELISA kit was purchased from R&D systems (Minneapolis, MN).

Preparation of heat-aggregated IgG: Heat aggregated IgG was prepared according to methods described previously[127]. In brief Chromopure mouse IgG at a concentration of 750 μg/ml was heated at 62°C for 30 minutes and then cooled on ice immediately and used directly to stimulate the cells.

Stimulation of cells, lysis and immunoblot: Resting and activated cells were lysed in TN1 lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 3 mM Na_3VO_4, 10 μg/ml each aprotinin and leupeptin, and 2 mM PMSF) for 10 minutes on ice. Proteins were separated by SDS/PAGE, transferred to nitrocellulose filters, probed with the antibody of interest and developed by enhanced chemiluminescence.

Immunoblot data quantitation: The ECL signal was quantitated using a scanner and a densitometry program (Scion Image). To quantitate the phospho-specific signals in the activated samples, we first subtracted background, normalized the signal to the amount of total protein in each lane, and plotted the values obtained as fold increase over the values in unstimulated samples. Values were obtained from a minimum of three independent experiments and statistical analysis was performed using the Student's t-test, setting a p value ≤ 0.05 as significant.

Transfection: Raw 264.7 cells were transfected using the Nucleofector (Amaxa) as previously described[47]. In brief 10 X 10^6 cells in 100 μl of solution V ("Kit V", Amaxa Biosystems) at room temperature were mixed with 3-8 μg of plasmids encoding hFcγRIIb1 or hFcγRIIb2 (generously provided by Dr. J. Ravetch, Rockefeller University, New York), along with 0.6-1.6 μg EGFP encoding plasmids. The cells were transfected using the program U-14. After transfection the samples were transferred to 6 well plates containing pre-warmed media. Transfectants were cultured for 24 hours and then used in phagocytosis assays.

Measurement of receptor expression by flow cytometry: Transfected Raw 264.7 cells were tested for expression of hFcγRII by incubating with anti-hFcγRII (mAb FLI8.26) (Pharmingen) at a concentration of 10 μg/ml for 30 minutes at 4°C. The cells were washed and incubated with FITC-labeled goat anti-mouse IgG secondary antibody for 30 minutes at 4°C. Cells were subsequently washed, fixed in 1% paraformaldehyde and analyzed by flow cytometry on BD FACSCalibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ). Data from 10,000 cells per condition were recorded to yield the percentage of cells expressing receptors (Figure 2.2A).

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

Phagocytosis Assays: IgG-coated SRBCs described above were added to Raw 264.7 cells or PBMs. The cells were pelleted by low speed centrifugation to increase contact between SRBCs and phagocytes. The samples were incubated for 1 hour at 37°C. Cells were subjected to brief hypotonic lysis with water to get rid of externally bound RBCs prior to fixation in 1% paraformaldehyde and mounted on slides to be viewed under a fluorescence microscope. Phagocytosis was measured by counting the total number of RBCs ingested by 100 phagocytes (Phagocytic Index). No phagocytosis was seen in any

of the samples treated with non-opsonized RBCs. Statistical analysis was performed using the Student's t-test and p value ≤ 0.05 was considered as significant.

Culture of murine bone marrow derived macrophages (BMMs): Bone marrow macrophages (BMMs) were derived as previously described[25]. Briefly, bone marrow cells were cultured in RPMI containing 10% fetal bovine serum and supplemented with 10 ng/ml CSF-1 and 5 μg/ml polymixin B for 7 days.

Measurement of TNFα by ELISA: Transfected Raw 264.7 cells or BMMs isolated from WT and FcγRII$^{-/-}$ mice were stimulated with 750 μg/ml immune-complexes for 2, 5 or 8 hours. The supernatants were harvested and analyzed by TNFα ELISA kit as per manufacturer's recommendations.

2.4 Results

Human FcγRIIb1 and b2 are both equivalently surface-expressed and phosphorylated

It is known that FcγRIIb downmodulates the process of phagocytosis. However it is not clear whether both hFcγRIIb1 and b2 isoforms can downregulate phagocytosis in myeloid cells. Also it is not known whether hFcγRIIb1 and b2 can both become tyrosine phosphorylated. Furthermore, the extracellular domains of FcγRIIb and FcγRIIa are virtually identical and all monoclonal antibodies that recognize hFcγRIIb also recognize hFcγRIIa. The lack of specific extracellular antibody has made it difficult to examine cell surface expression of FcγRIIb in human myeloid cells. To specifically assess the

functional capacity of the two isoforms of human FcγRIIb in myeloid cells, we transfected Raw 264.7 murine macrophage cells with plasmids encoding either hFcγRIIb1 or hFcγRIIb2. The transfectants were used in the following experiments. We first examined the ability of human FcγRIIb1 and b2 to be comparably surface-expressed. Here Raw 264.7 cells were transfected with vector alone or with 3 and 5 μg of hFcγRIIb1 or b2. Cells were harvested 24 hours post-transfection and were analyzed by flow cytometry to assess whether the two isoforms of hFcγRIIb are equivalently surface-expressed. For this cells were labeled with mAb FLI8.26 anti-hFcγRII antibody, followed by goat F(ab')2 anti-mouse IgG-FITC secondary antibody. Results indicated that both isoforms have comparable ability to be surface expressed (Figure 2.2A). Untransfected Raw 264.7 cells were labeled with both primary and secondary antibodies described above, and served to set the background for any non-specific binding of the antibodies. To confirm the expression of transfected plasmids, protein matched lysates were analyzed by Western blotting. The results shown in Figure 2.2B indicate that both b1 and b2 isoforms of hFcγRIIb are equivalently expressed in the cells.

Next, we assessed the ability of hFcγRIIb1 and b2 to become tyrosine phosphorylated in myeloid cells. Here, the transfected cells were stimulated for 2 minutes using heat-aggregated IgG as previously described[47]. Aggregated mouse IgG was used to co-cluster all FcγR (both endogenous murine FcγR and the transfected human FcγR) through ligand interaction. Human FcγRIIb was immuno-precipitated from unstimulated and stimulated cells using mAb FLI8.26, and analyzed by Western blotting with a rabbit polyclonal antibody specific for the phosphorylated ITIM of hFcγRIIb (Figure 2.3, upper

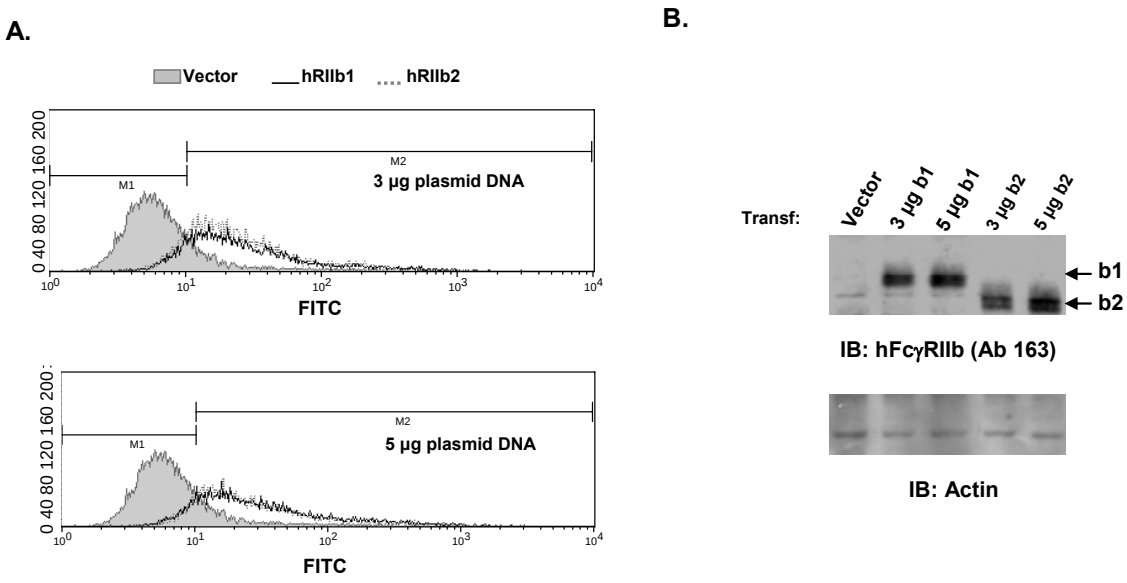


Figure 2.2: FcγRIIb1 and b2 show comparable ability to be surface expressed in macrophages. Raw 264.7 cells were transfected with varying concentrations of plasmid DNA encoding FcγRIIb1 or b2 as indicated in the figure. **A.** Expression of FcγRIIb was analyzed by flow cytometry and **B.** by Western blotting.

panel). The same membrane was reprobed with hFcγRIIb antibody (Figure 2.3, middle panel). Note that, as in the flow cytometry experiment above, there is no cross-reactivity of hFcγRIIb antibodies with murine FcγRIIb. Band intensity of phosphorylated FcγRIIb was quantitated and normalized to total FcγRIIb signals. The graph in Figure 2.3 is a composite of three independent experiments, and indicates that both hFcγRIIb1 and b2 become equivalently tyrosine phosphorylated in macrophages. This is in contrast to previously reported inability of hFcγRIIb2 to become tyrosine phosphorylated in B cells[126].

Both isoforms of human FcγRIIb induce phosphorylation of SHIP-1 and downregulate phagocytosis

The phosphorylated ITIM of FcγRIIb recruits the insoitol phosphatase SHIP-1, which is subsequently tyrosine phosphorylated by the membrane-associated src kinases[128]. We, therefore, next asked whether both the b1 and b2 isoforms of hFcγRIIb are capable of inducing SHIP-1 phosphorylation. In these experiments, Raw 264.7 transfectants were stimulated for 2 minutes with heat-aggregated IgG and protein-matched lysates were analyzed by Western blotting with phospho-SHIP antibody (Figure 2.4, upper panel). The middle panel is a reprobe of the same membrane with actin antibody to ensure equal loading of protein in all lanes. The results indicate that cells expressing either hFcγRIIb1 or b2 had significantly enhanced SHIP-1 phosphorylation upon stimulation (in comparison to cells transfected with vector only, lane 2), indicating

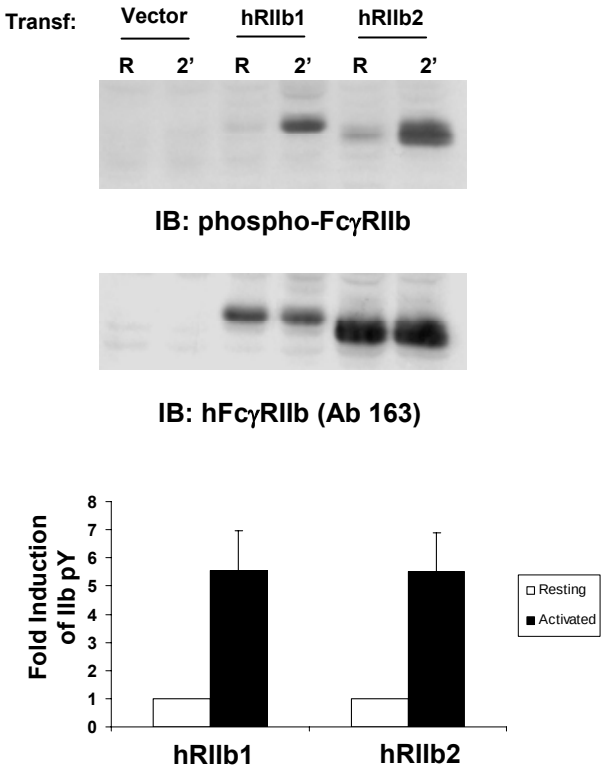


Figure 2.3: FcγRIIb1 and b2 are both tyrosine phosphorylated in macrophages. Raw 264.7 cells were transfected with 5 μg of plasmid DNA encoding FcγRIIb1 or b2. Transfected cells were stimulated with heat-aggregated IgG for 2 minutes, and FcγRII immunoprecipitates were analyzed by Western blotting with antibodies specific for phosphorylated FcγRIIb ITIM (upper panel). The middle panel is a reprobe of the same membrane with hFcγRIIb antibody (Ab 163). The graph shown in lower panel is a composite of three independent experiments.

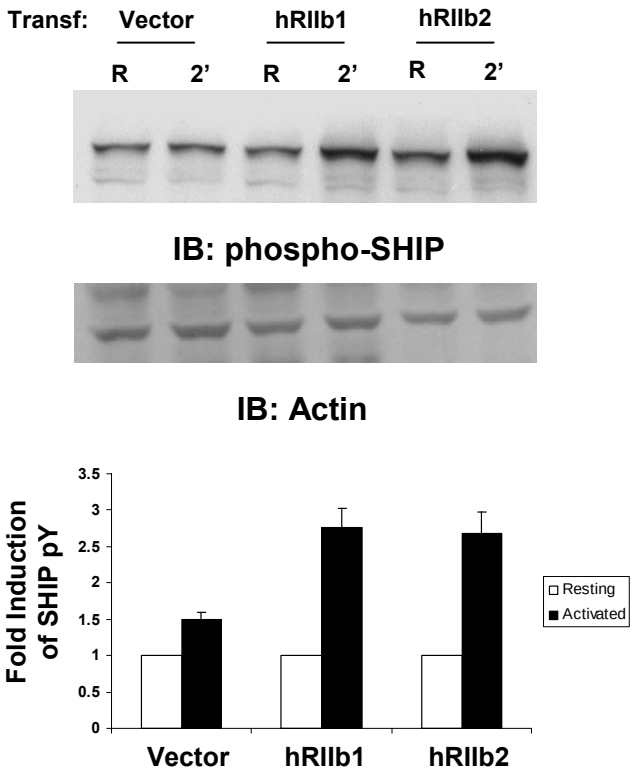


Figure 2.4: Both b1 and b2 isoforms of FcγRIIb are capable of inducing SHIP-1 phosphorylation. Raw 264.7 cells were transfected with vector alone or with plasmids encoding hFcγRIIb1 or b2. Cells were harvested 24 hours post-transfection and were either left unstimulated (resting) or stimulated for 2 minutes with heat-aggregated IgG. Protein matched lysates were analyzed by Western blotting with phospho-SHIP antibody (upper panel). The same membrane was reprobed with actin antibody (middle panel). The graph shown in lower panel is the quantitation of band intensity of phospho-SHIP band normalized to actin band intensity from three different experiments.

that both isoforms of hFcγRIIb are capable of inducing tyrosine phosphorylation of SHIP-1.

Finally, to examine the capacity of hFcγRIIb1 and b2 in regulating FcγR-mediated phagocytosis, Raw 264.7 cells were co-transfected with vector alone or with hFcγRIIb1 or b2, along with a fifth of the amount of plasmid encoding GFP. The transfectants were incubated with IgG-coated, PKH 26 Red-labeled SRBC, and analyzed for phagocytic efficiency. The number of SRBC ingested by 100 GFP-positive cells was set as the Phagocytic Index. Data were obtained in duplicate from three independent experiments. The results shown in Figure 2.5A indicate that both b1 and b2 isoforms of hFcγRIIb are capable of significantly downregulating phagocytosis (p value < 0.002). We conclude that, despite the 19 amino acid difference in the juxta-membrane region, both hFcγRIIb1 and b2 are functional inhibitory receptors in macrophages during FcγR-mediated phagocytosis. In addition, the inhibitory capacity of the receptors is attributable to the ITIM, as previously reported, since an ITIM tyrosine mutant of hFcγRIIb failed to inhibit phagocytosis (Figure 2.5B, upper panel). The whole cell lysates from Raw 264.7 cells transfected with hFcγRIIb1 and its tyrosine mutant were analyzed by Western Blotting. As shown in Figure 2.5B, lower panel, both hFcγRIIb1 and its tyrosine mutant are equivalently expressed in the cells.

The role of FcγRIIb in immune-complex induced TNFα production

It is known that the phagocytosis of IgG-immune complexes is often associated with the release of inflammatory mediators such as cytokines and reactive oxygen species

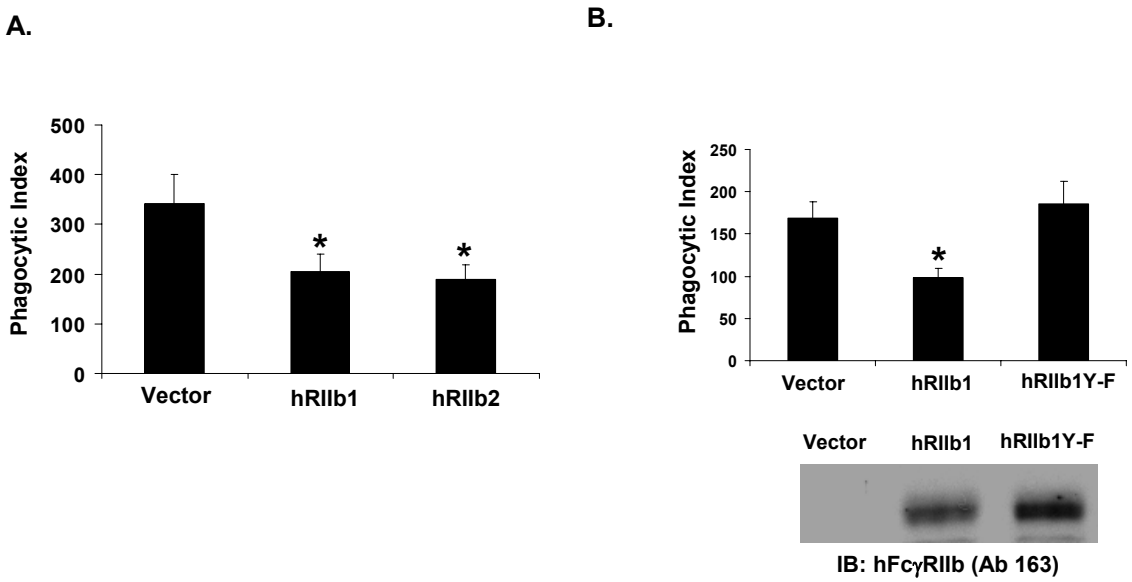


Figure 2.5: Both the isoforms of FcγRIIb downregulate FcγR-mediated phagocytosis. Raw 264.7 cells were transfected with vector alone or with plasmids encoding **A.** hFcγRIIb1 or b2 or **B.** hFcγRIIb1 or tyrosine mutant of hFcγRIIb1. Cells were harvested 24 hours post-transfection and were used in phagocytosis assays. Data shown represent duplicate values from three independent experiments.

(ROS) (reviewed in [129,130]). The immune-complex (IC) induced inflammatory responses are the major contributors to the pathogenesis of various autoimmune diseases and therefore need to be tightly regulated. FcγRIIb has been shown to regulate the extent of inflammatory response during IC induced alveolitis[71]. TNFα is a pro-inflammatory cytokine that has been found to be elevated in several autoimmune diseases and contributes to the pathophysiology of these diseases (reviewed in [131]). Although the role of FcγRIIb in down-modulating cellular responses such as antibody production, degranulation and phagocytosis is well studied; its role in the regulation of phagocytosis-associated events such as cytokines production is not well understood. A previous study in the mouse model of IC induced alveolitis indicated that levels of TNFα were elevated in bronchoalveolar lavage (BAL) fluid from FcγRII$^{-/-}$ mice[71].

To test whether the two isoforms of hFcγRIIb can modulate TNFα production by macrophages when stimulated with IC, Raw 264.7 cells were transfected with plasmids encoding either hFcγRIIb1 or hFcγRIIb2. Twenty hours post-transfection, cells were stimulated with heat-aggregated IgG (IC) for 2, 5, and 8 hours. Supernatants were harvested after every time point and the amount of TNFα was measured using ELISA. As shown in Figure 2.6A, over-expression of both the isoforms of hFcγRIIb in Raw 264.7 cells inhibited the amount of TNFα produced by macrophages. Interestingly, over-expression of tyrosine mutant of FcγRIIb1 restored the ability of Raw 264.7 cells to produce TNFα in response to IC stimulation indicating that the functionally intact ITIM motif is required for inhibition of TNFα (Figure 2.6B). The experiments were repeated more than three times and similar results were obtained in every experiment.

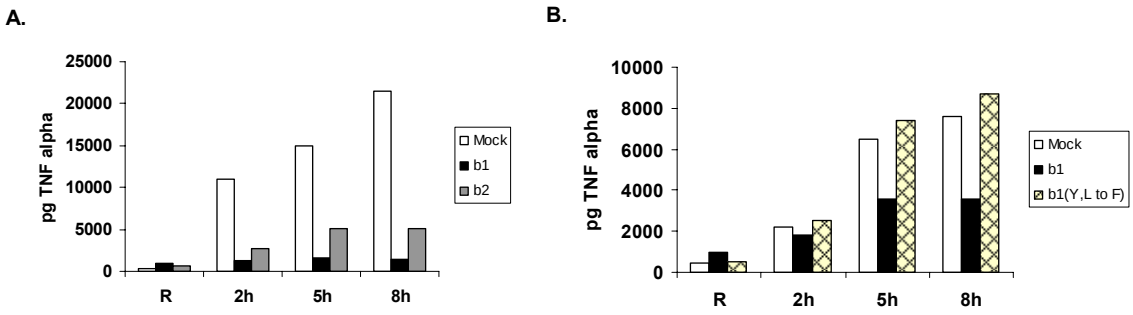


Figure 2.6: Both b1 and b2 isoforms of FcγRIIb downregulate FcγR-mediated TNFα production. Raw 264.7 cells were transfected with vector alone or with plasmids encoding **A.** hFcγRIIb1 or b2 **B.** hFcγRIIb1 or tyrosine mutant of hFcγRIIb1. Transfected cells were stimulated with 750 μg/ml immune-complex for 2, 5, and 8 hours. The amount of TNFα released in supernatants was measured by ELISA. The experiments were repeated four times and similar results were obtained every time.

We next compared the levels of TNFα produced by bone-marrow derived macrophages (BMMs) isolated from wild-type (WT) and FcγRII$^{-/-}$ mice. As shown in Figure 2.7A upper pannel, BMMs lacking FcγRIIb showed enhanced TNFα production upon stimulation with IC. This further confirms that FcγRIIb serves to regulate the production of pro-inflammatory cytokine TNFα. Protein matched cell lysates from WT and FcγRII$^{-/-}$ BMMs were resolved on SDS-PAGE and blotted with mouse FcγRIIb antibody to confirm the lack of expression of FcγRIIb (Figure 2.7A lower panel). Raw 264.7 whole cell lysate was used as a positive control.

MAP kinases, specifically p38 MAP kinase and Erk MAP kinase, are involved in the regulation of TNFα production (reviewed in [132]). We compared the activation of these two MAP kinases in WT and FcγRII$^{-/-}$ BMMs. For this, WT and FcγRII$^{-/-}$ BMMs were activated by clustering FcγR with anti-mouse FcγRIII/II 2.4G2 antibody followed by mouse F(ab')2 anti-rat antibody for indicated time points. Protein-matched whole cell lysates were separated by SDS-PAGE and probed with phospho-Erk (Figure 2.7B, upper panel) and phospho-p38 antibody (Figure 2.7B, middle panel). The same membrane was then reprobed with actin antibody to ensure equal loading of proteins (Figure 2.7B, lower panel). Results shown in Figure 2.7B indicate that Erk phosphorylation is significantly higher in FcγRII$^{-/-}$ BMMs as compared to WT BMMs. However, phosphorylation of p38 upon FcγR clustering was not different between WT and FcγRII$^{-/-}$ BMMs.

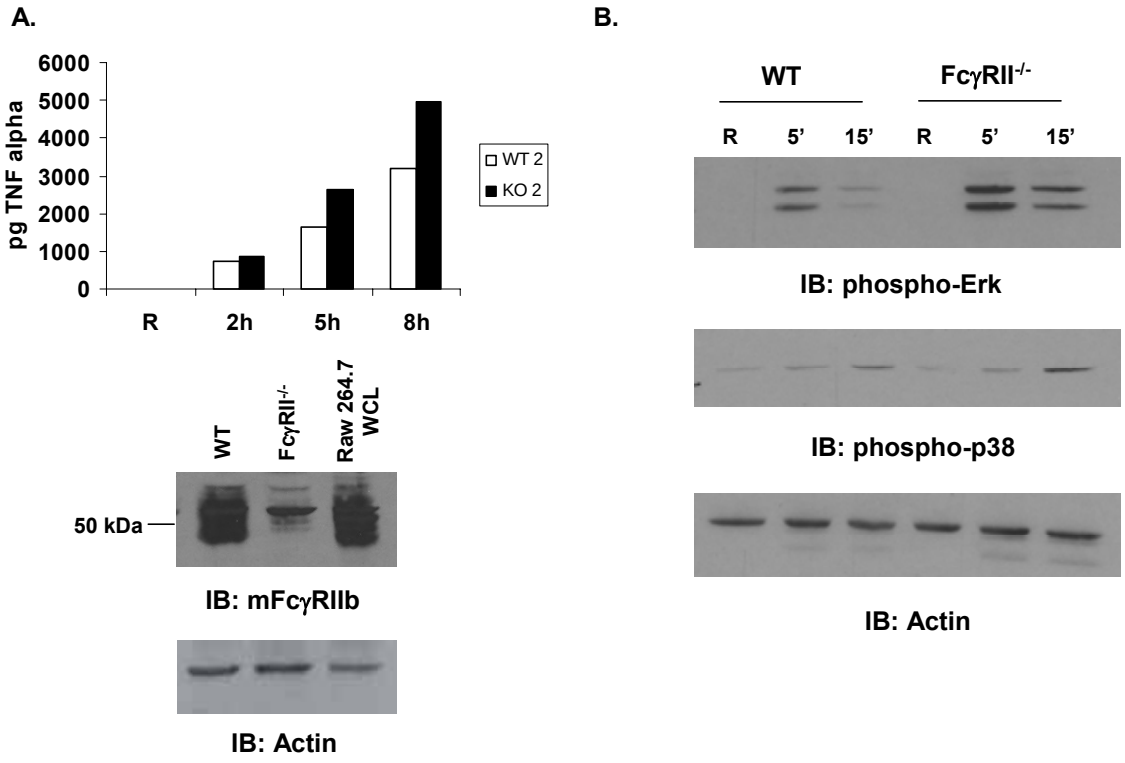


Figure 2.7: FcγRII$^{-/-}$ BMMs show enhanced TNFα production and Erk phosphorylation. A. BMMs isolated from WT and FcγRII$^{-/-}$ mice were stimulated with immune-complex for indicated time points. The amount of TNFα in the supernatants was measured by ELISA. Protein matched cell lysates from WT and FcγRII$^{-/-}$ BMMs were analyzed by Western Blotting with mouse FcγRIIb antibody. The same blot was then reprobed with actin antibody. **B.** WT and FcγRII$^{-/-}$ BMMs were activated by clustering FcγR for indicated time points. Protein matched cell lysates were resolved on SDS-PAGE and analyzed with phospho-Erk and phospho-p38 antibodies. The same membrane was then reprobed with actin antibody.

2.5 Discussion

FcγR-mediated phagocytosis involves the engagement of both activating and inhibiting FcγR. The ratio of activating to inhibiting receptors likely determines the magnitude of the phagocytic response. We, and others, have previously reported that the expression of FcγRIIb on phagocytes is highly variable depending on the cytokine milieu in which the cells are present. Recent studies also indicated that both b1 and b2 isoforms of FcγRIIb are expressed in monocytes, although the functional capacity or the regulation of these isoforms was not fully understood[75]. In this study we have demonstrated that both b1 and b2 isoforms are functional inhibitory receptors insofar as FcγR-mediated phagocytosis is concerned.

Three splice variants of hFcγRIIb, b1, b2 and b3, were first reported in the1980's. These all displayed comparable ligand binding capacity when expressed in fibroblasts[116]. Since then several studies have identified the expression of mRNA for the b1 and b2 isoforms in various hematopoeitic cells. Functional studies soon followed, primarily in B cells, analyzing the differences between b1 and b2 isoforms of both murine and human FcγRIIb. It is clear from these earlier studies that the b2 isoform of both murine and human FcγRIIb undergoes endocytosis upon ligand binding[17,124-126]. In contrast, the b1 splice variant is excluded from clathrin- coated pits and is not endocytosed[124,125]. Interestingly, upon receptor clustering, only b1 but not b2 was found to form caps[17]. Although, the exact reason for the above effect is not known, it is apparent that the cytoplasmic tail differences in the two isoforms of FcγRIIb translate to differences in the functional capacity of these receptors. More recently, it was shown that a chromosomal

translocation in malignant lymphoma results in overexpression of the b2 isoform of FcγRIIb in B cells, which normally express predominantly FcγRIIb1[133,134]. While the exact consequence of this deregulation is not known, this switch in isoform is thought to contribute to tumor progression.

The ITIM tyrosine of FcγRIIb is phosphorylated by src kinases to generate a docking site for the SH2 domain of SHIP-1[135,136]. These events are critical for the inhibitory function of FcγRIIb. Previous reports suggested that in B cells, hFcγRIIb1 but not hFcγRIIb2 is tyrosine phosphorylated[126]. In addition, specific src kinases were found to be able to phosphorylate the two isoforms[137]. Thus, while hFcγRIIb1 could be phosphorylated by Lyn and Fyn *in vitro*, only Blk was able to phosphorylate FcγRIIb2. Paradoxically, despite the lack of tyrosine phosphorylation, FcγRIIb2 was found to suppress calcium mobilization induced by B cell antigen receptor in a manner comparable to FcγRIIb1[126]. Thus there remain several inconsistencies in our current understanding of these two isoforms of FcγRIIb.

In contrast to the above findings, our data indicate that in macrophages, both FcγRIIb1 and b2 are tyrosine phosphorylated and induce enhanced SHIP-1 phosphorylation. Since SHIP-1 is a cytosolic enzyme that becomes tyrosine phosphorylated by membrane-associated src kinases only upon translocation to the membrane, phosphorylation of SHIP-1 is often used as a correlate for SHIP-1 activation. Our interpretation of the enhanced SHIP-1 phosphorylation in cells expressing hFcγRIIb1 and b2 is that these two receptors are equally capable of SHIP-1 recruitment via their phosphorylated ITIM domains. Consistent with this notion, expression of an ITIM

tyrosine mutant of FcγRIIb in Raw 264.7 cells failed to induce enhanced SHIP-1 phosphorylation (data not shown) or to downregulate phagocytosis. Thus both isoforms of FcγRIIb were equally capable of serving as inhibitory receptors in macrophages during the phagocytic process. Further studies examining the role of two isoforms of hFcγRIIb in the generation of pro-inflammatory cytokine TNFα revealed that both the isoforms of FcγRIIb downregulate IC induced TNFα production in macrophages. Although we have found no functional differences between the two isoforms in these studies, whether these two isoforms can differentially regulate other functional outcomes of FcγR clustering such as the generation of reactive oxygen remains to be tested. The 19 amino acid insert in b1 contains a potential tyrosine phosphorylation site that may be involved in either recruitment of unique signaling molecules or in stabilizing the association of proteins associated with FcγRIIb. For example, a non-ITIM tyrosine in the C-terminal end FcγRIIb cytoplasmic tail has been recently shown to be involved in associating with adapter molecules Grb2/Grap to stabilize the association of SHIPs with FcγRIIb[138]. A similar function may be served by the tyrosine residue present in the 19 amino acid insert, leading to functional differences between FcγRIIb1 and b2.

In conclusion, we have analyzed, for the first time, the function of the b1 and b2 isoforms of hFcγRIIb in macrophages. We report that, despite the difference of the 19 amino acid insert, the two isoforms are capable of equivalent surface expression and inhibition of FcγR-mediated phagocytosis and TNFα production.

CHAPTER 3

FcγR-INDUCED PRODUCTION OF SUPEROXIDE AND INFLAMMATORY CYTOKINES IS REGULATED BY SHIP-1 THROUGH ITS INFLUENCE ON PI 3-KINASE AND/OR RAS/ERK PATHWAY

3.1 Abstract

Phagocytosis of IgG-coated particles via FcγR is accompanied by the generation of superoxide and inflammatory cytokines, which can cause collateral tissue damage in the absence of regulation. Molecular mechanisms regulating these phagocytosis-associated events are not known. SHIP-1 is an inositol phosphatase that downregulates FcγR-mediated phagocytosis and PI 3-kinase - mediated activation events. Here, we have examined the role of SHIP-1 in FcγR-induced production of superoxide and inflammatory cytokines. Our results indicate that primary SHIP-1-deficient bone marrow macrophages produce elevated levels of superoxide upon FcγR clustering. Analysis of the molecular mechanism revealed that SHIP-1 regulates upstream Rac-GTP binding, an obligatory event for superoxide production. Likewise, SHIP-1-deficient macrophages

displayed enhanced IL-1β and IL-6 production in response to FcγR clustering. Interestingly, whereas IL-6 production required activation of both PI 3-kinase and Ras/Erk pathways, IL-1β production was dependent only on Ras/Erk activation, suggesting that SHIP-1 may also regulate the Ras/Erk pathway in macrophages. Consistent with this, Erk activation was significantly enhanced in SHIP-1-deficient macrophages upon FcγR clustering. Inhibition of Ras/Erk or PI 3-kinase suppressed the enhanced production of IL-6 in SHIP-1-deficient macrophages. In contrast, inhibition of Ras/Erk, but not PI 3-K, suppressed IL-1β production in these cells. Taken together, these findings demonstrate that SHIP-1 regulates phagocytosis-associated events through the inhibition of PI 3-kinase and Ras/Erk pathways.

3.2 Introduction

IgG-coated particles (immune-complexes) engage FcγR on the surface of macrophages and monocytes and initiate a series of signaling events that result in the phagocytosis/destruction of the immune complex (reviewed in[112,113]). This process is often accompanied by the generation of superoxide radicals and inflammatory cytokines, which are produced in an effort to clear the antibody-coated target. These phagocytosis-associated events can cause collateral tissue damage and therefore need to be tightly regulated.

Murine macrophages express both activating FcγR (FcγRI, FcγRIIIa and FcγRIV) and inhibiting FcγR (FcγRII) (reviewed in [11-13]). Clustering of the activating FcγR by immune complexes results in phosphorylation of the receptor ITAMs (immunoreceptor

tyrosine-based activation motif) by membrane-associated Src kinases[23,24]. The phosphorylated ITAMs serve as docking sites for SH2 domain containing cytoplasmic enzymes and enzyme/adapter complexes. This leads to the activation of two predominant pathways, namely Ras/Erk MAP kinase pathway and PI 3-kinase/Akt pathway. The Ras/Erk pathway is activated by recruitment of the Shc/Grb2/Sos complex to the membrane while docking of the p85 adapter subunit of PI 3-kinase to phosphorylated FcγR results in the generation of 3' phosphorylated inositol phospholipids such as PtdIns3,4,5P_3[76,79,86]. PtdIns3,4,5P_3 generated by the enzymatic activity of PI 3-kinase is an important second messenger which is necessary for the recruitment and activation of PH domain containing enzymes such as Vav, the guanine nucleotide exchange factor for Rac, Btk, the tec family tyrosine kinase involved in calcium mobilization, and the serine/threonine kinase Akt[139]. In addition to the activating enzymes recruited by the phosphorylated ITAM, negative regulatory phosphatases such as SHIP-1 are recruited by both the phosphorylated ITAM as well as the phosphorylated ITIM of FcγRIIb[77,81,82].

FcγR-mediated phagocytosis is a complex signaling cascade that involves several events, including rearrangement of cytoskeleton and production of superoxide (reviewed in [130,140]). Extensive studies in the last few years have shown that a small Rho family GTPase called Rac plays an important role in both of these events[56,141-144].

Superoxide production is catalyzed by NADPH oxidase complex which is a multi-subunit enzyme present in phagocytic cells. It is composed of two membrane bound subunits, gp91phox and p22phox and four cytosolic subunits, namely p40phox, p47phox, p67phox and small GTPase Rac (reviewed in [145]) (Figure 3.1). The complex of gp91phox and p22phox is called flavocytochrome b_{558}. This complex is inactive when

cells are in resting condition. The activation of cells with microorganisms or other stimuli such as antibody-opsonized particles leads to the assembly of cytosolic components of NADPH oxidase with flavocytochrome b_{558} making the complex functionally active. Thus Rac is essential for complete assembly of NADPH oxidase and its activation[56,141,144]. It has been reported that Rac-deficient neutrophils and macrophages show reduced superoxide production and phagocytic ability[146].

In addition to superoxide release, phagocytosis of immune-complexes involves production of pro-inflammatory cytokines such as IL-1β and IL-6[71,107,113]. Previous studies have suggested a role for the Ras/Erk pathway in the induction of cytokine gene expression in response to FcγR clustering[113]. However, precise signaling events involved in cytokine production associated with phagocytosis of IgG-coated particles are not yet known. Likewise, mechanisms that regulate these phagocytosis-associated events are not known.

SHIP-1 is an inositol 5' phosphatase expressed exclusively in hematopoietic cells, where it serves as a negative regulator of cell proliferation, activation and survival (reviewed in [147]). It is a multi-domain cytosolic protein that has an N-terminal SH2 domain, central catalytic domain, two NPXY motifs and a C-terminal proline-rich domain. The catalytic activity of SHIP-1 hydrolyzes PtdIns3,4,5P_3 to PtdIns3,4P_2 thereby downregulating PtdIns3,4,5P_3 dependent activation events described earlier. The other non-catalytic domains of SHIP-1 are involved in interactions with other proteins. For example, SH2 domain of SHIP-1 associates with phosphorylated ITAM or ITIM motifs of FcγR[77,79,82].

It has been shown that bone marrow-derived macrophages (BMMs) from

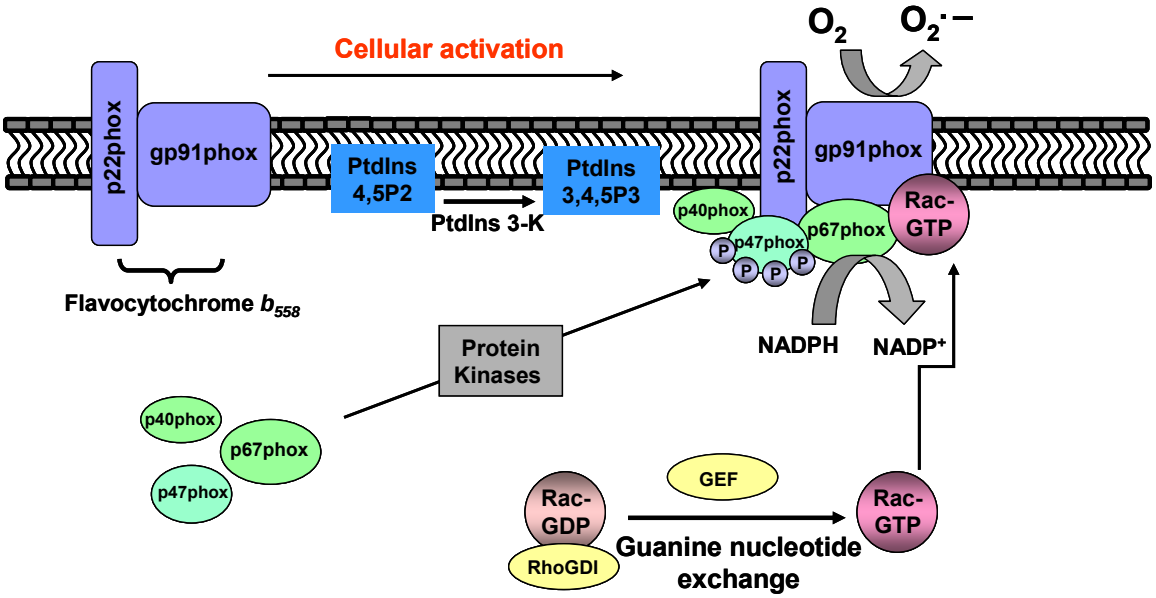


Figure 3.1: Assembly and activation of NADPH oxidase complex. NADPH oxidase is a multi-subunit enzyme. The two subunits, p22phox and gp91phox, together form a complex called flavocytochrome b_{558} which always resides in the membrane. Under resting condition, other subunits, p40phox, p47phox, p67phox and Rac live in the cytosol. Upon cellular activation, protein kinases such as Protein Kinase C (PKC) and Akt phosphorylate p47phox which now binds to membrane-residing p22phox subunit. Phosphorylated p47phox also binds to p40phox and p67phox thereby assembling these proteins at the membrane. In addition, p47phox and p40phox bind to the membrane lipids produced by PI 3-K and phospholipase D. Finally Rac is activated through exchange of GDP by GTP and translocates to the membrane where it binds to gp91phox and p67phox resulting in functional activation of NADPX oxidase complex (reviewed in reference 155).

SHIP-1 $^{-/-}$ mice and cells expressing dominant negative catalytic mutants of SHIP-1 exhibit enhanced phagocytic ability compared to SHIP-1$^{+/+}$ cells[81,97]. Interestingly, studies in B cells demonstrated that the non-catalytic, interaction domains of SHIP-1 are responsible for the regulation of the Ras/Erk pathway by virtue of their ability to interact with molecules such as p62dok and Shc[79,87,88,148]. However, there are no studies to date examining the influence of SHIP-1 on phagocytosis-associated events such as superoxide generation and inflammatory cytokine production.

In this study, we demonstrate that SHIP-1 down-regulates superoxide production when macrophages are stimulated with immune-complexes. An analysis of signaling events involved in reactive oxygen species (ROS) production revealed that SHIP-1 negatively regulates upstream Rac activity. Likewise, we also observed that SHIP-1 exerts negative influence on FcγR-induced IL-1β and IL-6 production. Interestingly, our results indicated a differential requirement for the activation of the Ras/Erk and PI 3-kinase pathways in the generation of IL-1β and IL-6 in response to FcγR clustering. Analysis of the mechanism of SHIP-1 regulation of FcγR-induced IL-1β and IL-6 production revealed that SHIP-1 influences the production of these cytokines through the regulation of the Ras/Erk and the PI 3-kinase pathways. Based on these findings, we propose that SHIP-1 is a key regulator of the FcγR-mediated inflammatory response.

3.3 Materials and Methods

Cells, antibodies and reagents: Raw 264.7 cells were obtained from ATCC (Manassas, VA) and maintained in RPMI supplemented with 5% fetal bovine serum. Rac antibody was purchased from Chemicon International. Ras and all phospho-specific antibodies

were from Cell Signaling Technology. Rabbit polyclonal SHIP-1 antibody was a generous gift from Dr. K. M. Coggeshall (Oklahoma Medical Research Foundation, Oklahoma City, OK). Actin and Akt antibodies were from Santa Cruz Biotechnology. Anti-mouse CD16/32 (FcγRIII/II) was purchased from BD Pharmingen (Franklin Lakes, NJ). Mouse anti-rat antibody was from Jackson ImmunoResearch (West Grove, PA). FITC-conjugated rat anti-mouse Mac-1 antibody was from BD Biosciences (Franklin Lakes, NJ).

Culture of murine bone marrow macrophages: Bone marrow macrophages (BMMs) were derived as previously described[25]. Briefly, bone marrow cells were cultured in RPMI containing 10% fetal bovine serum and supplemented with 10 ng/ml CSF-1 and 5 μg/ml polymixin B for 7 days. The cells obtained in this manner were greater than 99% positive for Mac-1 as determined by flow cytometry.

Checking purity of BMMs by flow cytometry: BMMs were harvested after 7 days of culture and were tested for macrophage-specific marker Mac-1 to check the purity of the population. For this, BMMs were incubated with FITC-conjugated Mac-1 antibody at a concentration of 10 μg/ml for 30 minutes at 4°C. The cells were washed and fixed in 1% paraformaldehyde and analyzed by flow cytometry on BD FACS Calibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ). Data from 10,000 cells per condition were recorded to yield the percentage of cells expressing Mac-1.

Generation of stable cell lines expressing SHIP-1: PINCO–wild-type (PINCO-wt) SHIP-1 retroviral vector was kindly provided by Dr Martin Sattler (Dana-Farber Cancer Institute, Harvard University, Boston, MA)[149-151]. PINCO-catalytic-deficient D675A SHIP-1 was generated as previously described[151]. Retroviral infections of Raw 264.7 cells were performed following previously published standards. Briefly, infectious supernatants from PINCO (vector only), PINCO-wt SHIP-1, or PINCO-catalytic-deficient D675A SHIP-1-transfected Phoenix cells were collected 48 hours after transfection and used for 3 cycles of infection. Upon infection, Raw 264.7 cells were sorted (FACS Vantage; BD Biosciences, San Jose, CA) for green fluorescent protein (GFP) expression and stable lines were raised.

Preparation of heat-aggregated IgG: Heat aggregated IgG was prepared according to methods described previously[127]. In brief, chromopure mouse IgG at a concentration of 350 μg /ml was heated at 62°C for 30 minutes, then cooled on ice immediately and used to stimulate cells.

Measurement of superoxide production in BMMs: Superoxide produced in cells was measured using the cell-permeable dye dihydroethidium (DHE, Sigma), which binds to nuclear DNA when oxidized by superoxide and emits red fluorescence[152]. Briefly, BMMs were seeded in 6 well plates at a concentration of 1.5 million cells per well. The next day cells were washed once with PBS followed by addition of RPMI medium without phenol red. The cells were stimulated with heat-aggregated IgG for two hours, in the dark at 37°C, in the presence of 10 μM of fluorescent probe dihydroethidium (DHE, Sigma).

After two hours the cells were washed with ice-cold PBS, scraped and lysed by brief sonication. Lysates and supernatants were loaded in 96 well plates and the fluorescence was measured in a microplate fluorimeter (SpectraMax Gemini, Molecular Devices) using excitation/emission filters 520/590 nm.

Rac and Ras activity assay: Glutathione agarose beads coated either with GST-PAK1-PBD (p21 binding domain) for Rac assay or with GST-Raf1-RBD (Ras binding domain) for Ras assay were prepared as described[153,154]. The p21 binding domain (PBD) of p21-Activated Kinase 1 (PAK1) and Ras binding domain of RAf1 bind and pull down specifically the GTP-bound (active form) and not the GDP-bound form of Rac (See Figure 3.2 for schematic representation of the assay) and Ras respectively. Here, BMMs, or Raw 264.7 transfectants were activated by clustering FcγR with anti-mouse FcγRIII/II 2.4G2 antibody followed by mouse-anti rat antibody for indicated time points. Cells were lysed in TN1 buffer (50 mM Tris pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 10 mM Na_3VO_4, 10 μg/ml each aprotinin and leupeptin). Protein-matched cell lysates were incubated with GST-PAK1-PBD beads or GST-Raf1-RBD beads for 1 hour at 4°C. After 1 hour, beads were washed with TN1 and then boiled in 1X SDS sample buffer (60 mM Tris pH 6.8. 2.3% SDS, 10% glycerol, 0.01% bromophenol blue, and 1% 2-ME) for 10 minutes. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, probed with Rac or Ras antibody and developed by enhanced chemiluminescence.

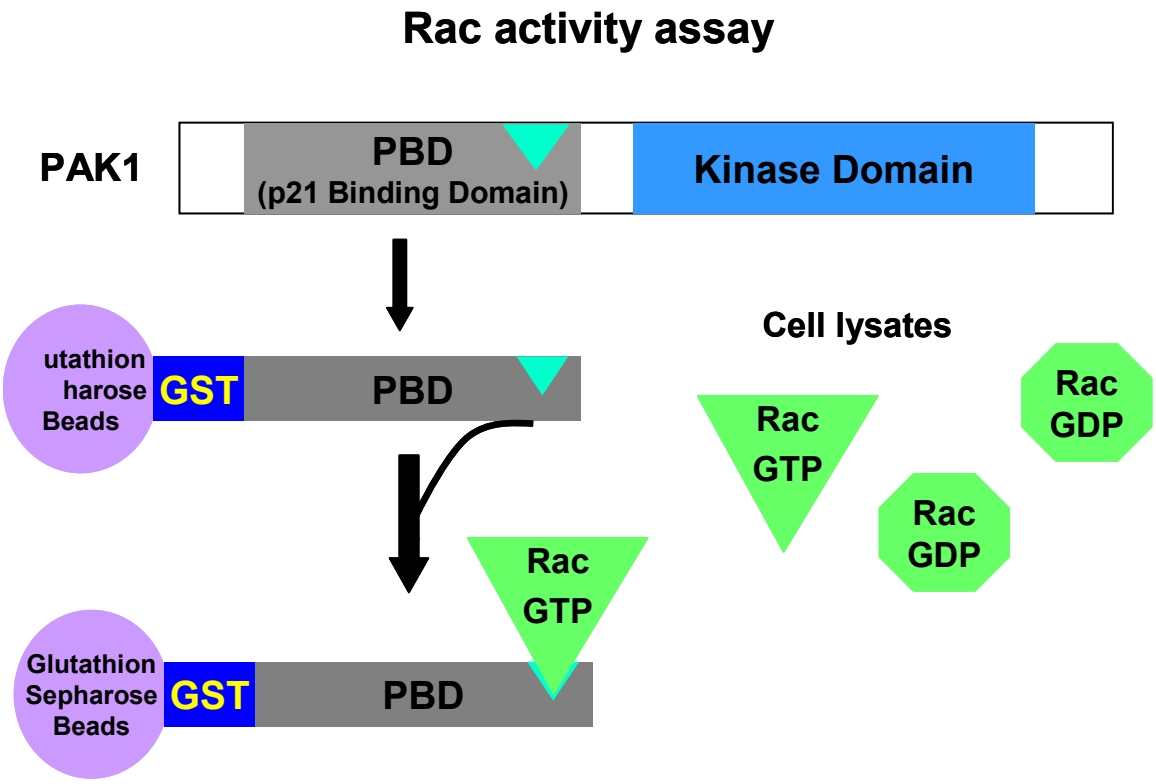


Figure 3.2: Schematic of Rac activity assay. GTP-bound form of Rac is active and binds to p21-binding domain (PBD) of p21-activated kinase 1 (PAK1) whereas Rac-GDP is inactive and can not bind PBD. Thus, the fusion protein GST-PAK1-PBD bound to glutathione sepharose beads is used to specifically pull down Rac-GTP and not Rac-GDP.

Western blot data quantitation: The ECL signal was quantitated using a scanner and a densitometry program (Scion Image). In experiments measuring phosphorylation, we first subtracted background, normalized the signal to the amount of actin or total target protein in the lysate, and plotted the values as fold increase over unstimulated samples.

Measurement of cytokines by ELISA: Cells were cultured for varying time points ranging from 2 hours to 8 hours, in the presence or absence of heat-aggregated IgG. Cell lysates and supernatants were harvested, centrifuged to remove dead cells and analyzed by ELISA using cytokine specific kits from R & D Systems (Minneapolis, MN). Data were analyzed using a paired t-test, and a *p* value less than 0.05 was considered significant.

3.4 Results

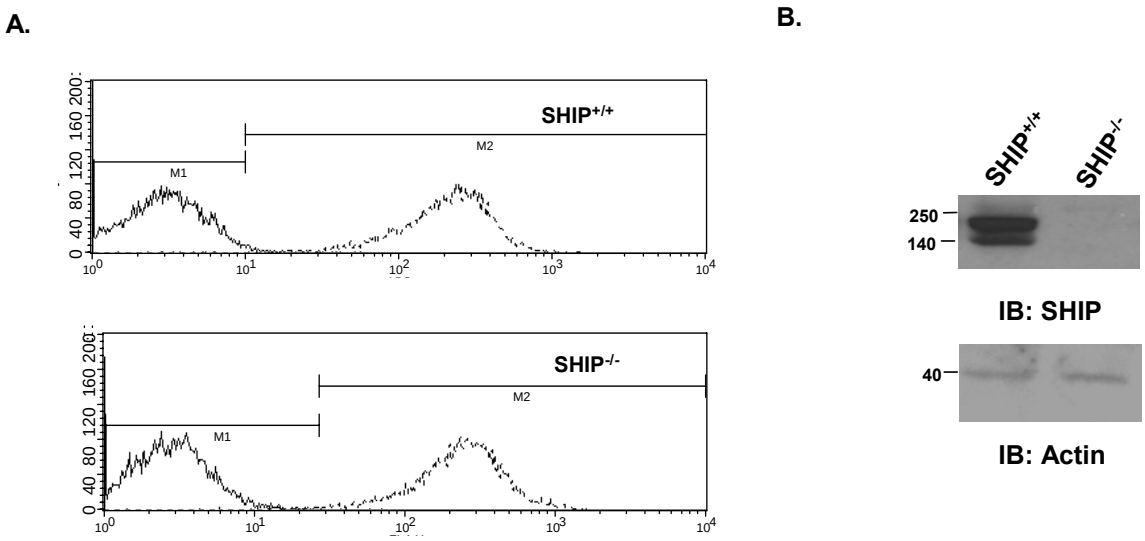
Culture of bone-marrow cells for 7 days in presence of MCSF yields pure population of macrophages

In this study we have extensively used bone-marrow derived macrophages (BMMs) isolated from SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ mice. To obtain BMMs, bone-marrow cells were cultured in RPMI containing 10% fetal bovine serum, supplemented with 10 ng/ml CSF-1 and 5 μg/ml polymixin B for 7 days. We then checked the percentage of cells expressing macrophage-specific marker Mac-1. For this, BMMs were harvested at the end of day 7, labeled with FITC-conjugated Mac-1 and cells were analyzed by flow cytometry. As shown in Figure 3.3A upper and lower panel, more than 99% of both SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ BMMs were positive for Mac-1 indicating that all cells used were indeed macrophages.

To check the expression of SHIP-1 protein, whole cells lysates from SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ cells were analyzed by Western blotting. Figure 3.3B upper panel demonstrates the presence and absence of SHIP-1 in SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ BMM respectively. The same membrane was then reprobed with actin to ensure equal loading of protein (Figure 3.3B, lower panel).

Immune complex-induced production of superoxide is regulated by SHIP-1

FcγR clustering activates the assembly of the NADPH oxidase complex and the generation of superoxide[130]. However, mechanisms of regulation of FcγR-induced superoxide generation are not known. To examine whether SHIP-1 regulates FcγR-induced superoxide production, BMMs from SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ animals were stimulated with heat-aggregated IgG for varying time points ranging from 0 to 120 minutes in the presence of dihydroethidium (DHE). As shown in Figure 3.4A, FcγR - mediated superoxide production was detectable only after one hour of stimulation in wild-type cells. Interestingly, superoxide production in SHIP-$1^{-/-}$ cells was enhanced over the SHIP-$1^{+/+}$ cells at all time points tested post stimulation. For further analysis, the 120 minute time point was chosen, and four independent experiments were performed. Results confirmed that SHIP-1-deficient BMMs make significantly higher amounts of superoxide in response to immune-complex stimulation (Figure 3.4B).



<u>Figure 3.3:</u> Culture of bone-marrow cells for 7 days in presence of MCSF yields pure population of macrophages. A. Mac-1 expression on the SHIP-1$^{+/+}$ and SHIP-1$^{-/-}$ BMMs was analyzed by flow cytometry. For this, the cells were labeled with FITC-labeled Mac-1 antibody in the presence of the anti-FcγRII/III mAb 2.4G2 (to block Fcγ receptors) (dashed line). As a negative control, cells were stained with FITC-labeled isotype control antibody (solid line). **B.** Protein-matched whole cell lysates were analyzed by western blotting with SHIP-1 antibody (upper panel). The same membrane was reprobed with actin antibody (lower panel).

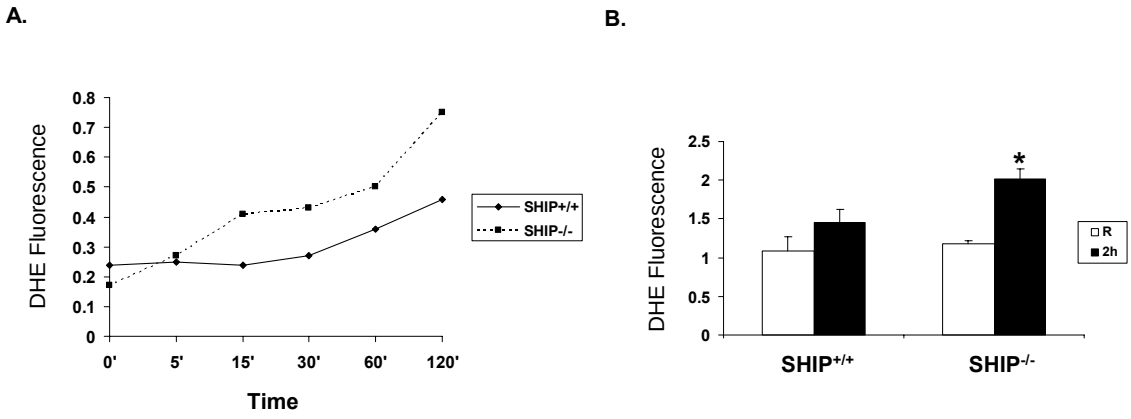


Figure 3.4: SHIP-1 downregulates FcγR-induced superoxide production. A. BMMs derived from SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ mice were activated with heat-aggregated IgG for the time points indicated in the Figure. Generation of superoxide was measured using 10 μM fluorescent probe DHE (dihydroethidium). DHE fluorescence intensity is plotted in the graph. **B.** BMMs derived from SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ mice were activated with heat-aggregated IgG for 2 hours. Generation of superoxide was measured using fluorescent probe DHE (dihydroethidium). Data represent mean and SEM of 4 independent experiments. Data were analyzed by student's t-test. ∗ p value < 0.05.

SHIP-1 regulates activation of upstream Rac

Activation of the low-molecular weight GTP-binding protein Rac is essential for the assembly of NADPH oxidase complex and superoxide generation[56,144]. The activation of Rac involves the exchange of GDP by GTP which is facilitated by the guanine nucleotide exchange factor Vav[48]. Vav activation in turn depends upon the products of PI 3-kinase[91,155]. Since the catalytic activity of SHIP-1 results in the hydrolysis of PI 3-kinase products and the subsequent downregulation of downstream enzymes that are dependent on PI 3-kinase products, we next asked whether SHIP-1 influences Rac activation. To test this, SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ BMMs were stimulated by clustering FcγR and protein-matched lysates were incubated with GST-Pak1 PBD as bait protein to capture GTP-bound Rac. The bound proteins were separated by SDS/PAGE and probed with anti-Rac antibody. Results indicated that FcγR clustering induces Rac GTP-binding and that SHIP-$1^{-/-}$ macrophages show enhanced Rac activation (Figure 3.5). The band intensity from three different experiments was quantitated and was expressed as fold increase over resting (Figure 3.5, lower panel). The graph indicates that Rac activity is significantly higher in SHIP-$1^{-/-}$ BMMs compared to SHIP-$1^{+/+}$ BMMs.

As a second approach to verify that SHIP-1 regulates activity of Rac, stable cell lines over-expressing wild-type SHIP-1, catalytic-deficient D675A SHIP-1 or vector alone were generated by retroviral infection of Raw 264.7 murine macrophage cells. These stable transfectants were stimulated by clustering FcγR, and assayed for Rac GTP-binding. The results shown in Figure 3.6A demonstrate that over-expression of wild-type SHIP-1 downregulates Rac activation by FcγR clustering. The graph shown in Figure

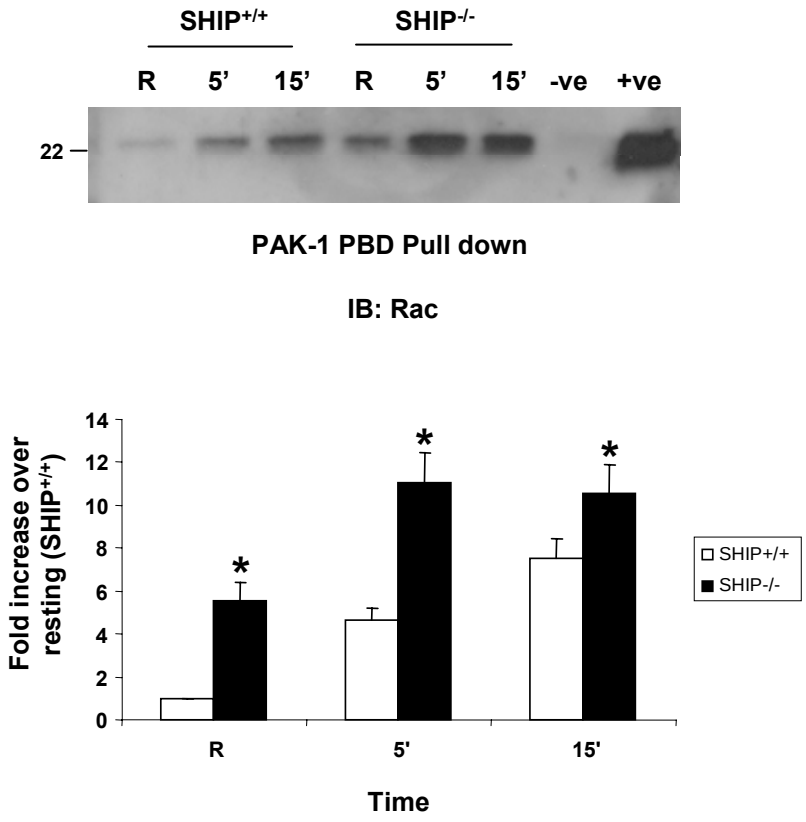


Figure 3.5: FcγR-induced Rac activation is enhanced in SHIP-1-deficient macrophages. SHIP-1$^{+/+}$ and SHIP-1$^{-/-}$ macrophages were activated by clustering Fcγ receptors for the indicated time points. GTP bound Rac was captured with PAK-1 PBD beads as bait from protein-matched cell lysates and visualized by Western blotting with Rac antibody (upper panel). Unhydrolizable GTP and GDP analogs (obtained from Chemicon International), were used as +ve and -ve controls respectively. The lower panel represents fold induction of GTP Rac in the activated samples over resting. The graph represents mean and SEM of three independent experiments. Data were analyzed by student's t-test (* = p value $\leq$ 0.05).

3.6A lower panel represents results obtained from three independent experiments. To ensure that the SHIP-1 constructs were indeed over-expressed in the transfectants, protein-matched lysates from transfected cells were subjected to immunoprecipitation with SHIP-1 antibody and analyzed by Western blotting with SHIP-1 antibody (Figure 3.6B). Taken together, these results demonstrate that SHIP-1 negatively regulates Rac activity.

Immune complex-induced production of inflammatory cytokines is regulated by SHIP

The phagocytic process is also accompanied by the production of inflammatory cytokines such as IL-1β and IL-6[71,107,113]. While extensive analyses have been performed to understand mechanisms involved in the process of particle engulfment, little is known about signaling pathways involved in cytokine generation and its regulation in macrophages. We first examined whether FcγR-induced cytokine production is influenced by SHIP-1. In these experiments, SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ BMMs were stimulated with heat-aggregated IgG for the time points indicated in Figure 3.7. Cell lysates and supernatants were collected and assayed for the presence of IL-1β and IL-6 by ELISA (Figure 3.7A and B). Results from three independent experiments revealed that SHIP-$1^{-/-}$ BMMs produce significantly higher levels of IL-1β and IL-6 compared to SHIP-$1^{+/+}$ BMMs upon FcγR activation. These results indicate that SHIP-1 is a negative regulator of FcγR-induced IL-1β and IL-6 production. Of note, macrophages release minimal amounts of IL-1β into the supernatant (Figure 3.7A, lower panel), since this

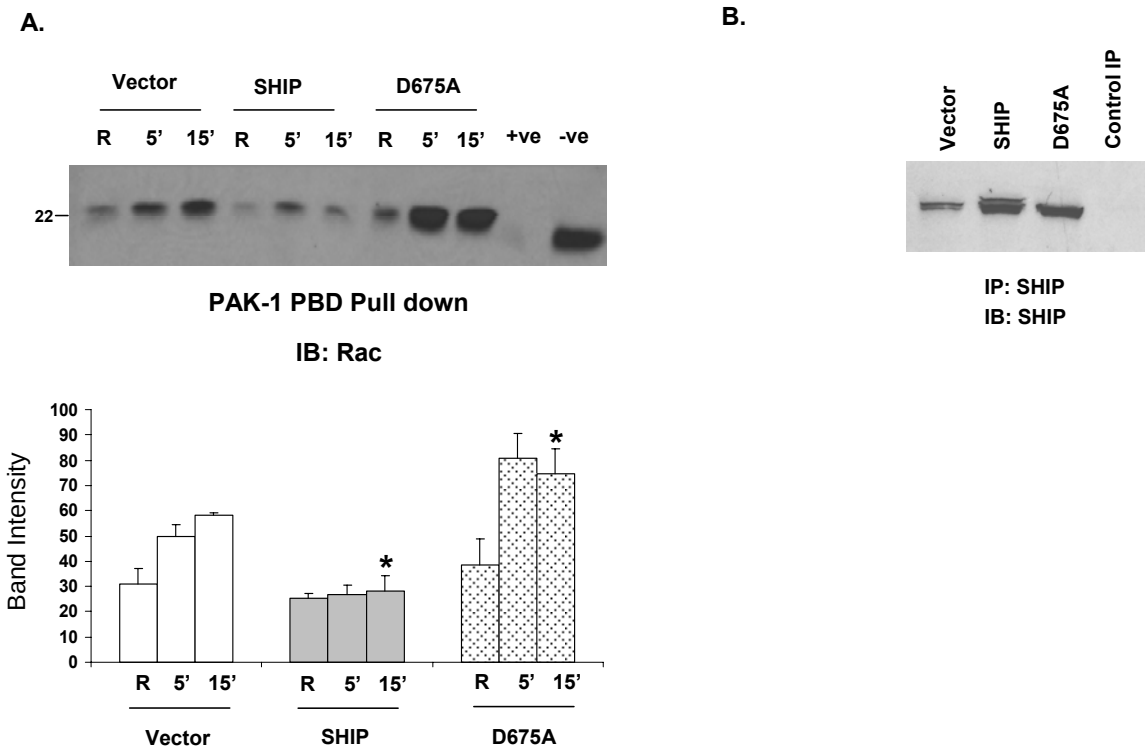
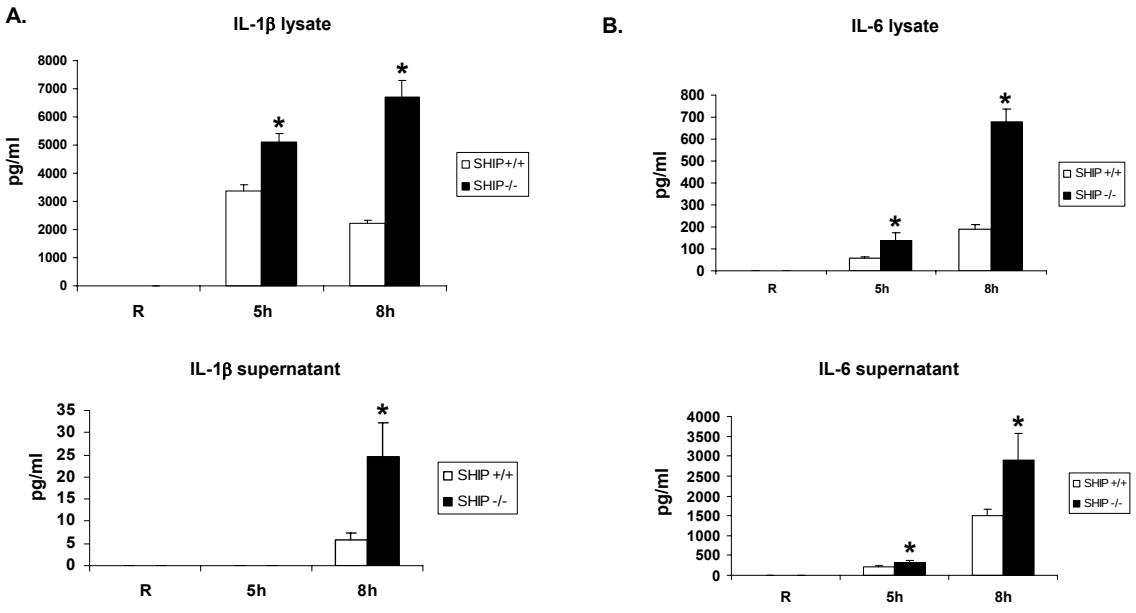


Figure 3.6: Overexpression of wild-type SHIP-1 downregulates FcγR-induced Rac activation. A. Raw 264.7 cells were retrovirally infected using vector alone, with wild-type SHIP-1 or with catalytic-deficient D675A SHIP-1**.** GTP bound Rac was measured in these transfectants after FcγR clustering. The lower panel represents the band intensity of the Rac-GTP. Values obtained from three independent experiments are represented as mean and SEM. **B**. The stable transfectants were analyzed for SHIP-1 expression by immunoprecipitating SHIP-1 with rabbit polyclonal SHIP-1 antibody and immunoblotting with the same SHIP-1 antibody. The last lane is a control immunoprecipitate with normal rabbit serum. Data were analyzed by student's t-test (* = p value $\leq$ 0.05).

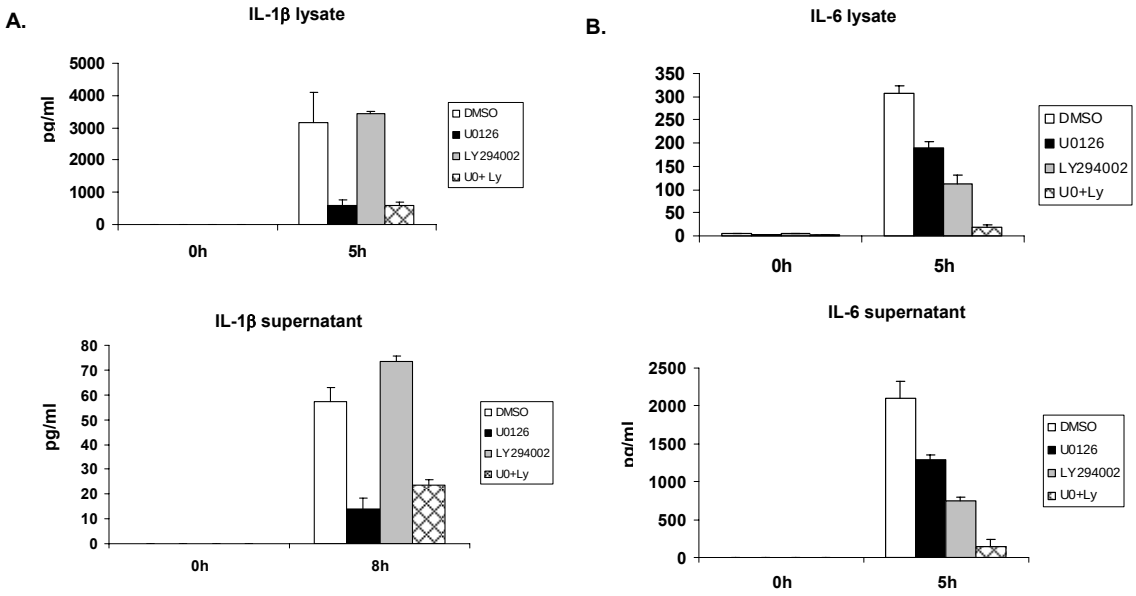


<u>Figure 3.7:</u> SHIP-1 downregulates FcγR-induced production of IL-1β and IL-6. BMMs obtained from SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ mice were stimulated for the time points indicated in the Figure with heat-aggregated IgG. **A.** The levels of IL-1β and **B.** IL-6 in lysates and supernatants were measured by ELISA. The graphs represent the mean and SEM of values obtained from three independent experiments. Data were analyzed by student's t-test (* = p value $\leq$ 0.05).

cytokine, unlike IL-6, requires post-translational modification, which is reported to be dramatically impaired in macrophages compared to monocytes[156].

Differential requirement for the PI 3-kinase and Ras/Erk MAP kinase pathways in FcγR-induced IL-1β production and IL-6 production

In order to examine the mechanism by which SHIP-1 influences cytokine production in macrophages, we first analyzed the signaling pathways involved in FcγR-induced cytokine production. It is known that the enzymatic activity of SHIP-1 hydrolyzes PI 3-kinase products and downregulates downstream signaling. In addition to its catalytic activity, SHIP-1 has been previously demonstrated to downregulate the Ras/Erk pathway in B cells by associating with the Ras GAP-binding protein p62dok and accelerating hydrolysis of Ras GTP, and by competing with the Grb2-Shc adapter complex necessary for Ras activation. We therefore examined the role of the Ras/Erk and PI 3-kinase pathways on FcγR-induced cytokine production using pharmacological inhibitors. In these experiments BMMs were pre-incubated with either vehicle control or with inhibitors of the Ras/Erk pathway (U0126) or PI 3-K pathway (LY294002) and subsequently stimulated with heat-aggregated IgG. Production of IL-1β and IL-6 was measured by ELISA. As shown in Figure 3.8A, IL-1β production was completely inhibited upon inhibition of the Ras/Erk pathway whereas blocking of PI 3-kinase pathway did not suppress the production of IL-1β. In contrast, inhibition of either the Ras/Erk pathway or the PI 3-kinase pathway resulted in partial downregulation of IL-6 production (Figure 3.8B). Simultaneous inhibition of both the PI 3-kinase and the



<u>Figure 3.8:</u> Differential requirement for the PI 3-K and Ras/Erk pathways in FcγR-induced IL-1β and IL-6 production. BMMs were treated with DMSO or 10 μM LY294002 or 2.5 μM UO126 for 30 minutes at 37°C prior to stimulation with heat-aggregated IgG. **A.** The levels of IL-1β and **B**. IL-6 in cell lysates and supernatants were measured by ELISA. The graphs represent the mean and SEM of three independent experiments. Data were analyzed by student's t-test. ∗ p value < 0.05.

Ras/Erk pathways resulted in further attenuation of IL-6 production. These results indicate that IL-1β production is predominantly mediated by Ras/Erk pathway while both Ras/Erk pathway and the PI 3-kinase pathway contribute to IL-6 production. To ensure the efficacy/specificity of the inhibitors, protein-matched lysates from resting and stimulated cells were probed with antibodies to phospho-Erk and phospho-Akt (upper panels, Figure 3.9A and B respectively). These results show that U0126 specifically inhibits Erk phosphorylation and not Akt phosphorylation while Ly294002 blocks phosphorylation of Akt but not of Erk. The lower panels in Figure 3.9 are reprobes with Erk and Akt antibody, respectively, to ensure equal loading of protein in all lanes.

Collectively, these results demonstrate several novel points: a) that there is no cross talk between the Ras/Erk pathway and the PI 3-kinase pathway during FcγR signaling in BMMs; b) that the two signaling pathways play differential roles in FcγR-induced cytokine production; c) that SHIP-1 may influence the Ras/Erk pathway as well as the PI 3-kinase pathway during FcγR signaling in BMM, independently of each other.

FcγR-induced activation of the Ras/Erk pathway is downregulated by SHIP-1

The above experiments suggest that SHIP-1 influences FcγR-induced activation of the Ras/Erk pathway in macrophages. To directly test this, SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ BMMs were stimulated by clustering FcγR, and assessed for the activity of the Ras signaling pathway. First, Ras activation assays were performed by capturing GTP-bound Ras with GST-Raf1-RBD as bait. The bound proteins were separated by SDS/PAGE and analyzed by Western blotting with Ras antibody. As shown in Figure 3.10A, Ras

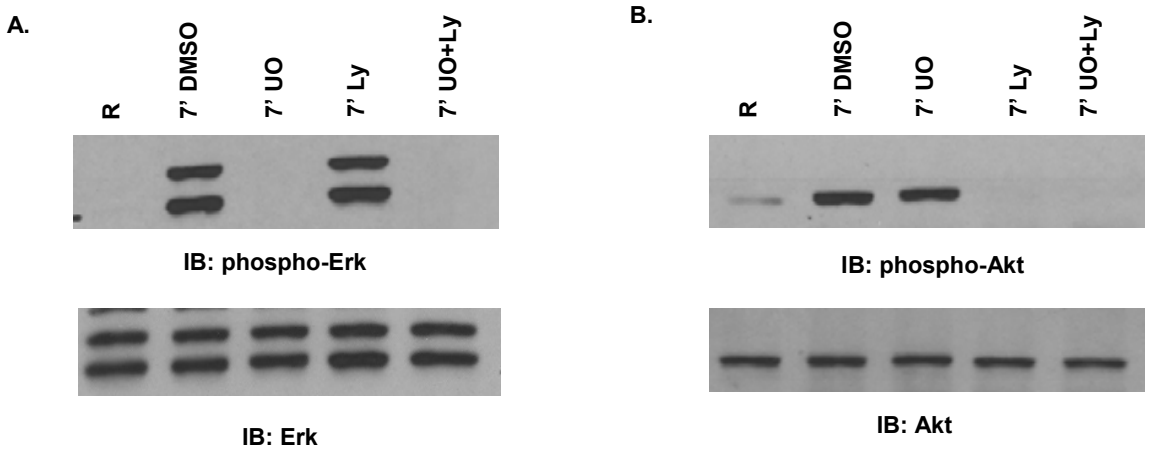


Figure 3.9: Inhibition of Erk and PI 3-K pathways by pharmacological inhibitors. **A.** Protein-matched lysates from unstimulated and stimulated cells (stimulated for 7 minutes) were analyzed by Western blotting with phospho-Erk antibody. The lower panel is a reprobe of the same membrane with Erk antibody. **B.** Parallel samples were probed with phospho-Akt antibody, and the membrane was reprobed with Akt antibody (lower panel).

activation was enhanced in SHIP-1$^{-/-}$ BMMs compared to SHIP-1$^{+/+}$ BMMs. The graph shown in Figure 3.10A, lower panel represents mean and SEM of Ras-GTP band intensity from three independent experiments. As an additional approach, similar assays were performed in stable transfectants over-expressing wild-type SHIP-1 (Figure 3.10B). The cells over-expressing wild-type SHIP-1 showed reduced Ras activation in comparison to vector-transfected cells.

Next, we assessed activation of Raf1, MAP Kinase kinase kinase (MKKK) for Ras/Erk pathway which is activated downstream of Ras, in SHIP-1$^{+/+}$ and SHIP-1$^{-/-}$ BMMs by Western blotting protein-matched cell lysates with phospho-specific antibodies to Raf1. Results indicated that Raf1 phosphorylation was enhanced in SHIP-1$^{-/-}$ BMMs (Figure 3.11A). The graphs shown in Figure 3.11 are quantitation of Raf1 phosphorylation from three independent experiments. Likewise, cells over-expressing SHIP-1 displayed attenuated Raf1 phosphorylation in response to FcγR clustering (Figure 3.11B).

We also examined FcγR-induced Erk phosphorylation in SHIP-1$^{+/+}$ and SHIP-1$^{-/-}$ BMMs. As shown in Figure 3.12A, Erk phosphorylation is significantly enhanced in SHIP-1$^{-/-}$ BMMs. Lastly we examined Erk phosphorylation in the Raw 264.7 transfectants expressing vector alone, wild-type SHIP-1 or catalytic-deficient SHIP-1 (Figure3.12B). Results from three independent experiments were quantitated in the graph shown in Figure 3.12B and demonstrate that over-expression of either a wild-type SHIP-1 or a catalytic-deficient SHIP-1 inhibits the induction in phosphorylation of Erk following FcγR clustering. These results demonstrate that SHIP-1 downregulates

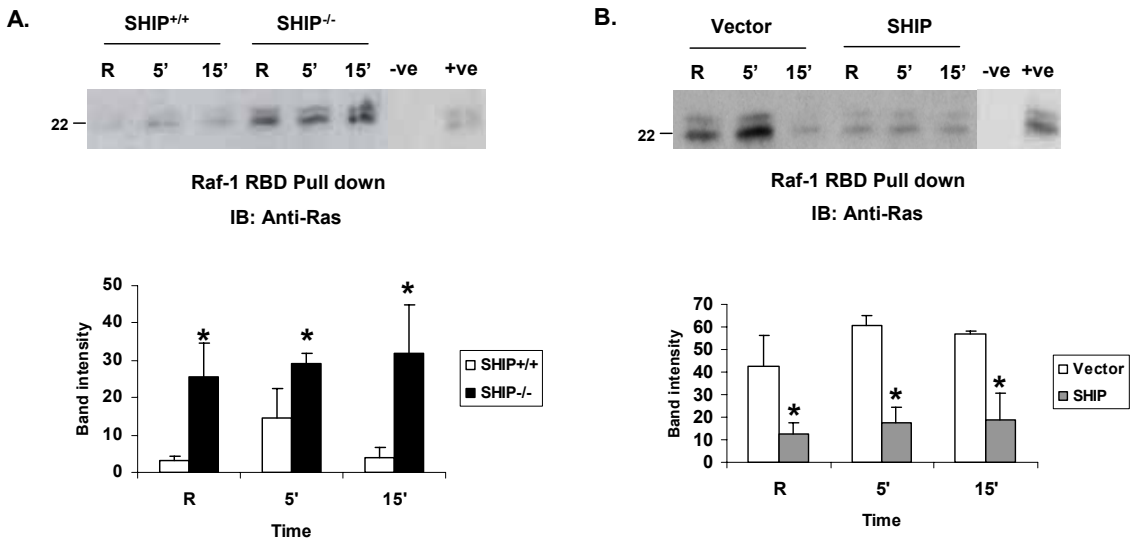
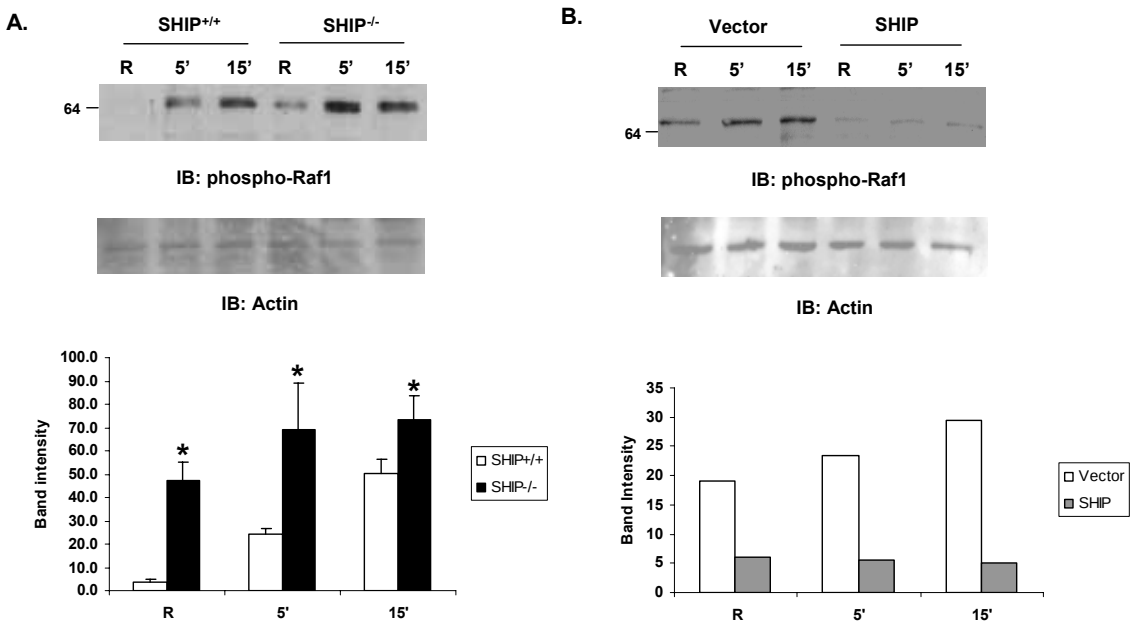


Figure 3.10: Influence of SHIP-1 on FcγR-mediated Ras activation. A. SHIP-1$^{+/+}$ and SHIP-1$^{-/-}$ macrophages were activated by FcγR clustering for the indicated time points. GTP bound Ras was captured with Raf1-RBD beads as bait from protein-matched lysates and visualized by Western blotting with Ras antibody (upper panel). The graph in lower panel is a composite of three independent experiments representing mean and SEM of Ras-GTP band intensity. **B.** Ras activation was likewise examined in stable transfectants overexpressing vector alone or wild-type SHIP-1 (upper panel). The lower panel represents the band intensity of Ras-GTP band. Values obtained from three independent experiments are represented as mean and SEM. Data were analyzed by student's t-test (* = p value $\leq$ 0.05).



<u>Figure 3.11:</u> SHIP-1 downregulates FcγR-induced phosphorylation of Raf1. A. SHIP-1$^{+/+}$ and SHIP-1$^{-/-}$ macrophages were activated by clustering of FcγR. Protein-matched cell lysates were probed with phospho-Raf1 antibody (upper panel) followed by actin antibody (middle panel). The lower panel represents quantitative analysis of phospho-Raf1 band intensity obtained from three independent experiments. Data were analyzed by student's t-test (* = p value $\leq$ 0.05). **B.** Similar analyses were performed in stable transfectants over-expressing vector alone or wild-type SHIP-1. The graph shown in the lower panel represents mean of phospho-Raf1 band intensity.

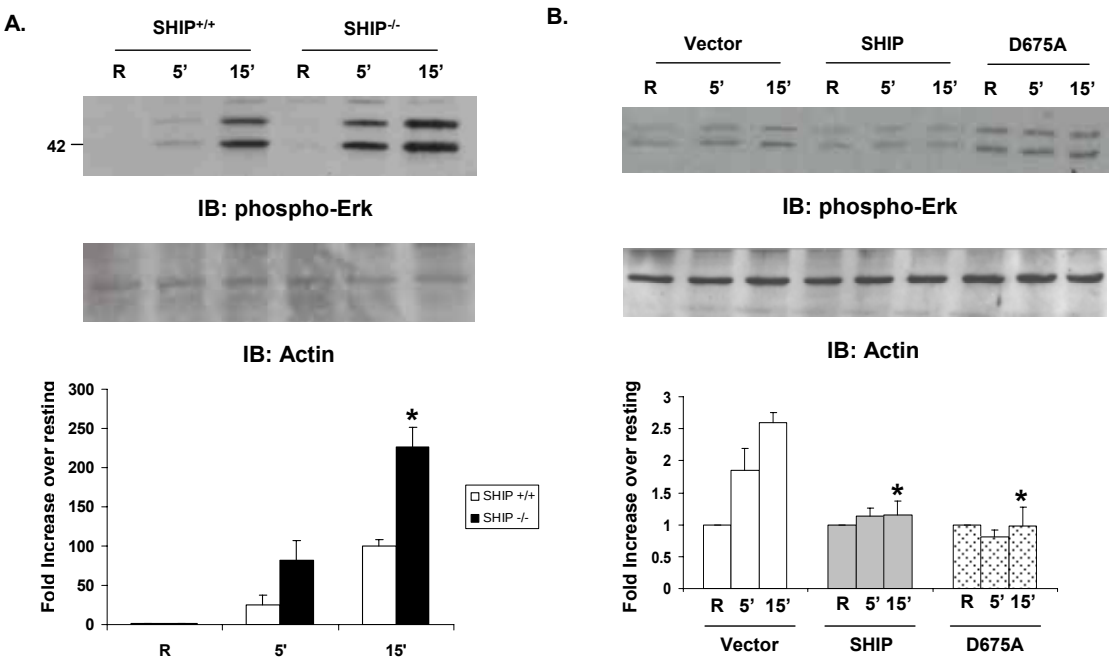


Figure 3.12: SHIP-1 downregulates FcγR-induced activation of Erk. A. SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ macrophages were activated by clustering Fcγ receptors for the indicated time points. Protein-matched lysates were probed with phospho-ERK and reprobed with antibody against actin (lower panel). The graph shown below is a quantitative estimate of Erk phosphorylation and represents the mean and SEM of values obtained from three independent experiments. Data were analyzed by student's t-test. ∗ = p value < 0.05. **B.** Erk phosphorylation was likewise measured in stable transfectants over-expressing vector alone, wild-type SHIP-1, or catalytic-deficient D675A SHIP-1.

activation of the Ras/Erk pathway by FcγR clustering, in a manner that is independent of SHIP-1's catalytic function.

SHIP-1 downregulates FcγR-induced inflammatory cytokine production through the inhibition of the Ras/Erk and PI 3-kinase pathways

The above experiments demonstrate that SHIP-1 regulates FcγR-induced production of IL-1β and IL-6, and also that SHIP-1 regulates the PI 3-kinase and the Ras/Erk pathways. We next wanted to determine whether the influence of SHIP-1 on cytokine production is due to its influence on the PI 3-kinase and the Ras/Erk pathways. For these experiments, SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ BMMs were pre-incubated with either vehicle alone (DMSO), the MEK inhibitor U0126, or the PI 3-kinase inhibitor LY294002. Cells were subsequently stimulated with heat-aggregated IgG for 5 hours. Cell lysates and supernatants were assayed for IL-1β and IL-6 by ELISA. To measure IL-1β in the supernatants, BMMs were stimulated for 8 hours with immune-complex. As seen in Figure 3.13A, IL-1β production was enhanced in SHIP-$1^{-/-}$ BMMs in comparison to SHIP-$1^{+/+}$ BMMs. Treatment with the MEK inhibitor significantly downregulated IL-1β production, even in cells deficient in SHIP-1. This suggests that SHIP-1 influences IL-1β production through its influence on the Ras/Erk pathway. On the other hand, IL-1β production was not influenced upon inhibition of PI 3-kinase (Figure 3.13A, lower panel). The results shown in Figure 3.13B demonstrate that SHIP-$1^{-/-}$ BMMs produce significantly higher levels of IL-6 and this production is downregulated in the presence of both the MEK inhibitor (upper panel) and the PI 3-kinase inhibitor (lower panel). These

results demonstrate that SHIP-1 influences FcγR-induced IL-6 production through its influence on both the PI 3-kinase and the Ras/Erk pathways and IL-1β production through its effect on Ras/Erk pathway.

As a second approach, IL-1β and IL-6 production was measured in Raw 264.7 transfectants over-expressing wild-type SHIP-1 or catalytic-deficient SHIP-1 (Figure 3.14). Results shown in Figure 3.14A indicated that over-expression of either the wild-type or the catalytic-deficient version of SHIP-1 significantly dampened the production of IL-1β production in response to FcγR clustering, indicating that the inhibition of IL-1β production is independent of the catalytic-function of SHIP-1. These results are consistent with the findings that the influence of SHIP-1 on the Ras/Erk pathway is independent of its catalytic-function. In contrast, although IL-6 production was almost completely abrogated when wild-type SHIP-1 was over-expressed, there was only partial inhibition of IL-6 when the catalytic mutant of SHIP-1 was over-expressed (Figure 3.14B). These results demonstrate that both the catalytic function and the non-catalytic domains of SHIP-1 are necessary for the inhibition of IL-6 production.

Thus we conclude that SHIP-1 influences FcγR-induced production of IL-1β and IL-6 through the inhibition of the Ras/Erk and the PI 3-kinase pathways.

3.5 Discussion

In this study we have analyzed for the first time the influence of SHIP-1 on phagocytosis-associated events, i.e., superoxide generation and inflammatory cytokine production. Our findings demonstrate several new aspects of the regulation of these

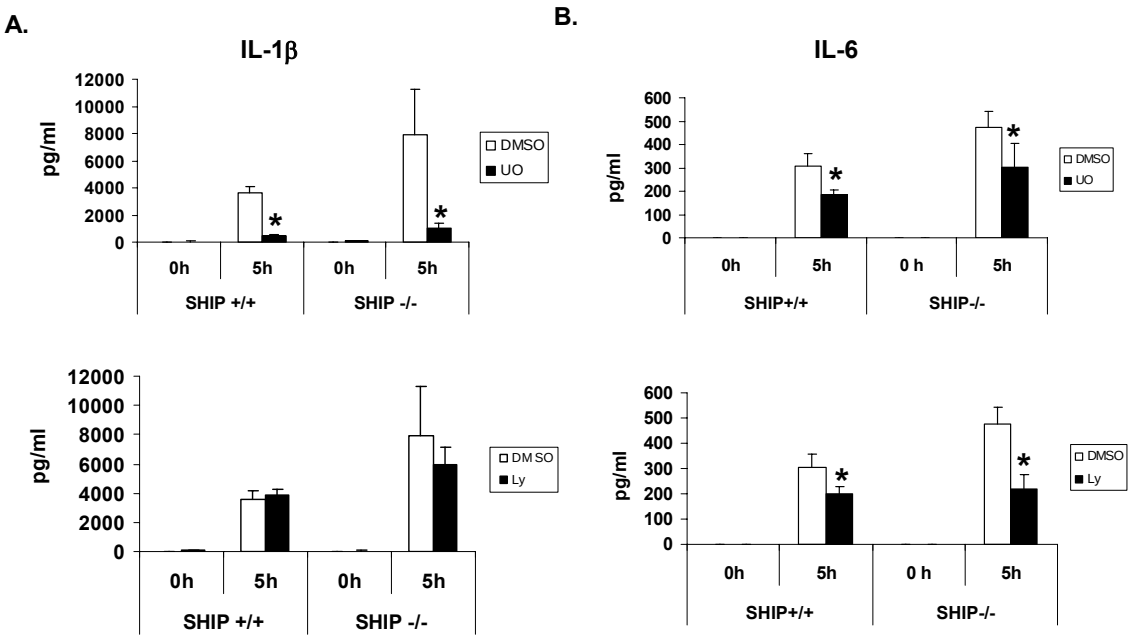


Figure 3.13: SHIP-1 downregulates FcγR-induced IL-1β and IL-6 production through the inhibition of PI 3-K and Ras/Erk pathways. SHIP-$1^{+/+}$ and SHIP-$1^{-/-}$ macrophages were treated with either DMSO, 10 μM LY294002 or 2.5 μM UO126 for 30 minutes at 37°C prior to stimulation with heat-aggregated IgG. **A.** The levels of IL-1β in cells treated with DMSO or UO126 (upper panel) and IL-1β in cells treated with DMSO or LY294002 (lower panel) were measured by ELISA. The graphs represent the mean and SEM of values obtained from three independent experiments. Data were analyzed by student's t-test. ∗ p value < 0.05. **B.** The levels of IL-6 in supernatants of cells treated with DMSO or UO126 (upper panel) and cells treated with DMSO or LY294002 (lower panel) were measured by ELISA. The graphs represent the mean and SEM of values obtained from three independent experiments. Data were analyzed by student's t-test. ∗ p value < 0.05.

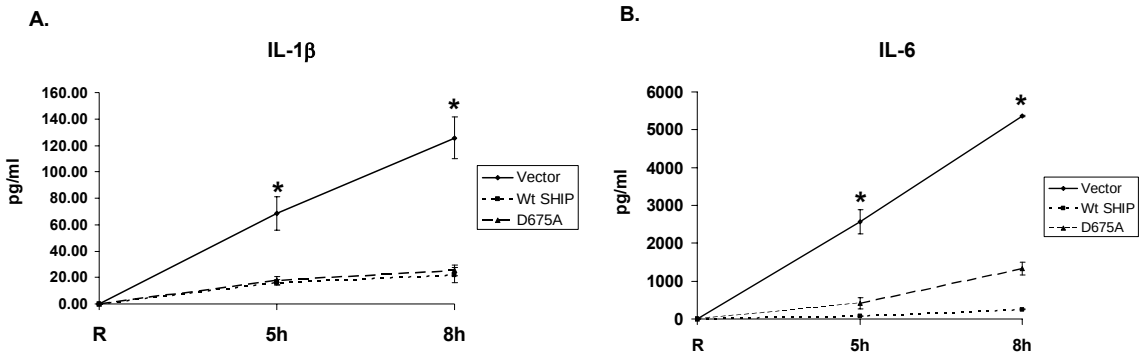


Figure 3.14: Influence of overexpressing wild-type SHIP-1 or catalytic deficient SHIP-1 on IL-1β and IL-6 production. Raw 264.7 stable transfectants over-expressing vector alone, wild-type SHIP-1, or catalytic-deficient D675A SHIP-1 were stimulated with heat-aggregated IgG. Production of **A.** IL-1β and **B.** IL-6 was analyzed by measuring lysates and supernatants, respectively, by ELISA at 5 and 8 hours post stimulation. Data were analyzed by student's t-test. ∗ p value < 0.05.

phagocytosis-associated events. Our results show that SHIP-1 downregulates immune-complex induced Rac activity and subsequent superoxide production. We observed high basal Rac activity in SHIP-1$^{-/-}$ BMMs. However, there was no detectable production of superoxide in the absence of Fc receptor stimulation, suggesting that, while the high Rac activity may contribute to enhanced superoxide production, there are other FcγR-induced activation events that are necessary for generation of superoxide. Activation of Rac is critical for particle ingestion as well as for the assembly of NADPH oxidase complex and superoxide generation[142,143]. While earlier reports have demonstrated that SHIP-1 negatively regulates phagocytosis[81,97], the mechanism by which SHIP-1 mediates its inhibitory effect was previously not known. Thus these findings represent a significant advance in our understanding of SHIP-1's influence on phagocytosis.

SHIP-1 is a cytosolic enzyme which requires activation-induced recruitment to the cell membrane, where it encounters its lipid substrates. Extensive studies from our group and others have demonstrated that SHIP-1 is recruited to the phosphorylated ITIM of FcγRIIb via the SHIP-1 SH2 domain[77,79]. Interestingly, although SHIP-1 associates more strongly with inhibitory motifs, phosphorylated ITAMs of FcγR γ-subunit and human FcγRIIa can also recruit SHIP-1, albeit with much less efficiency[81,82,157,158]. Association of SHIP-1 with the phosphorylated ITAMs occurs both directly as well as through the adapter protein Shc[81,82,157]. IgG-immune complexes engage both ITAM-containing and ITIM-containing FcγR simultaneously, thus recruitment of SHIP-1 to the plasma membrane, under these conditions, likely occurs through SHIP-1 association with both types of receptors. Our data suggest that SHIP-1 downregulates both the PI 3-kinase and the Ras/Erk pathways by virtue of its catalytic domain and its non-catalytic domains

respectively. The catalytic activity of SHIP-1 involves hydrolysis of 5' phosphate from 3' phosphorylated PI 3-kinase products. The non-catalytic domains of SHIP-1 are involved in interaction with a number of cytoplasmic proteins to inhibit signaling pathways. Among the best characterized interactions of SHIP-1 with other proteins is the influence of SHIP-1 on the Ras pathway. Two models have been proposed in this context in B cells: i) the SHIP-1 SH2 domain competes with Grb2/Sos complex for binding to phosphorylated Shc and thereby downregulates Ras activation[79,88] and ii) that SHIP-1 associates with p62dok, which results in hyperphosphorylation of dok, its association with RasGAP and the subsequent hydrolysis of Ras-GTP[87]. Future studies will tell whether there are other signaling pathways that are influenced by SHIP-1's association with additional cytoplasmic signaling molecules.

Although it is well established that FcγR-clustering results in the production of inflammatory cytokines, the signaling pathways involved in the induction of these cytokines are not well characterized. Our experiments demonstrate that the PI 3-kinase and Ras/Erk pathways are activated by FcγR clustering. Moreover, there is no cross-talk between these pathways as inhibition of the PI 3-kinase pathway has no effect on the activation of the Ras/Erk pathway and vice versa (Figure 3.9). This is in contrast to the signaling events initiated by cytokines and growth factors, where PI 3-kinase has been shown to be both upstream and downstream of Ras[159,160].

Interestingly, our current findings also indicate a differential requirement for the PI 3-kinase and Ras/Erk pathways in induction of the inflammatory cytokines IL-1β and IL-6. Thus, while activation of both PI 3-kinase and Ras /Erk pathways was necessary for the induction of IL-6, activation of only Ras/Erk, but not PI 3-kinase, was sufficient for

the induction FcγR-induced IL-β production (Figures 3.8 and 3.13). These observations are important because they allow for the distinction between the catalytic function of SHIP-1 (which hydrolyzes PtdIns3,4,5P_3 and downregulates the PI 3-kinase pathway) and the non-catalytic function of SHIP-1 (which downregulates the Ras/Erk pathway).

In summary, these findings demonstrate that phagocytosis-associated inflammatory responses are regulated by SHIP-1 in addition to the previously reported regulation of particle engulfment. Thus the entire phagocytic process appears to be tightly regulated by the cumulative actions of the kinases and phosphatases in order to maintain homeostasis and prevent collateral tissue damage.

CHAPTER 4

THE PI 3-KINASE/AKT PATHWAY REGULATES MACROPHAGE-MEDIATED ADCC AGAINST B CELL LYMPHOMA THROUGH ITS INFLUENCE ON CYTOSKELETAL REMODELING

4.1 Abstract

Macrophages are important effectors in the clearance of antibody-coated tumor cells through phagocytosis and/or by antibody-dependent cell-mediated cytotoxicity (ADCC). Previous findings suggest that macrophages may destroy tumor cells by the release of TNFα and nitric oxide (NO). The purpose of this study was to characterize the signaling pathways and mechanisms that regulate macrophage-mediated cytotoxicity.

Using a human B cell lymphoma coated with Rituximab as a target, we have examined the molecular details of macrophage response. We first demonstrate that the interaction of macrophages with antibody-coated tumor targets leads to the activation of multiple signaling events including the activation of tyrosine kinases and PI 3-Kinase. Inhibition of PI 3-K activation completely abolished cytotoxicity indicating that the PI 3-

K/Akt pathway is critical for macrophage-mediated ADCC. Consistent with this, murine peritoneal macrophages expressing overactive Akt (Myr-Akt) showed significantly enhanced ADCC.

Further analysis revealed that in this model of ADCC cytotoxicity is dependent upon the release of nitric oxide (NO). However, PI 3-kinase does not appear to regulate NO production. An examination of the role of PI 3-kinase/Akt in regulating conjugate formation indicated that macrophages treated with an inhibitor of PI 3-kinase fail to polarize the cytoskeleton at the synapse and show a significant reduction in the number of conjugates formed with tumor targets. Further, inhibition of PI 3-kinase also reduced macrophage spreading on Rituximab-coated surfaces. On the other hand, Myr-Akt expressing macrophages displayed a significantly greater ability to form conjugates with tumor cells. Taken together, these findings illustrate that the PI 3-kinase/Akt pathway plays a critical role in macrophage ADCC through its influence on conjugate formation between macrophages and antibody-coated tumor cells.

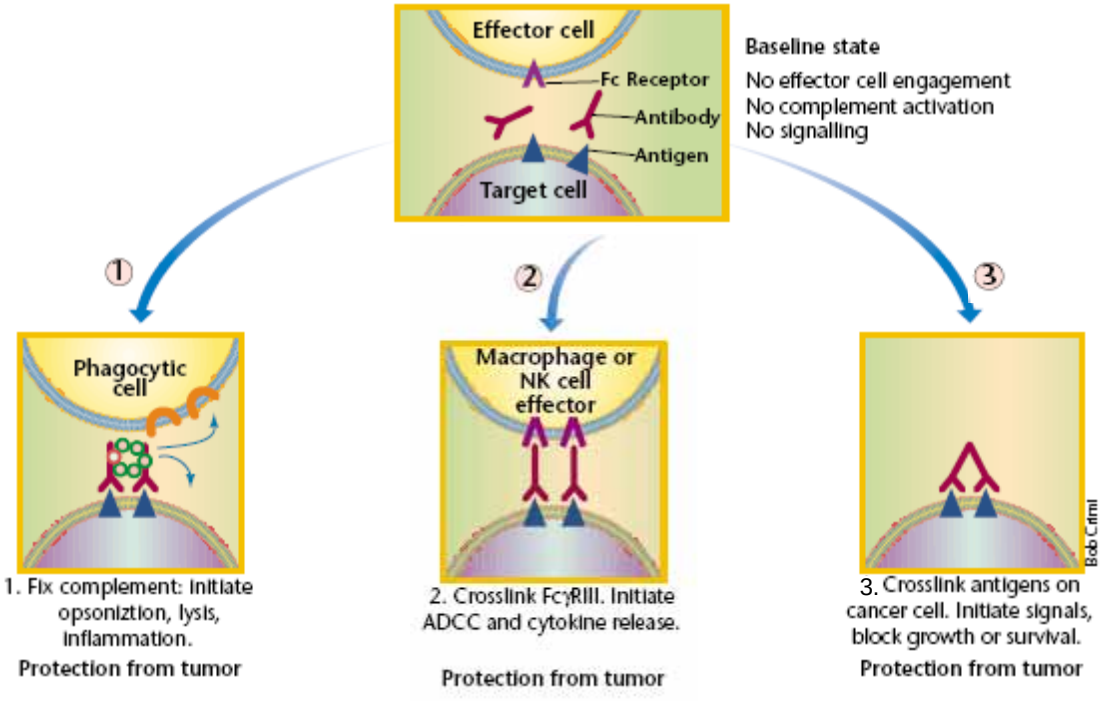
4.2 Introduction

In recent years, monoclonal antibodies (mAb) such as rituximab and alemtuzumab have emerged as effective therapeutic agents in the treatment of human malignancies (reviewed in [161]). Several mechanisms have been proposed to be involved in the elimination of tumor cells during antibody therapy such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or alteration of signaling pathways within the tumor cell by blocking cell surface receptors (reviewed in [162]) (Figure 4.1). The first two mechanisms, ADCC and CDC are mediated

by the binding of the constant Fc region of the antibody to immune effector cells while disruption of signaling events in tumor cells is mediated by binding of F(ab')2 variable region of the monoclonal antibody. The binding of antibody to cell surface antigens on tumor cells can regulate cell survival, proliferation or death by promoting cellular signaling pathways or by blocking access to growth factors. One of the best characterized examples of interference with signaling events by therapeutic antibodies is that of Herceptin. Herceptin is an mAb used in the treatment of breast cancers that over-express *her2/neu* oncogene. The *her2* gene encodes for epidermal growth factor receptor (EGFR) which belongs to the tyrosine kinase receptor family. The binding of Herceptin to this receptor on breast cancer cells inhibits the activation of PI 3-Kinase and MAP kinase pathways, thereby inhibiting cell proliferation (reviewed in[163]).

Complement-dependent cytotoxicity (CDC) is another mechanism by which therapeutic antibodies may eliminate target tumor cells. In this process, binding of antibodies to tumor cells expose the multiple C1q (one of three subunits of complement c1) binding sites on these antibodies. This triggers the activation of the complement cascade that ultimately forms a membrane attack complex on the tumor cell and damages the cell membrane permanently. Some reports suggest that a part of tumor cell killing with anti-CD20 mAb Rituximab may involve the process of CDC[164,165].

Antibody-dependent cell-mediated cytotoxicity (ADCC) constitutes one of the most important mechanisms of elimination of tumor cells by mAbs. This process is initiated when Fc regions of antibodies bound to tumor cells engage the Fc receptors present on immune effector cells (Figure 4.2). The contribution of Fcγ receptors on immune effector cells in the clearance of tumor cells during antibody therapy was



Nature Medicine, 2000; 6: 373-374

<u>Figure 4.1:</u> Mechanisms involved in clearance of tumor cells by monoclonal antibodies. The binding of monoclonal antibodies can induce cell death through complement-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC) or through disruption of signaling cascades within the tumor cell.

conclusively demonstrated by Clynes et al[9]. Consistent with a role for FcγR, it has also been reported that lymphoma patients with 'high responder' Fc-receptor polymorphisms show a better response to Rituximab treatment[166,167].

The population of immune cells that bear Fcγ receptors include monocytes, macrophages, neutrophils and NK cells. However, in a murine model, Uchida et al demonstrated that macrophages are the principal mediators of CD20 antibody-dependent depletion of B cells[168]. There are three classes of FcγR expressed by human and murine macrophages: FcγRI, FcγRIIb and FcγRIII. FcγRI and RIII are activating receptors while FcγRIIb is an inhibitory receptor. In addition, human macrophages express FcγRIIa while murine cells express FcγRIV, both of which are activating receptors (reviewed in [11-13]).

The mechanism by which macrophages cause the cytolysis of tumor cells depends on the nature of the target tumor cell. Several studies have reported the release of TNFα and nitric oxide (NO) as dominant mediators of macrophage induced cytotoxicity[169-173], although the release of certain proteases[174] and reactive oxygen species (ROS) by macrophages has also been reported in a few cases[175]. The activation of macrophages for cytolytic function involves a priming event during which macrophages acquire the ability to respond to tumor cells upon subsequent exposure. IFNγ is a potent inducer of macrophage tumoricidal activity[176-178]. The priming of macrophages with IFNγ leads to the up-regulation of Fcγ receptors, TNFα, and inducible nitric oxide synthase (iNOS); all of which can enhance the killing of tumor cells[178,179]. To this end, the administration of *ex-vivo* IFNγ-activated macrophages is being tested as an approach to augment traditional cancer therapies[180,181].

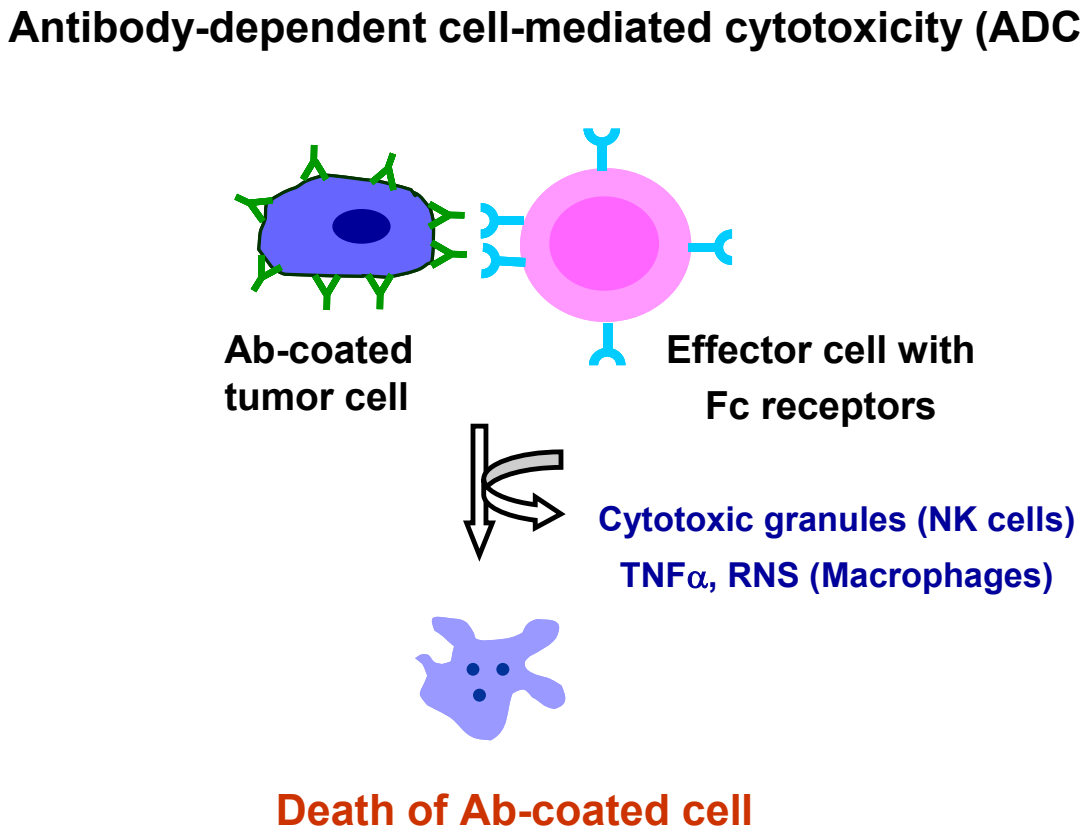


Figure 4.2: Schematic of antibody-dependent cell-mediated cytotoxicity (ADCC). Monoclonal antibodies bind to target tumor cells through F(ab')2 region while their constant Fc region binds and activates Fc receptors expressed on monocytes, macrophages or NK cells. This initiates the release of cytotoxic granules from NK cells or the release of TNFα and reactive nitrogen species (RNS) from monocytes and macrophages and ultimately lead to the death of the tumor cell.

The signaling pathways involved in NK cell-mediated cytotoxicity have been extensively studied and the activation of protein tyrosine kinases such as lck and Syk kinase are shown to be the initial signaling events in NK cells[182,183]. The stimulation of both PI 3-kinase and extracellular signal regulatory kinase 1/2 (Erk 1/2) has been shown to play a critical role during NK cell-mediated cytotoxicity[184,185]. Erk 1/2 control the lytic function of NK cells by regulating the movement of cytosolic perforin and granzyme B towards the target tumor cell[185]. Further, it has been shown that Erk 1/2 activation is independent of the traditional Ras-dependent pathway and that PI 3-kinase mediates the activation of Erk 1/2 through sequential involvement of Rac1, PAK1 and MEK[184]. In addition, although the release of cytotoxic granules containing perforin and granzyme by NK cells mediates the lysis of tumor cell, an efficient conjugate formation between the NK cell and the target tumor cell is critical for the process of cytotoxicity[186,187]. This process involves the formation of an immunologic synapse between NK cell and target cell[188,189]. During the synapse formation, there is a rapid reorganization of the NK cell cytoskeleton including the reorientation of the Golgi complex and the microtubule-organizing center (MTOC) as well as the remodeling of actin cytoskeleton[190,191].

As opposed to NK cell cytotoxicity, the signaling events that regulate macrophage mediated ADCC are yet to be characterized. The aim of this study was to identify the signaling pathways that play a crucial role in macrophage response to antibody-coated tumor cells. For this purpose, we have used Rituximab-coated human Burkitt's B cell lymphoma cell line Raji cells as tumor targets. Rituximab is a human-mouse chimeric monoclonal antibody specific for CD20 antigen expressed on B cells. Rituximab has been shown to be effective in the treatment of various forms of B cell malignancies including

the aggressive and indolent B cell non-Hodgkin's lymphoma (NHL) as well as B-cell chronic lymphocytic leukemia (CLL) (reviewed in [192]).

We demonstrate a critical role for the PI 3-kinase/Akt pathway in macrophage mediated ADCC against tumor cells. We first show that IFNγ primed macrophages mediate Rituximab-dependent killing of B cell lymphoma. The interaction of macrophages with antibody-coated tumor targets leads to the activation of multiple signaling events including the activation of tyrosine kinases and PI 3-kinase/Akt. Pharmacological inhibition of PI 3-kinase/Akt completely abolished macrophage cytotoxicity indicating that the activation of PI 3-kinase/Akt is critical for macrophage cytotoxicity. Consistent with this, murine peritoneal macrophages expressing overactive Akt (Myr-Akt) showed significantly enhanced ADCC compared to their wild-type counterparts. Further analysis of the role of PI 3-kinase/Akt in macrophage ADCC reveals that this pathway regulates macrophage cytotoxicity at the level of conjugate formation between the effector macrophages and antibody-coated tumor cells.

4. 3 Materials and Methods

Cells, antibodies and reagents: Raw 264.7 cells and Raji cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 5% fetal bovine serum. Recombinant mouse IFNγ and TNFα neutralizing antibody were purchased from R&D systems (Minneapolis, MN). All phospho-specific antibodies were from Cell Signaling Technology (Danvers, MA), iNOS antibody was from BD Transduction laboratories (Franklin Lakes, NJ), mouse anti-human CD37 antibody was from BD Biosciences and actin antibody was from Santa

Cruz Biotechnology (Santa Cruz, CA). Rituximab and Herceptin antibodies were produced by Genentech (San Francisco, CA). All pharmacological inhibitors were purchased from Calbiochem. Griess Reagent kit was from Molecular Probes (G-7921) (Eugene, OR). Goat anti-mouse Alexa Fluor 594 and FITC-phalloidin were purchased from Molecular probes. TNFα ELISA kit was purchased from R&D systems.

Isolation of peritoneal macrophages: Transgenic mice with macrophage-specific expression of Myr-Akt have been described earlier[47]. Peritoneal macrophages from transgenic mice and their wild-type littermates were induced by i.p. injection of 1.5 ml of 2.9% Brewer's thioglycolate broth. Macrophages were harvested 4-5 days post-injection by peritoneal lavage using RPMI supplemented with 10% FBS. RBCs were lysed by incubating peritoneal exudate cells with 3-5 ml of RBC lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA [pH 8.0]) for 3 minutes on ice.

ADCC assay: A ^{51}Cr-release assay was performed as described elsewhere[193] with some modifications. Macrophages were plated in 96-well V-bottom tissue culture plates in 10% RPMI medium supplemented with mIFNγ (25 ng/ml) and incubated overnight at 37°C. Raji cells were labeled with ^{51}Cr followed by coating with Rituximab. Rituximab was used at a final concentration of 10 μg/ml in all assays except those where dose response with Rituximab was measured. Rituximab-coated, ^{51}Cr-labeled Raji targets were added to macrophages at various E:T ratios. After 8 hours incubation at 37°C, supernatants were harvested and the amount of ^{51}Cr released was measured using a gamma counter. The percent relative cytotoxicity was determined as [(experimental cpm – spontaneous cpm) /

(total cpm – spontaneous cpm)] *100. For assays involving inhibitors, IFNγ-primed macrophages were incubated with the appropriate inhibitors for 30 minutes before addition of target cells. Both, L-NMMA, a competitive inhibitor of nitric oxide production and TNFα neutralizing antibody were added to macrophage cultures along with IFNγ and incubated overnight before addition of targets.

Preparation of target cells for signaling and cytokine experiments: Raji cells were first coated with Rituximab (10 μg/ml) at 37°C for 20 minutes. Unbound antibody was removed by washing cells once in PBS. Cells were then fixed on ice in 1% paraformaldehyde (PFA) in PBS for 10 minutes. The cells were finally washed with PBS thoroughly to remove PFA. Uncoated or control antibody (Herceptin) treated cells were processed in an identical manner.

Cell stimulation, lysis and Western blotting: Raw 264.7 cells or peritoneal macrophages were plated in 48-well tissue culture plates and primed with mIFNγ (25 ng/ml) overnight. Cells were then stimulated with fixed Rituximab-coated or uncoated Raji targets at 1:1 E:T ratio for indicated time points. The plate was spun down at 1400 rpm for 5 minutes to increase the contact between fixed Raji cells and macrophages. Cells were lysed in TN1 lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 3 mM Na_3VO_4, 10 μg/ml each aprotinin and leupeptin). Protein-matched whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with the antibody of interest and developed with enhanced chemiluminescence (ECL).

Western Blot Data Quantitation: The ECL signal was quantitated using a scanner and a densitometry program (Scion Image; Scion, Frederick, MD). Background pixel values were subtracted, the signal was normalized to the amount of actin and the normalized band intensity values were plotted.

Preparation of heat-aggregated IgG (IgG immune-complexes): Heat-aggregated IgG was prepared according to methods described previously[127]. In brief, chromopure mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a concentration of 350 μg/ml was heated at 62°C for 30 minutes, then cooled on ice immediately and used to stimulate cells.

Measurement of TNFα by ELISA: Raw 264.7 cells or peritoneal macrophages were incubated with or without IFNγ (25 ng/ml) overnight, treated with DMSO or 10 μM Ly294002 and then stimulated with paraformaldehyde-fixed Rituximab-coated Raji targets for 8 hours at E:T ratio of 1:1. The supernatants were harvested and analyzed by ELISA. To test the neutralizing ability of TNFα blocking antibody, similar samples were run in parallel in the presence of TNFα blocking antibody.

Measurement of nitrite by Griess Reagent: The levels of NO were measured by assaying the culture supernatants for NO_2^-, a stable product of NO with molecular oxygen. The assay was performed with a modification of previously described method[194] using Griess Reagent kit from Molecular Probes. For the assay, macrophages were plated in RPMI containing no phenol red and no serum. To test the effect of Fc receptor clustering on NO

production, macrophages were primed with IFNγ for 16 hours, treated with DMSO or 10 μM Ly294002 for 30 minutes followed by stimulation with immune-complexes (350 μg/ml) for 8 hours (IFNγ was left in the medium during immune-complex stimulation). For the nitrite assay, 100 μl of supernatant was mixed with 50 μl of Griess Reagent (Molecular Probes G-7921) and incubated at room temperature in dark for 30 minutes. The absorbance was measured at 520 nm and nitrite concentrations were calculated from a standard curve obtained using standards containing increasing concentration of $NaNO_2$.

Analysis of conjugate formation: IFNγ-primed macrophages and Raji tumor cells were stained for analysis of conjugates using a method described elsewhere[189]. The IFNγ-primed macrophages were treated with DMSO or inhibitors (10 μM Ly294002 or 20 nM rapamycin) for 30 minutes and then mixed with Rituximab-coated Raji cells at 5:1 ratio for 30 minutes at 37°C in suspension. The cell suspension was transferred to poly-L-lysine coated cover-slips and the plate was centrifuged at 1600 rpm for 5 minutes. The plate was further incubated at 37°C for 1 hour. The cells were then fixed using 4% formaldehyde, washed twice and incubated with mouse anti-human CD37 antibody overnight followed by Alexa Fluor 594-conjugated goat anti-mouse for 4 hours. The cells were then permeabilized using 0.2% Triton-X100 and stained with FITC-phalloidin.

Cell conjugates were analyzed using Olympus microscope (Olympus, BX40F-3, Melville, NY) and images were captured using digital video camera (Olympus U-CMAD-2, Optronics, Galeta, CA). 100 red cells were analyzed per cover-slip and a total of 300 red cells per condition were scored for conjugate formation. The results were expressed as % conjugates formed.

Spreading of macrophages on Rituximab-coated surfaces: The spreading of macrophages on antibody-coated surfaces was measured as described elsewhere[43]. Thioglycollate-elicited wild-type peritoneal macrophages were treated with DMSO or 10 μM Ly294002 for 30 minutes at 37°C. They were then allowed to spread on cover-slips pre-coated with 1 mg/ml Rituximab for different time points. The spreading of cells was analyzed using Nikon E800 microscope and images were captured using a Nikon FDX-35 camera. The cells were then fixed with 4% formaldehyde. The surface area occupied by cells was measured using Morphometry software (version 6) and expressed as arbitrary units (AU).

Statistical Analysis: All data were analyzed using Student's t-test and p value of ≤ 0.05 was considered significant.

4.4 Results

IFNγ-primed macrophages show Rituximab-dependent lysis of B cell lymphoma Raji cells

We first tested whether macrophage-mediated ADCC could be elicited by Rituximab-coated B cell lymphoma cells. For this, we co-cultured IFNγ-primed Raw 264.7 macrophages with ^{51}Cr-labeled Raji cells opsonized with either an irrelevant control antibody (Herceptin, 10μg/ml) or increasing concentrations of Rituximab. The macrophages showed lysis of Raji targets only upon coating with Rituximab (Figure 4.3A). An increase in relative cytotoxicity towards Raji cells corresponded with an increasing dose of Rituximab. The complete absence of cytotoxicity towards Raji cells

coated with a control antibody (indicated by C in the Figure 4.3A) further confirmed that the cytotoxicity is indeed antibody-dependent.

We also analyzed the cytolysis of Rituximab-Raji cells at different effector to target (E:T) ratios. As indicated in Figure 4.3B, there was a progressive increase in cytotoxicity with increasing E:T ratio. As mentioned earlier, priming of macrophages is required for tumoricidal function. To test the effect of IFNγ priming on macrophage ADCC, we performed cytotoxicity assays with macrophages primed with varying doses of IFNγ. The cytotoxicity of Raw 264.7 cells increased gradually with increasing doses of IFNγ (Figure 4.3C).

Rituximab-coated targets induce significant activation of Syk kinase and the PI 3-kinase/Akt pathway in macrophages

To investigate the signaling pathways activated in macrophages in response to antibody-coated tumor targets, IFNγ-primed peritoneal macrophages were stimulated with Rituximab-coated Raji targets for various time points. Raji cells coated with Herceptin antibody were used as control. As shown in Figure 4.4A upper panel, strong phosphorylation of Syk kinase was seen at 30 minutes with Rituximab-coated targets but not with control antibody-coated targets. The graph shown in lower panel is a quantitation of band intensity from three independent experiments. The stimulation of macrophages with Rituximab-coated tumor cells also resulted in the activation of PI 3-kinase/Akt pathway as seen by serine phosphorylation of Akt while tumor cells coated with control antibody failed to induce Akt activation (Figure 4.4B). The graph shown in

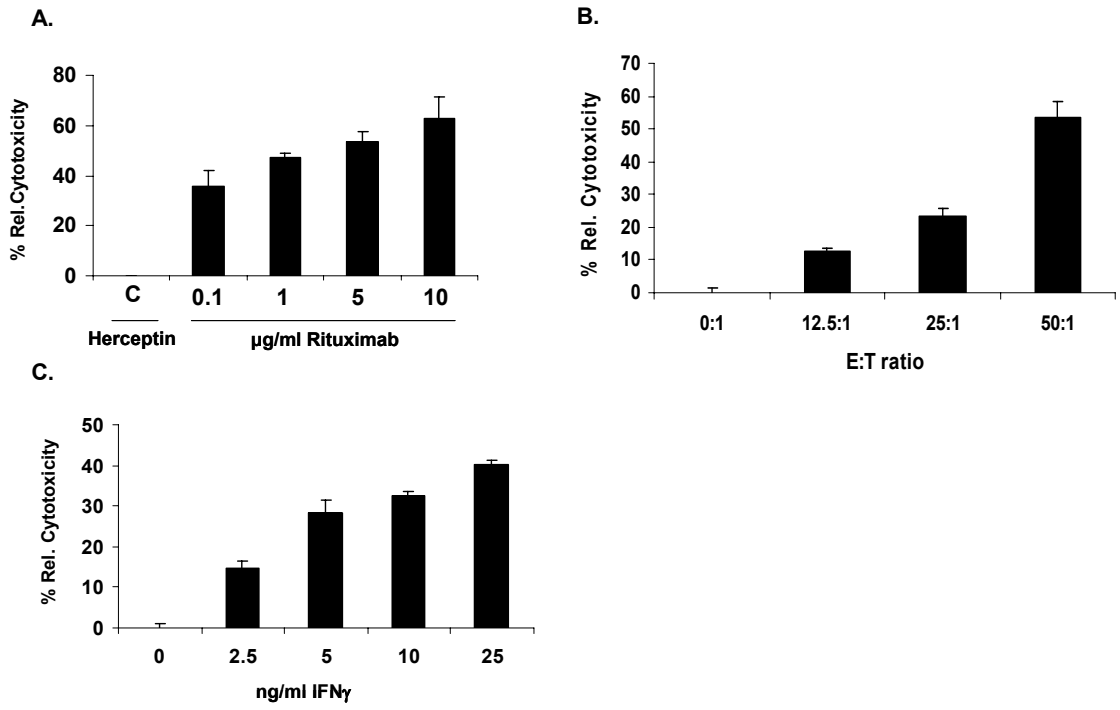


Figure 4.3: IFNγ-primed macrophages show Rituximab-dependent lysis of B cell lymphoma Raji cells. A. Raw 264.7 cells were primed with 25ng/ml IFNγ over-night. ^{51}Cr- labeled Raji cells coated with increasing concentrations of Rituximab or treated with Herceptin (10 µg/ml) were added to primed Raw 264.7 cells at various E:T ratio. At the end of 8 hours, supernatants were harvested, amount of ^{51}Cr released was measured and % relative cytotoxicity was calculated. The graph shows values obtained at 50:1 E:T ratio. **B.** ^{51}Cr- labeled Rituximab-coated Raji cells were added to IFNγ primed Raw 264.7 cells at E:T ratios indicated in the Figure. Amount of ^{51}Cr released was measured at the end of 8 hours and is shown as % relative cytotoxicity. **C.** To test the effect on IFNγ priming, Raw 264.7 cells were primed with increasing concentration of IFNγ and co-cultured with ^{51}Cr- labeled Rituximab-coated Raji targets for 8 hours. % relative cytotoxicity values obtained at E:T ratio of 50:1 are shown in the graph.

lower panel represents the average of phospho-Akt band intensity from three experiments. In addition to the activation of these pathways, we also observed an increase in phosphorylation of Erk/MAPK with targets opsonized with Rituximab. No activation of p38 or JNK MAPKs was seen (data not shown). Collectively, these results indicate that macrophages upon interaction with tumor cells coated with the appropriate antibody display activation of specific signaling pathways.

Activation of Syk kinase and the PI 3-kinase/Akt pathway is critical for macrophage ADCC

The above experiments demonstrated that there is an activation of Syk kinase and the PI 3-kinase/Akt pathway in macrophages upon ligation with antibody-coated targets. To examine whether the activation of these pathways is important for cytotoxicity function, we used piceatannol, a pharmacological inhibitor of Syk and Ly294002, a specific pharmacological inhibitor of PI 3-kinase. Previous work from our group has demonstrated that Akt promotes Fc receptor-mediated phagocytosis through activation of p70S6 kinase (p70S6K)[47]. To test the requirement of downstream p70S6K in macrophage mediated ADCC, we incorporated rapamycin, an inhibitor of mTOR, in these experiments. IFNγ primed Raw 264.7 cells were pre-treated with either DMSO or 10 μM Ly294002 or 25 μg/ml piceatannol or 20 nM rapamycin for 30 minutes. They were then co-cultured with ^{51}Cr-labeled, Rituximab-coated Raji targets for 8 hours. At the end of 8 hours, supernatants were harvested and the amount of ^{51}Cr released was measured. As seen in Figure 4.5A, pre-treatment of macrophages with piceatannol and Ly294002 resulted in complete inhibition of antibody-dependent cytotoxicity indicating that both

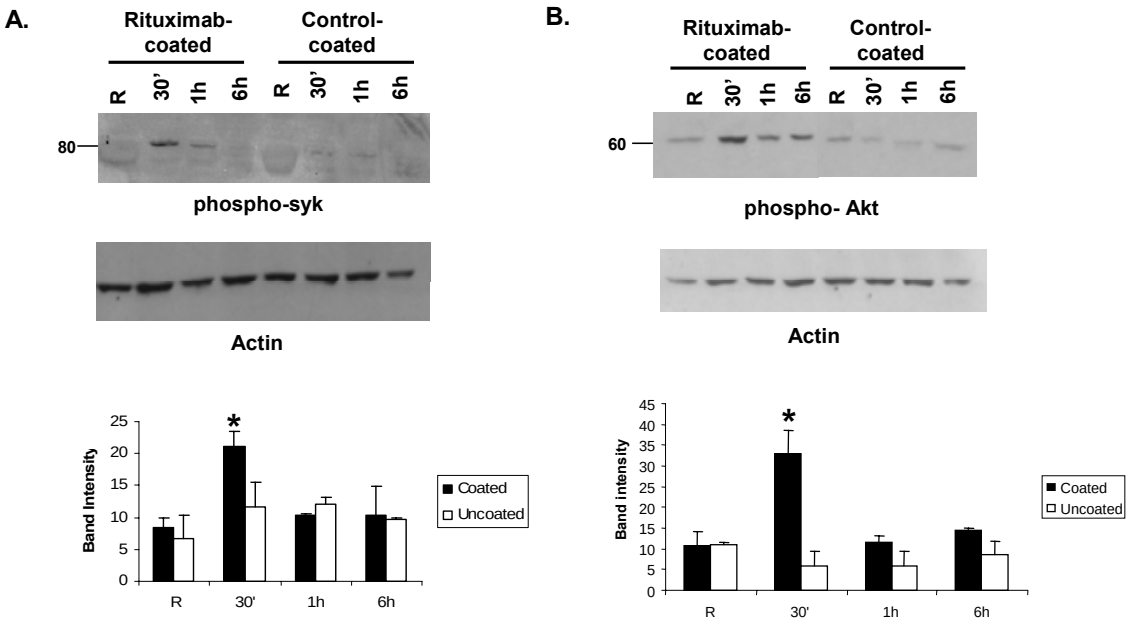


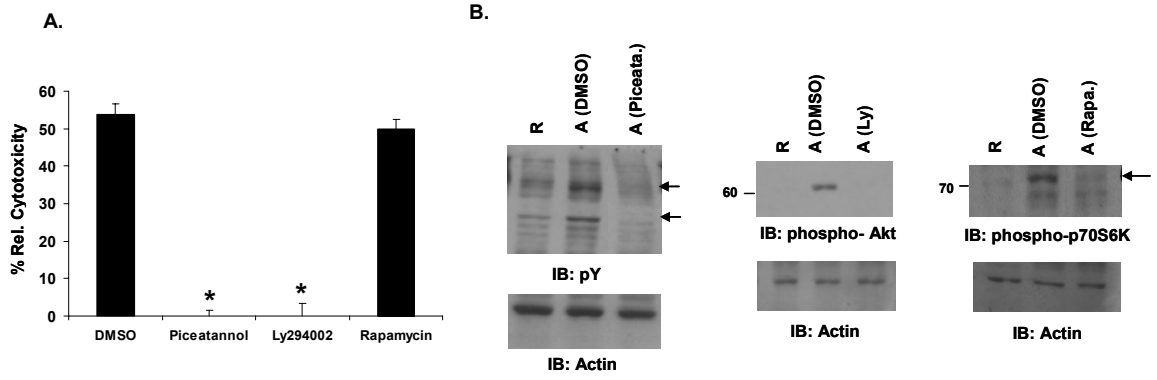
Figure 4.4: Rituximab-coated targets induce significant activation of Syk kinase and the PI 3-kinase/Akt pathway in macrophages. IFNγ-primed peritoneal macrophages were stimulated with paraformaldehyde-fixed Rituximab-coated or control antibody-coated Raji cells at E:T ratio of 1:1 for indicated time points. Protein-matched whole cell lysates were analyzed by western blotting and probed with **A.** phospho-Syk antibody (upper) and **B.** phospho-Akt antibody (upper). The same membranes were reprobed with actin antibody (lower). The graphs in lower panels show mean and SD of the band intensity of phospho-Syk and phospho-Akt from three independent experiments. Data were analyzed by student's t-test (* = p value ≤ 0.05).

Syk and the PI 3-kinase/Akt pathways play a critical role in macrophage response towards antibody-coated targets. On the other hand, the cytotoxic ability of macrophages treated with rapamycin remained unaffected. This suggests that unlike phagocytosis, activation of p70S6K is dispensable during FcγR-mediated macrophage cytotoxicity.

To ensure the specificity of the inhibitors, Raw 264.7 cells were first incubated with respective inhibitors for 30 minutes and then stimulated with immune-complex (heat-aggregated IgG) for 7 minutes. The protein-matched whole cell lysates were analyzed using phospho-tyrosine antibody for piceatannol, phospho-p70S6K antibody for rapamycin and phospho-Akt antibody for Ly294002. The cells treated with vehicle-control (DMSO) showed significant induction in tyrosine phosphorylation (pY) as well as phosphorylation of Akt and p70S6K whereas the treatment of cells with piceatannol, Ly294002 and rapamycin resulted in complete inhibition of pY and phosphorylation of Akt and p70S6K respectively (Figure 4.5B).

Over-expression of active Akt enhances macrophage ADCC

The above data indicated that Rituximab-coated Raji cells stimulate Akt activation in macrophages and that activation of PI 3-kinase upstream of Akt is necessary for macrophage ADCC. Also a previous report from our group has demonstrated that constitutively active Akt significantly up-regulates FcγR-mediated phagocytosis by macrophages[47]. To determine whether the activation of Akt downstream of PI 3-kinase plays a role during macrophage mediated ADCC, we tested the cytotoxic ability of peritoneal macrophages from previously described transgenic mice over-expressing macrophage specific, constitutively active Akt (Myr-Akt). The results shown in Figure



<u>Figure 4.5:</u> Activation of the PI 3-kinase/Akt pathway is critical for macrophage-mediated ADCC. A. IFNγ-primed Raw 264.7 cells were treated with DMSO, 10 μM Ly294002, 25 μg/ml piceatannol or 20 nM Rapamycin for 30 minutes. They were then incubated with ^{51}Cr-labeled Rituximab-coated Raji cells for 8 hours. The graph shows % relative cytotoxicity at 50:1 E:T ratio. **B.** To test the specificity of the inhibitors used, Raw 264.7 cells were incubated with DMSO, 10 μM Ly294002, 25 μg/ml piceatannol or 20 nM Rapamycin for 30 minutes. The cells were then stimulated with immune-complexes for 7 minutes. Protein-matched cell lysates were analyzed by Western blotting and probed with phospho-Akt, phospho-tyrosine and phospho-p70S6K antibody. R indicates resting samples and A indicates cells activated with immune-complexes. The same membranes were reprobed with actin antibody (lower panel).

4.6A indicate that Myr-Akt macrophages show significantly enhanced cytolysis of Rituximab-coated Raji cells as compared to their wild-type counterparts. As described previously, there is no difference in the expression of FcγR on the surface of Myr-Akt and wild-type macrophages[47] indicating that the enhanced ADCC is indeed a function of over-active Akt and not attributable to the changes in Fc receptor expression. To confirm the expression of Myr-Akt transgene, whole cell lysates from wild-type and Myr-Akt macrophages were resolved on SDS-PAGE and blotted with phospho-Akt antibody (Figure 4.6B, upper panel). The lower panel of Figure 4.6B is a reprobe of the same membrane with actin antibody to show equal loading of protein. Together these results demonstrate that downstream of PI 3-kinase, Akt activation plays an important role in macrophage response to antibody-coated tumor cells.

Influence of the PI 3-kinase/Akt pathway on TNFα production

Several previous studies have indicated that TNFα and nitric oxide produced by activated monocytes/macrophages (NO) are predominant effectors of cytotoxicity[169-171]. These studies further suggest that although TNFα possesses cytolytic activity, some tumor cells appear to be resistant to TNFα-mediated lysis[195,196]. Having established the role of PI 3-kinase/Akt pathway in macrophage-mediated ADCC, we next wanted to understand the mechanism by which this pathway regulates cytotoxicity. We hypothesized that PI 3-kinase/Akt may enhance macrophage induced tumoricidal activity by promoting the production of TNFα and/or nitric oxide. To test this hypothesis, we measured the amount of TNFα produced by IFNγ-primed macrophages in response to

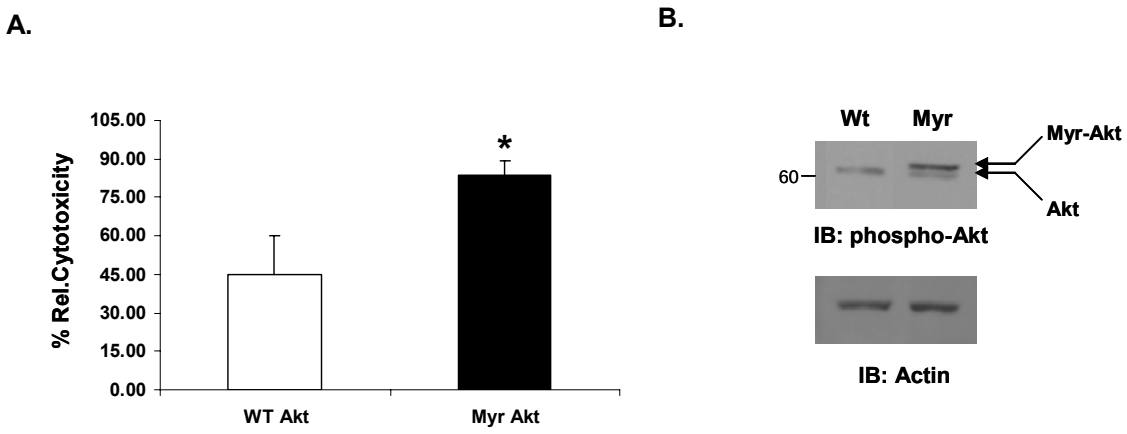


Figure 4.6: Over-expression of active Akt enhances macrophage ADCC. A. Wild-type and Myr-Akt expressing peritoneal macrophages were activated with IFNγ over-night. ^{51}Cr- labeled Rituximab-coated Raji targets were added and supernatants harvested at the end of 8 hours. The graph shows % relative cytotoxicity E:T ratio of 50:1. Data was analyzed using student's t-test (* = p value ≤ 0.05). **B.** To confirm the expression of the Myr-Akt transgene, cell lysates from wild-type and Myr-Akt peritoneal macrophages were analyzed by western blotting and probed with phospho-Akt antibody (upper panel). The same membrane was reprobed with actin antibody (lower panel).

antibody-coated tumor cells in presence or absence of PI 3-kinase/Akt inhibitor. For these experiments, Raw 264.7 cells were primed with IFNγ (25 ng/ml) for 16 hours, treated with either DMSO or 10 μM Ly294002 for 30 minutes and then stimulated with formaldehyde-fixed Rituximab-coated Raji cells at E:T ratio of 1:1 for 8 hours. Raw 264.7 cells left untreated with IFNγ, or stimulated with IFNγ alone or Rituximab-coated Raji cells alone served as control conditions for the experiment. Supernatants were harvested and levels of TNFα were measured using ELISA. Consistent with previous reports, the results shown in Figure 4.7A indicate that macrophages primed with IFNγ alone produced a small amount of TNFα[179,197]. Further activation of IFNγ-primed macrophages with antibody-coated tumor cells (indicated as RR in the figure) resulted in significantly higher levels of TNFα in presence of DMSO. However, macrophages treated with Ly294002 did not show an increase in TNFα production upon ligation with antibody-coated tumor cells indicating that PI 3-kinase/Akt is involved in FcγR-mediated TNFα production.

As mentioned earlier, certain tumor cells have been shown to be resistant to TNFα-mediated lysis[195,196]. Therefore, we next tested the requirement of TNFα in cytolysis of Rituximab-opsonized Raji cells. As seen in Figure 4.7B, incubation of macrophages with TNFα neutralizing antibody had no effect on their cytotoxic ability towards Rituximab-coated Raji cells suggesting that Raji cells are resistant to lysis by TNFα. We also confirmed the blocking activity of TNFα neutralizing antibody in a parallel experiment using ELISA (Figure 4.7C).

Thus, although the PI 3-kinase/Akt pathway regulates TNFα production, TNFα is not required for cytolysis of Raji cells.

IFNγ priming of macrophages induces the production of nitric oxide (NO)

Next, we wanted to determine the role of NO in macrophage-mediated ADCC against Rituximab-coated B cell lymphoma cells. Resting macrophages do not produce nitric oxide since inducible nitric oxide synthase (iNOS), the enzyme involved in NO synthesis by macrophages, is not constitutively expressed[198,199]. However, priming of macrophages with IFNγ induces the expression of iNOS and thereby leads to NO production[178,200].

We first examined the dose-response and time-course of NO production in Raw 264.7 cells. For dose-response experiments, Raw 264.7 cells were primed with increasing concentrations of mIFNγ (0, 2.5, 5, 10 and 25 ng/ml) for 24 hours. Supernatants were harvested and the amount of NO produced was measured by Griess Reagent assay. As shown in Figure 4.8A, macrophages left untreated did not show any NO production while maximum NO production was observed at the dose of 10 ng/ml. The whole cell lysates from the same experiments were analyzed for the expression of iNOS using Western blotting. Results shown in Figure 4.8B indicate that the expression of iNOS protein was induced only upon IFNγ treatment and was increased with increasing dose of IFNγ. The same membrane was then reprobed with actin antibody to ensure equal loading of proteins in all lanes. For time-course experiments, Raw 264.7 cells were treated with 25 ng/ml IFNγ for time-points indicated in Figure 4.8C and the levels of NO were measured

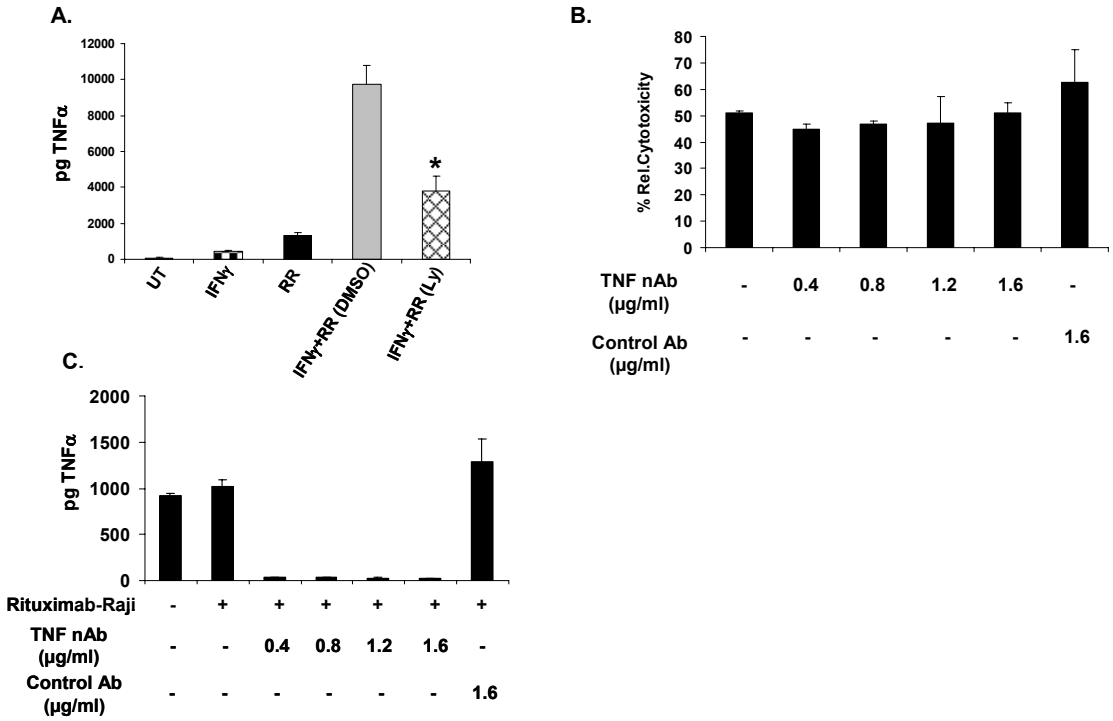


Figure 4.7: Influence of the PI 3-kinase/Akt pathway TNFα production. A. Raw 264.7 cells were left untreated or treated with IFNγ for 16 hours. They were then incubated with DMSO or 10 μM Ly294002 for 30 minutes followed by stimulation with Rituximab-coated Raji cells (indicated as RR in the figure) at E:T ratio of 1:1 for 8 hours. Controls conditions consisted of Raw 264.7 cells stimulated with media alone (UT) or IFNγ (25 ng/ml) alone or Rituximab-coated Raji cells alone. Supernatants were harvested and levels of TNFα were measured using ELISA. **B.** Raw 264.7 cells were cultured in presence of IFNγ and increasing concentrations of TNFα neutralizing antibody (0-1.6 μg/ml) or control antibody (1.6 μg/ml). After 16 hours of IFNγ priming, ^{51}Cr- labeled Rituximab-coated Raji targets were added to Raw 264.7 cells and incubated for 8 hours. The graph shows % relative cytotoxicity at E:T ratio of 50:1. **C.** A parallel assay was set up without labeling tumor cells with ^{51}Cr and the levels of TNFα were measured using ELISA to test the blocking ability of TNFα neutralizing antibody.

in the supernatants. The measurable amount of NO was observed at 16 hours time point and NO production increased with increasing time intervals. The levels of iNOS expression in cell lysates corresponded with the levels of NO in the supernatants (Figure 4.8D).

NO production is required for macrophage-mediated cytotoxicity against Rituximab-coated Raji cells

To examine the role of NO in lysis of Raji cells, we measured the cytotoxic ability of macrophages in presence of a competitive inhibitor of NO production. Since L-arginine is used as a substrate by iNOS for NO synthesis, analogs of L-arginine such as L-NMMA act as competitive inhibitors of iNOS activity and thereby block NO production[201]. For this, Raw264.7 cells were plated with mIFNγ (25 ng/ml) in presence of increasing concentrations of L-NMMA. The next day, ^{51}Cr-labeled Rituximab-coated Raji targets were added to these macrophages and at the end of 8 hours, the amount of ^{51}Cr released was measured. The results shown in Figure 4.9A demonstrate that macrophages treated with L-NMMA lose their cytolytic ability towards Rituximab-coated Raji cells in a dose-dependent manner. These results demonstrate that nitric oxide is necessary for lysis of Rituximab-coated Raji cells by macrophages.

To ensure that L-NMMA indeed blocked NO production, Raw 264.7 cells were treated with 25 ng/ml IFNγ and 10 mM L-NMMA for 24 hours. The supernatants were analyzed for NO while cell lysates were examined for iNOS expression. As shown in Figure 4.9B left panel, NO production was blocked in presence of L-NMMA. L-NMMA

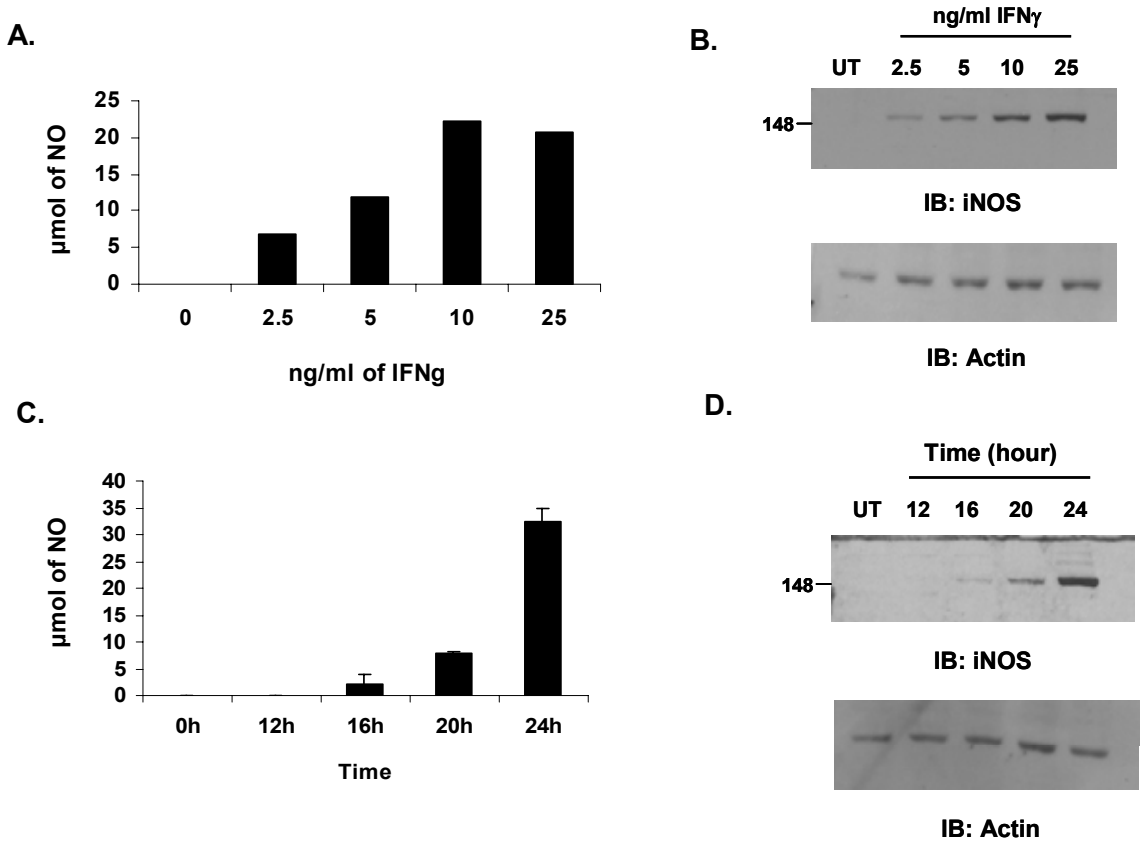


Figure 4.8: IFNγ priming of macrophages induces production of nitric oxide (NO). **A.** Raw 264.7 cells were treated with increasing concentrations of mIFNγ and supernatants were collected at the end of 24 hours and assayed for NO levels. **B.** Protein-matched cell lysates from A were analyzed by Western blotting for iNOS expression. **C.** Raw 264.7 cells were treated with 25 ng/ml mIFNγ for indicated time points. Supernatants were analyzed for NO production. **D.** Cell lysates from C were analyzed for iNOS expression using Western blotting.

being a competitive inhibitor of iNOS enzyme activity, the expression of iNOS protein was not affected by L-NMMA (Figure 4.9B, right panel).

Influence of the PI 3-kinase/Akt pathway on NO production

Having established the requirement for NO production, we next analyzed the role of PI 3-kinase in NO production. Here, Raw 264.7 cells were primed with IFNγ (25 ng/ml) alone or further stimulated by IgG immune-complexes in presence of either DMSO or 10 μM Ly294002 for 8 hours and the amount of NO present in supernatants was measured. As shown in Figure 4.10A, macrophages when primed with IFNγ produced NO. NO production was further enhanced significantly following the activation of Fcγ receptors by immune-complexes. However, inhibition of PI 3-kinase had no effect on the production of NO upon FcγR clustering indicating that the PI 3-kinase/Akt pathway plays no role in FcγR-induced NO production by macrophages. The expression of iNOS protein was examined in these experiments by resolving whole cell lysates using SDS-PAGE and then blotting with iNOS antibody. Consistent with NO levels in the supernatants, iNOS expression was not altered by Ly294002.

These results can be summarized as follows: a) FcγR-mediated TNFα production is regulated by the PI 3-kinase/Akt pathway; however, TNFα is not involved in lysis of Rituximab-coated Raji cells, b) Although nitric oxide is required for macrophage ADCC against Raji cells, activation of the PI 3-kinase/Akt pathway does not contribute to NO synthesis.

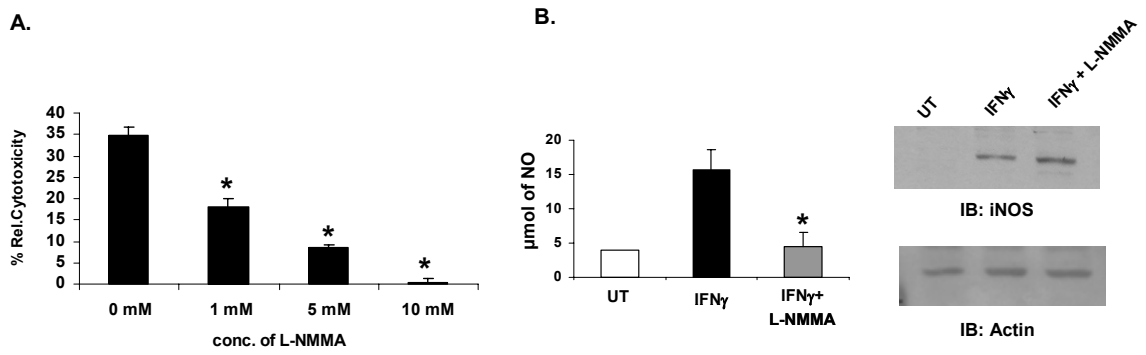


Figure 4.9: NO production is required for macrophage-mediated cytotoxicity against Rituximab-coated Raji cells. A. Raw 264.7 cells were primed with IFNγ in presence of varying concentrations of L-NMMA (0-10 mM). The next day ^{51}Cr- labeled Rituximab-coated Raji targets were added and amount of ^{51}Cr released was measured at the end of 8 hours. The graph shows % relative cytotoxicity at E:T ratio of 50:1. **B.** Raw 264.7 cells were primed with IFNγ in presence of 10 mM L-NMMA for 24 hours. Supernatants were analyzed for levels of NO (left panel) while cell lysates were analyzed for iNOS protein using Western blotting (right panel).

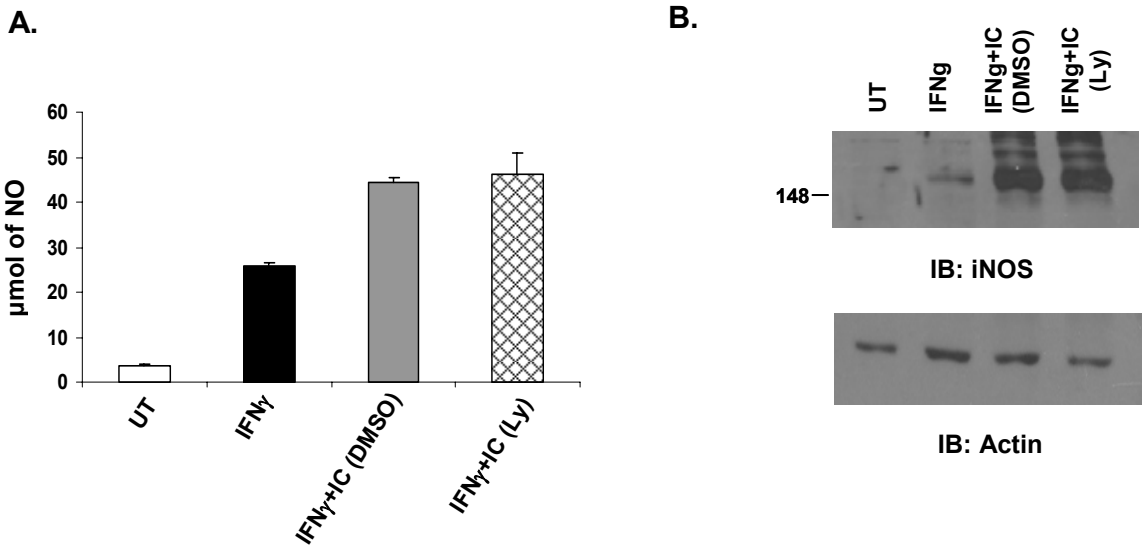


Figure 4.10: Influence of the PI 3-kinase/Akt pathway on NO production. A. Raw 264.7 cells were pre-treated with IFNγ (25 ng/ml) for 16 hours. They were then stimulated with immune-complex (indicated as IC in the figure) at concentration of 350 µg/ml for 8 hours in presence of DMSO or 10 µM Ly294002. Controls conditions consisted of Raw 264.7 cells cultured with media alone (UT) or IFNγ (25ng/ml) alone. Supernatant were harvested at the end of 24 hours and levels of NO produced were measured. **B.** Protein-matched cell lysates of samples from A were analyzed for iNOS expression using Western blotting.

The PI 3-kinase/Akt pathway promotes conjugate formation between macrophages and tumor cells

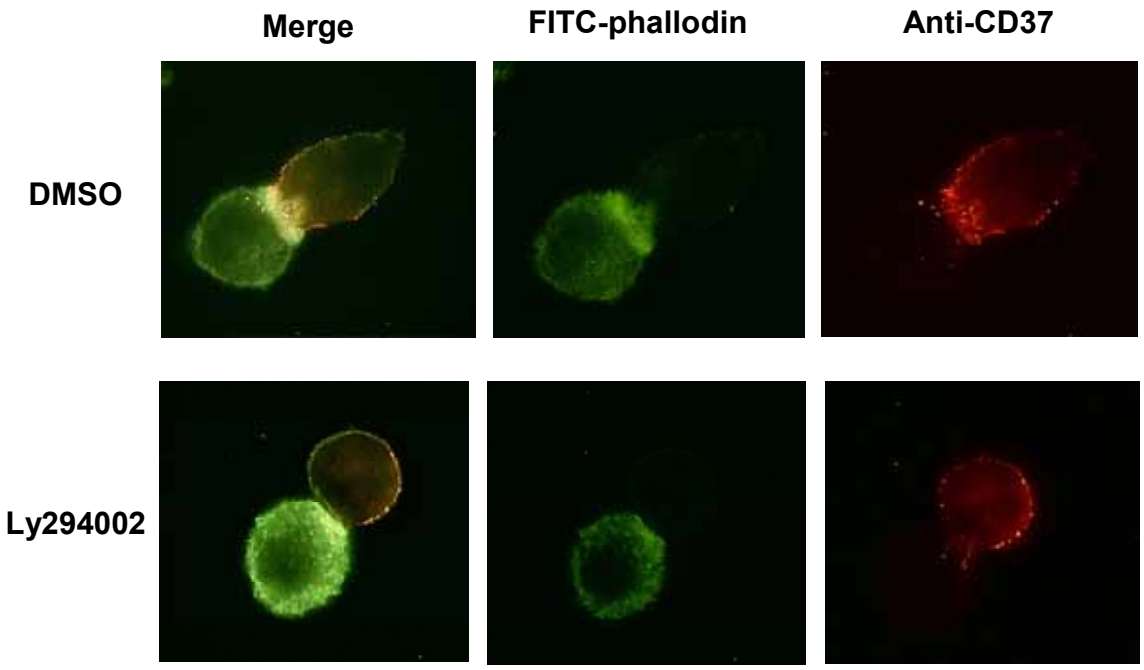
The above results suggest that an event other than the release of cytotoxic mediators is a requisite for cytotoxicity and is probably influenced by the PI 3-kinase/Akt pathway. Many studies have demonstrated that in the case of NK cell cytotoxicity, the process of conjugate formation between the effector NK cell and the target tumor cell is critical and determines the efficacy of killing[186,187]. The two important events involved in conjugate formation include reorganization of the actin cytoskeleton and polarization of microtubule organizing center (MTOC) at the synapse[190,191,202]. It has been shown that actin remodeling is required for the maintenance of conjugate formation and also for the localized release of cytotoxic mediators (reviewed in [203,204]). Several other reports have demonstrated the role of PI 3-kinase in cytoskeletal rearrangements[205-207].

We therefore analyzed whether the loss in macrophage ADCC upon inhibition of the PI 3-kinase/Akt pathway was due to defects in conjugate formation with tumor cells. To visualize the conjugates, we allowed macrophages (pre-treated with DMSO, Ly294002 or rapamycin) and tumor cells mixed at E:T ratio of 5:1 to adhere to poly-L-lysine coated cover-slips. Rituximab-coated Raji tumor cells were then labeled with mouse anti-human CD37 antibody (a B cell-specific cell surface marker) followed by goat anti-mouse Alexa Fluor 594 (red fluorescence) and IFNγ-primed macrophages were stained with FITC-phalloidin (green fluorescence). The visualization of actin polarization at the junction between the macrophage and the Rituximab-coated Raji cell indicated that vehicle treated macrophages showed polarization of actin at the synapse formed with tumor cell, whereas Ly294002 treated macrophages failed to show significant actin

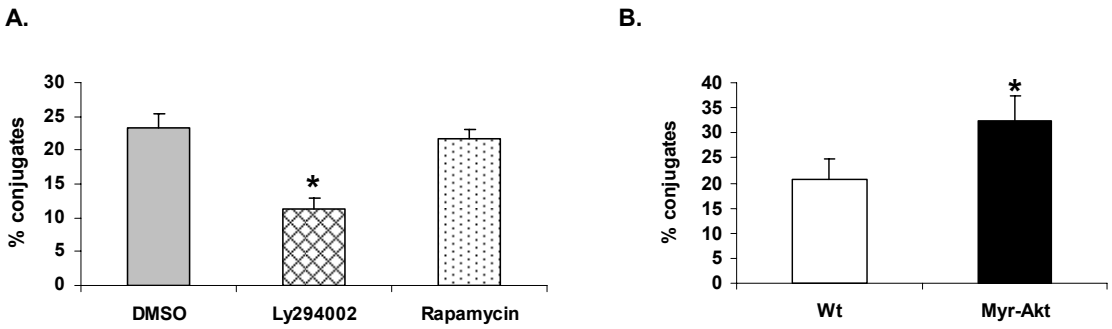
polarization (Figure 4.11). To measure the number of conjugates, 300 tumor cells were counted for each condition and were scored for conjugate formation. The experiment was repeated at least three times. As shown in Figure 4.12A, pre-treatment of macrophages with Ly294002 reduced their ability to form conjugates with tumor cells. There was no reduction in percent conjugates formed upon rapamycin treatment. Similar experiments were performed using peritoneal macrophages isolated from WT and Myr-Akt mice. Myr-Akt macrophages displayed significantly increased conjugate formation with antibody-coated tumor cells (Figure 4.12B). These data indicate that the PI 3-kinase/Akt pathway plays a role in promoting the contact between macrophages and antibody-coated tumor cells.

The PI 3-Kinase/Akt pathway enhances the spreading of macrophages on antibody-coated surfaces

As an alternative approach, we analyzed the ability of macrophages to spread on Rituximab-coated surfaces. Earlier studies have indicated that spreading of macrophages on human IgG-coated surfaces involves the insertion of membrane from intra-cellular source and is dependent upon PI 3-kinase[43]. When we compared the surface area occupied by peritoneal macrophages treated with DMSO or Ly294002 on Rituximab-coated surfaces, it was clearly evident that the spreading of macrophages upon inhibition of PI 3-kinase was restricted to a significant extent (Figure 4.13A). The surface area occupied by these cells was measured using Morphometry software and showed a significant difference in the areas occupied by DMSO treated cells as opposed to those



<u>Figure 4.11:</u> The PI 3-kinase/Akt pathway influences actin polarization at the contact point between macrophages and tumor cells. Raw 264.7 cells were primed with IFNγ overnight followed by treatment with DMSO or 10 μM Ly294002 for 30 minutes. They were then mixed with Rituximab-coated Raji cells at E:T ratio of 5:1 and adhered to poly-L-lysine coated cover-slips for 1 hour. Raji cells were labeled with CD37 antibody followed by anti-mouse Alexa Fluor 594 (red fluorescence). F-actin was labeled with FITC-phalloidin. Stained cells were observed under the microscope for action polarization at the synapse.



<u>Figure 4.12:</u> The PI 3-kinase/Akt pathway promotes conjugate formation between macrophages and tumor cells. Raw 264.7 cells were primed with IFNγ overnight followed by treatment with DMSO or 10 μM Ly294002 for 30 minutes. They were then mixed with Rituximab-coated Raji cells at E:T ratio of 5:1 and adhered to poly-L-lysine coated cover-slips for 1 hour. Raji cells were labeled with CD37 antibody followed by anti-mouse Alexa Fluor 594 (red fluorescence). F-actin was labeled with FITC-phalloidin. Stained cells were observed under the microscope and 300 red cells were scored per condition for conjugate formation. The data indicate % conjugates formed in one experiment. Similar results were obtained in three independent experiments. **B.** Conjugate formation by WT and Myr-Akt expressing peritoneal macrophages was determined by processing the samples as described in A.

treated by Ly294002 (Figure 4.13B). On the other hand, Myr-Akt macrophages occupied a significantly greater surface area as compared to WT macrophages on Rituximab-coated surfaces (Figure 4.13C).

Taken together, these findings illustrate that the PI 3-kinase/Akt pathway plays a critical role in macrophage response to Rituximab-coated tumor cells at least in part through its influence on conjugate formation between the macrophages and the target tumor cells.

4.5 Discussion

In this chapter, we demonstrate that the activation of Akt by PI 3-kinase plays an important role in macrophage mediated ADCC against Rituximab-coated Raji cells. The PI 3-kinase/Akt pathway was found to be required for efficient conjugate formation as well as spreading of macrophages on antibody-coated surfaces. In the case of NK cells, studies have shown that conjugate formation is a critical step for the lysis of tumor cells[186,187]. Our findings similarly indicate that cytoskeletal rearrangements and thereby conjugate formation constitute a prerequisite for effective cytolysis of antibody-coated tumor cells by monocytes and macrophages as well.

PI 3-kinase regulates a diverse array of cellular activities such as cell growth, survival, cytoskeletal changes and trafficking of intra-cellular organelles. The activation of PI 3-kinase leads to changes in the actin cytoskeleton through the activation of various downstream proteins. PI 3-kinase-dependent activation of Vav leads to the activation of Rac1 which has been shown to be involved in actin reorganization[38,208]. During FcγR-mediated phagocytosis, our group has demonstrated that activation of Akt downstream of

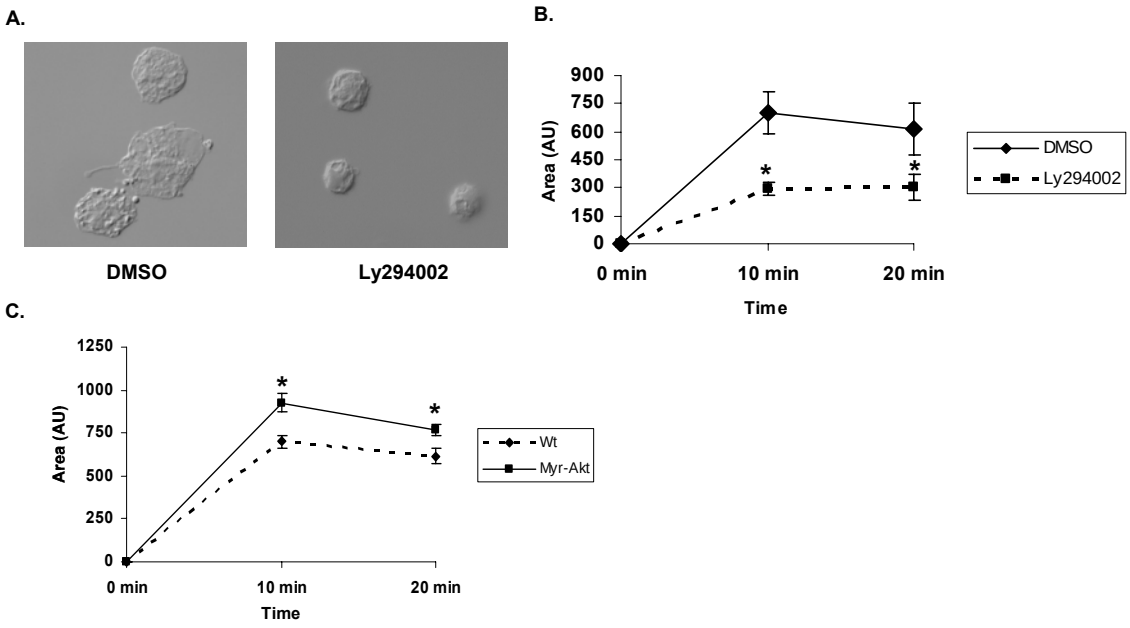


Figure 4.13: The PI 3-Kinase/Akt pathway enhances the spreading of macrophages on antibody-coated surfaces. A. Spreading of thioglycollate-elicited peritoneal macrophages on Rituximab-coated cover-slips in the presence of DMSO or 10μM Ly294002. **B.** Peritoneal macrophages, pre-incubated with DMSO or 10 μM Ly294002, were allowed to spread on Rituximab-coated cover-slips at 37°C for indicated times before fixation. Data are presented as the mean surface area occupied by cells. Shown are data from one experiment. Similar results were obtained from three independent experiments. **C.** Mean surface area occupied by Wt and Myr-Akt peritoneal macrophages.

PI 3-kinase up-regulates phagocytic process through the activation of p70S6 kinase[47]. Arf6 is reported to be essential for actin assembly during FcγR-mediated phagocytosis as well as for NK cell ADCC and the activation of Arf6 depends on PI 3-kinase[45,188]. Further, it has been shown that PI 3-kinase activity is also involved in the regulation of membrane availability during engulfment of large size particles[43,209]. In this report, we show that PI 3-kinase activation during macrophage response to antibody-coated tumor cells leads to the activation of Akt and thereby regulates the cytotoxic response, at least in part, by promoting effective contact between macrophages and tumor cells. In addition to the activation of Akt, PI 3-kinase may also be involved in the regulation of macrophage-mediated cytotoxicity through its influence on other proteins involved in actin remodeling as discussed earlier. Further studies are required to test the possible involvement of PI 3-kinase with respect to these pathways.

The role of PI 3-kinase during actin remodeling is well established. However, few studies report the involvement of Akt as a modulator of cytoskeletal changes. Studies in chicken embryonic fibroblasts indicated that activation of Akt by PI 3-kinase induced actin reorganization promoting cell migration[210]. In the same study, authors demonstrated that constitutive activation of Akt was sufficient to activate actin assembly and resulted in increased cell migration even in the presence of Ly294002. A previous study from our group showed that constitutively active Akt increased the phagocytic efficiency of macrophages suggesting that over-active Akt enhances the cytoskeletal rearrangement[47]. In both studies, it was further demonstrated that Akt stimulates actin cytoskeleton through p70S6 kinase. Interestingly, our findings reported here show that inhibition of p70S6K by rapamycin has no effect on the ability of macrophages to lyse Rituximab-

coated B cells. This suggests that Akt can modulate actin assembly in other ways. Recently, it has been shown that Akt directly interacts with actin leading to phoshorylation of actin at Akt consensus sites in Ly294002-dependent manner[211]. Akt has been shown to associate with and phosphorylate yet another protein involved in actin remodeling called Girdin[212].

IFNγ is known to be a potent activator of macrophage tumoricidal activities[176,177]. The treatment of macrophages with IFNγ has been reported to stimulate TNFα and nitric oxide production by macrophages[178,179]. Our data indicate that activation of FcγR upon IFNγ priming results in enhanced TNFα and nitric oxide production. Fc receptor clustering is known to induce TNFα production even in absence of IFNγ priming. Thus a synergistic increase in TNFα production upon co-stimulation with IFNγ and antibody-coated tumor cells was not surprising. However, to our knowledge, this is the first report demonstrating enhanced NO production by IFNγ-primed macrophages when stimulated further by immune-complexes.

The inability of IFNγ-primed macrophages to lyse B cells in the absence of antibody opsonization has several implications. It indicates that FcγR clustering on macrophages by Rituximab-coated B cells is an important event and contributes to the conjugate formation with tumor cells. The priming of macrophages by IFNγ alone leads to the production of nitric oxide, which is essential for the cytotoxicity towards B cells. However, mere release of nitric oxide is not sufficient for cytolysis of B cells if they are not occupied by a specific antibody. The activation of the PI 3-kinase/Akt pathway by

antibody-coated tumor cells results in stable ligation of tumor cells with macrophages thereby allowing the killing of targets by nitric oxide.

Collectively, our findings demonstrate a critical role for the PI 3-kinase/Akt pathway in macrophage-mediated cytotoxicity against Rituximab-coated B cell lymphomas. We show that the PI 3-kinase/Akt pathway is required for occupation of greater surface areas on antibody-coated surfaces. Moreover, the formation of efficient cytolytic synapse between macrophages and Rituximab-coated B cells is completely dependent upon the PI 3-kinase/Akt pathway.

CHAPTER 5

SUMMARY AND PERSPECTIVES

Macrophages play an important role in the clearance of antibody-coated targets such as immune-complexes and antibody-coated tumor cells via the engagement of Fcγ receptors. These processes are often accompanied by the release of inflammatory mediators such as pro-inflammatory cytokines and reactive oxygen and nitrogen species, which if released in excess, can cause collateral tissue damage. Therefore, FcγR-mediated responses are subjected to tight regulation. The regulation is mainly achieved through simultaneous engagement of activating and inhibitory Fcγ receptors that associate preferentially with kinases and phosphatases respectively.

Work in recent years has shown that the engagement of Fcγ receptors, particularly on monocytes and macrophages, forms the basis of *in vivo* cytotoxicity of monoclonal antibodies administered for the treatment of malignant disorders[9]. In this study, mice that lacked activating FcγR could not clear the tumor load upon antibody treatment. On the other hand, mice deficient in inhibitory FcγRIIb receptor showed complete clearance of tumor upon antibody therapy. This implies that a condition that favors the expression of activating Fcγ receptors and downmodulates the expression of inhibitory Fcγ receptors is

beneficial for the elimination of tumors. On the contrary, such a situation is detrimental during autoimmune diseases. The lack of inhibitory FcγRIIb in mice rendered them susceptible to autoimmune diseases such as collagen-induced arthritis (CIA) whereas mice that lack activating FcγR were protected from CIA[213,214]. Taken together, these findings suggest that modulation of the Fcγ receptor expression towards activating or inhibitory side may not prove beneficial as a therapeutic strategy. On the other hand, knowledge of the signaling pathways that are activated only under the specific set of conditions may help us in monitoring the disease states better. Thus, a thorough understanding of Fcɣ receptor-mediated responses and their regulation can help improve the efficacy of therapeutic interventions for cancer and autoimmune diseases. In this study we have analyzed the molecular mechanisms involved in regulation of FcγR-mediated responses in macrophages.

In the first part, we investigated the functional differences between the two isoforms of FcγRIIb, namely FcγRIIb1 and FcγRIIb2, generated by alternative splicing. The sole difference between these two isoforms is that the b1 isoform contains a 19 amino acid insert in its cytoplasmic tail. Several studies in the past have examined the differences between the two isoforms of FcγRIIb in B cells. However, this is the first study examining the functional differences between FcγRIIb1 and FcγRIIb2 in macrophages. Since both the isoforms of FcγRIIb are almost identical, there are no antibodies that can differentiate between the two isoforms, thus making it difficult to study them in isolation. To overcome this problem, we transfected mouse macrophages with plasmids encoding either human FcγRIIb1 or b2, allowing us to study the functional

capabilities of the two isoforms in isolation. Our findings demonstrated that both FcγRIIb1 and FcγRIIb2 inhibit the macrophage-mediated phagocytosis and TNFα production.

Earlier studies in B cells indicated that the b2 isoform is endocytosed in contrast to b1[17,124-126]. The process of endocytosis is of interest; particularly in macrophages as they present a part of endocytosed antigen to initiate adaptive immune responses. However, whether there exist any differences in the endocytic potential of these two isoforms in myeloid cells remains to be tested.

Our data indicate that both isoforms are capable of inhibiting phagocytosis; however it is possible that the efficiency and the mechanisms involved during inhibition may differ between the two isoforms. The presence of the extra 19 amino acids in FcγRIIb1 isoform may lead to more stabilized interactions between the b1 isoform and the associating proteins or may result in the recruitment of additional effector proteins. To this end, the sequence of 19 amino acids in FcγRIIb1 shows some interesting features. The sequence is as follows: - GYPECREMGETLPEKPANP. Thus the insert contains a tyrosine residue which can potentially be phosphorylated and may recruit SH2 domain-containing proteins. In a recent study using phosphotyrosil peptides, Imhof et al demonstrated that the presence of a hydrophobic (argenine or histidine) or a positively charged amino acid (lysine) at +4 position from the position of pY favors the binding of N-terminal SH2 domain of protein tyrosine phosphatase SHP-1[215]. Analysis of the 19 amino acids sequence indeed indicates the presence of arginine (indicated by R) at +4 position from the tyrosine. Thus, if FcγRIIb1, but not FcγRIIb2, associates with SHP-1 in vivo, it may confer additional inhibitory potential to b1 in comparison to b2. Thus it

would be interesting to see if there are any differences in the associations of proteins with these two receptor isoforms.

The inositol phosphatase SHIP-1 is the primary effector of FcγRIIb-mediated inhibition. The lack of SHIP-1 in macrophages has been shown to upregulate FcγR-mediated and complement receptor 3 (CR3) mediated phagocytosis[97]. Kalesnikoff et al demonstrated that SHIP-1 negatively regulates IgE + Ag-induced IL-6 production in mast cells[216]. However, whether SHIP-1 influences inflammatory cytokines production and ROS production that accompanies the process of phagocytosis in macrophages was not known. The present data show for the first time that SHIP-1 regulates the production of FcγR-induced IL-6 and IL-1β production and ROS generation in macrophages.

Our findings show that SHIP-1 downregulates immune-complex induced Rac activation and subsequent ROS generation. We demonstrate that activation of Ras/Erk pathway is required for the production of IL-1β whereas IL-6 production is accomplished by activation of both, the Ras/Erk pathway and the PI 3-kinase pathway. Interestingly, although both the Ras/Erk and the PI 3-kinase pathway contribute to IL-6 production, our data indicate that there is no cross-talk between these two pathways during FcγR-induced signaling. This is in contrast to growth factor induced signaling or that observed during NK cell-mediated natural cytotoxicity where PI 3-kinase acts upstream of Ras/Erk activation. This observation is worthy of note as it suggests that the effects mediated by one pathway can be modulated without affecting the other pathways.

The second remarkable observation that emerged from this study is that SHIP-1 regulates the activation of the Ras/Erk pathway through its non-catalytic domains while the activation of PI 3-kinase pathway is regulated by the catalytic activity of SHIP-1. The

catalytic activity of SHIP-1 hydrolyzes PtdIns3,4,5P_3 to PtdIns3,4P_2 and thereby antagonizes the effects of PI 3-kinase activation. The non-catalytic domains of SHIP-1 interact with several proteins, for example, SH2 domain interacts with tyrosine phosphorylated ITAM motifs of the receptor, proline-rich domain (PRD) interacts with SH3 domain containing proteins while NPXY motifs bind to phospho-tyrosine binding (PTB) domain containing proteins. In the case of B-cells, SHIP-1 has been shown to downregulate the Ras/Erk activation. Two models have been proposed in the case of B cells: i) the SHIP-1 SH2 domain competes with Grb2/Sos complex for binding to phosphorylated Shc and thereby downregulates Ras activation[79,88], and ii) that SHIP-1 associates with p62dok, which results in hyperphosphorylation of dok, its association with RasGAP and the subsequent hydrolysis of Ras-GTP[87]. The mechanism by which SHIP-1 downregulates the Ras/Erk pathway in macrophages needs to be tested. It is possible that one or both of these mechanisms may be active in macrophages or there may exist a completely different pathway by which SHIP-1 downregulates Erk activation. Co-immunoprecipitation, co-localization and kinetic studies of SHIP-1 association with signaling intermediates involved in the Ras/Erk pathway will be helpful in unraveling the mechanisms of SHIP-1 influence on this pathway.

We show that SHIP-1 negatively regulates the activation of Rac, a small GTPase required for the assembly of functional NADPH oxidase complex and that the catalytic domain of SHIP-1 is involved in this regulation. The activation of Rac is facilitated by a guanine nucleotide exchange factor Vav which is activated in PI 3-K dependent manner[38,208]. Thus SHIP-1 may modulate Rac activation by influencing the PI 3-K

dependent Vav activation upstream of Rac. Further studies are required to test the mechanism by which SHIP-1 influences Rac activation.

Previous studies have shown that other than Rac activation, phosphorylation of p47phox is also essential for the assembly of functional NADPH oxidase complex. Akt has been shown to phosphorylate Ser 304 and Ser 328 residues of p47phox[217,218]. Therefore the possibility exists that SHIP-1 may influence ROS production through its influence on p47phox activation in addition to its effect on Rac activation. One approach to test this would be to examine the levels of p47phox phosphorylation in SHIP-1 sufficient and SHIP-1 deficient macrophages as well as in macrophage cell lines stably transfected with SHIP-1 constructs. However, the lack of antibodies specific for the phosphorylated form of p47phox may become a limiting factor. In such a case, an alternative method employing incorporation of radioactive ^{32}P isotope may be tested.

Our findings described in this thesis provide new insights into the role of FcγRIIb and SHIP-1 in the phagocytosis of immune-complexes by macrophages. Macrophages are also capable of clearing tumor cells coated with antibodies through a process called antibody-dependent cell-mediated cytotoxicity (ADCC). The process of ADCC forms the basis of monoclonal antibody-based therapies in the treatment of cancer. The role of NK cells in mediating ADCC has been extensively studied. However, very little is known about macrophage-mediated ADCC. Also, although the role of macrophages in tumor clearance has been acknowledged, it has been confounded by recent studies where tumor-associated macrophages (TAM) have been shown to facilitate tumor progression. In these studies, factors derived from tumor cells have been proposed to manipulate the cytotoxic effects of macrophages[219]. We therefore believe that a detailed analysis of the signaling

pathways involved in macrophage-mediated cytolysis of tumor cells could help us in the modulation of macrophage responses towards antibody-coated tumor cells.

To study macrophage-mediated ADCC, we used human B cell lymphoma Raji cells coated with Rituximab as the tumor target and IFNγ primed mouse macrophages as the effectors. The priming of macrophages by IFNγ induces potent tumoricidal activity in macrophages. IFNγ upregulates the expression of several genes, however, enhanced expression of FcγRI, TNFα and iNOS is of particular importance in the context of ADCC. Our experiments analyzing the cytolysis of Rituximab-coated Raji cells by macrophages reconfirmed the requirement of IFNγ for macrophage tumoricidal activity. However, such requirement of priming stimulus by macrophages may become a limiting factor for their tumoricidal potential *in vivo*. For several years, it has been thought that NK cells and activated T cells act as the major sources of IFNγ. However, recent studies indicate that macrophages from different tissues are capable of producing IFNγ by themselves in presence of IL-12 or upon infection by bacteria (reviewed in [220]). Although not a scope of this work, it would be an interesting pursuit to see whether tumor-associated macrophages show such capability to produce IFNγ and if not, whether tumor-derived factors suppress their ability to do so.

The results summarized in Chapter 4 demonstrate a critical role for the PI 3-Kinase/Akt pathway in macrophage-mediated cytotoxicity towards Rituximab-coated Raji cells. We also show that NO production by macrophages is essential for lysis of tumor cells. As illustrated by the studies in Chapter 3, the inositol phosphatase SHIP-1 negatively regulates the PI 3-K/Akt pathway. Thus, it is reasonable to hypothesize that

macrophages deficient in SHIP-1 may show enhanced cytolysis of tumor cells. However, Rauh et al recently demonstrated that peritoneal and alveolar macrophages isolated from SHIP-$1^{-/-}$ mice show impaired LPS-induced NO production[221]. Thus, although SHIP-$1^{-/-}$ macrophages show enhanced Akt activation, reduced ability of these macrophages to produce NO may compromise their ability to kill tumor cells. Further studies are therefore required to test the effect of SHIP-1 on macrophage-mediated ADCC.

Our studies show that the PI 3-kinase/Akt pathway regulates macrophage ADCC through its influence on the conjugate formation event with tumor cells. Similarly, inhibition of Rac-1 in NK cells has been shown to inhibit NK cell cytotoxicity due to reduced conjugate formation[186]. Interestingly, the cytotoxic ability of NK cells isolated from patients with Wiskott-Aldrich syndrome is greatly compromised due to defective conjugate formation[187]. Studies analyzing the tumoricidal potential of monocytes and macrophages isolated from these patients will further enhance our understanding about the process of conjugate formation and its contribution to tumor cell killing.

We show that downstream of PI 3-kinase activation, Akt enhances the cytotoxic ability of macrophages. Studies in NK cells illustrate that Vav-Rac1 pathway and Arf6 activation play an important role in the regulation of cytotoxicity[186,188]. During FcγR-induced signaling in macrophages, PI 3-K has been shown to be upstream of both Vav-Rac1 and Arf6 activation[38,45,208]. Thus, in addition to its effect on Akt, PI-3K may enhance tumor cell killing through its effect on other signaling intermediates involved in actin cytoskeleton rearrangement.

The results described in Chapter 4 illustrate that activation of Akt in macrophages upregulate their cytotoxic potential. However, activation of Akt has also been implicated

in several malignant cells. Therefore, an utmost care is required for Akt to be considered as a therapeutic target. On the other hand, a signaling protein downstream of Akt or downstream of PI 3-K, other than Akt as described above that influences the conjugate formation event may serve as a better target for therapeutic intervention.

In conclusion the findings described in this dissertation have contributed significantly to our understanding of the molecular mechanisms involved in FcγR-induced phagocytosis and ADCC in macrophages.

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