

Development of imaging-based techniques to examine signaling properties of channel catfish
(*Ictalurus punctatus*) leukocyte immune-type receptors (IpLITRs)

by

Lena Ocampo Jones

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Department of Biological Sciences
University of Alberta

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ABSTRACT

Cells of the innate immune system have the ability to rapidly detect and eliminate pathogens using surface-expressed immunoregulatory receptors that translate extracellular binding into effector functions such as degranulation, cytokine secretion, cell-mediated cytotoxicity, and phagocytosis. Teleost leukocyte immune-type receptors (LITRs) are a family of immunoregulatory receptors capable of inducing effector responses when heterologously expressed in mammalian immune cells. The mechanisms by which these receptors induce effector responses remain unknown; however, these effector responses are all dependent on cytoskeletal rearrangements. Many of the intracellular signaling components that facilitate cytoskeleton remodelling are conserved through evolution due to the ubiquitous requirement for cells to change shape or move. The main objective of my thesis was to develop imaging-based assays to examine LITR-induced signaling activities that culminate in effector responses such as phagocytosis. Using the phagocytic process as a bioassay, I developed experimental strategies employing cellular transfections, imaging flow cytometry, and confocal microscopy to monitor the LITR-mediated phagocytic process and associated signaling. I showed that two LITR-types induced very distinct cytoskeletal remodelling patterns to induce a phagocytic response. Additionally, cytoskeletal remodelling was mirrored by the distribution of a phospholipid species known to participate in the induction of filamentous actin (the main cytoskeletal component) polymerization. Overall, my work set the stage for testing potential signaling activities that IpLITRs are believed to induce.

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LIST OF ABBREVIATIONS

Abp	Actin binding protein
AD293	Semi-adherent derivative of HEK293 cells
ADCC	Antibody-dependent cellular cytotoxicity
AF647	Fluorophore with 650/665nm excitation/emission peaks
AKT	Protein kinase B, serine/threonine-specific protein kinase
BB	Fluoresbrite® carboxy Bright Blue 4.5 µm microspheres
BLASTp	Protein basic local alignment search tool
C57BL/6	Inbred strain of laboratory mouse
CD150	Signaling lymphocytic activation molecule 1
CD3ζ-L	CD3 zeta-like subunit
CD94/NKG2	Family of C-type lectin receptors on NK cells
cDNA	Complementary deoxyribonucleic acid
CEACAM	Carcinoembryonic antigen-related cell adhesion molecules
CR3	Complement receptor 3
CSF	Colony stimulating factors
Csk	C-terminal Src kinase
Cy5	Indodicarbocyanine fluorophore, 650/670nm excitation/emission peaks
D-PBS	Dulbecco's phosphate buffered saline
D1-D5	Immunoglobulin domain 1-5
DAP12	ITAM-containing intracellular adapter molecule
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ERK	Extracellular signal-regulated kinase
EST	Expressed sequence tag
FYVE	Zinc-binding domain specific for PI(3)P
FBS	Heat-inactivated fetal bovine serum
Fc	Fragment crystallizable
FcR	Fragment crystallizable receptor
FCRL	Fc receptor-like
FcRγ	FcR γ-chain
FcRγ-L	FcR γ-chain-like
Fcγ	Fragment crystallizable portion of Igγ
Fcε	Fragment crystallizable portion of Igε
FcεRI	High-affinity Fc receptor for IgE
FcμR	Fc receptor for IgM
FLAG	Epitope tag encoding amino acid residues DYKDDDDK
Gab2	Grb2-associated binder
GFP	Green fluorescent protein

GST	Glutathione S-transferase
GαM Cy5	Cy5-conjugated antibody raised in goat against mouse Ig
GαM PE	Phycoerythrin-conjugated antibody raised in goat against mouse Ig
H + L	Heavy and light chain
HA	Hemagglutinin epitope tag
HEK-293	Human embryonic kidney cells
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IgSF	Immunoglobulin Superfamily
IgT/IgZ	Teleost-specific immunoglobulin (T or Z in depends on teleost species)
IL	Interleukin
IL-1β	Interleukin 1 beta
IpLITR	<i>Ictalurus punctatus</i> leukocyte immune-type receptors
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
itim	ITIM-related sequence
ITSM	Immunoreceptor tyrosine-based switch motif
KIR	Killer immunoglobulin-like receptor
LAT	Linker of activated T cells
LB	Luria-Bertani or Lysogeny broth
LILRs	Leukocyte immunoglobulin-like receptors
LITR	Leukocyte immune-type receptors
LRC	Leukocyte receptor complex
Ly49D	Mouse NK cell receptor with ITAMs
Ly49H	Mouse NK cell receptor with ITAMs
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MLC	Mixed leukocyte culture
mRNA	Messenger ribonucleic acid
NCC	Nonspecific cytotoxic cells
Nck1	Non-catalytic region of tyrosine kinase adaptor protein 1
nIL-1Fm	Novel IL-1 family member
NILT	Novel immunoglobulin-like transcript
NITR	Novel immune-type receptor
NITR11	Novel immune-type receptor 11
Nitr3.1	Novel immune-type receptor 3.1
Nitr9L	Novel immune-type receptor 9 long transcript

NK	Natural Killer cell
NK-92	Human NK cell line
NKG2C	C-type lectin, activating natural killer cell receptor
NKp44	Human NK cell-specific receptor
nm	Nanometers
pAb	Polyclonal antibody
PBL	Peripheral blood leukocyte
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
PECAM-1	Human platelet endothelial cell adhesion molecule
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI(3,4,5,)P ₃	Phosphatidylinositol 3,4,5-trisphosphate
PI(3,4)P ₂	Phosphatidylinositol 3,4-bisphosphate
PI(3,5)P ₂	Phosphatidylinositol 3,5-bisphosphate
PI(3)P	Phosphatidylinositol 3-phosphate
PI(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate
PI(4)P	Phosphatidylinositol 4-phosphate
PI(5)P	Phosphatidylinositol 5-phosphate
PI3K	Phosphatidylinositol 3-kinase
pIgA	Polymeric immunoglobulin receptor for IgA
PIGRL/pIgRs	Polymeric immunoglobulin receptor-like proteins
PKC	Protein kinase C
PLC	Phospholipase
RBL-2H3	Rat basophil leukemia cells
rcf	Relative centrifugal force
RαM AF647	Alexa Fluor® 647-conjugated antibody raised in rabbit against mouse Ig
SH2	Src homology 2
SH2D1A	SH2-containing adaptor protein 1A
SHIP 1	SH2-containing inositol-5'-phosphatase 1
SHIP2	SH2-containing inositol-5'-phosphatase 2
SHP-1	SH2 domain-containing phosphatase 1
SHP-2	SH2 domain-containing phosphatase 2
Siglecs	Sialic acid-binding Ig-type lectins
Syk	Spleen tyrosine kinase
TAE	Tris base, acetic acid, EDTA
TCR	T cell receptor
TGF	Transforming growth factor
TKB1	<i>Escherichia coli</i> strain with inducible tyrosine kinase gene
TM	Transmembrane
TNF	Tumor necrosis factor
TREM	Triggering receptor expressed on myeloid cells

v/v	Volume to volume
w/v	Mass to volume
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verpolin-homologous protein
WT	Wild type (or full-length)
α HA	Anti-hemagglutinin tag

CHAPTER 1

Introduction

1.1 Introduction

The innate immune system is the first line of defense against infection in all animals.

Cells of the innate immune system are found ubiquitously throughout the body to serve as first responders. Using germline-encoded genes, innate immune cells are capable of rapidly detecting and eliminating foreign invaders by inducing effector responses such as degranulation (release of granules containing potent antimicrobial factors); phagocytosis (internalization of large particles, such as pathogens, followed by their intracellular destruction); cellular cytotoxicity (direct killing of virally infected, sick, or transformed host cells); and secretion of cytokines (soluble proteins used for immune cell communication). Innate immune cells express immunoregulatory receptors on their cell surface that allow them to sense their environment and induce effector responses. In addition, there are subsets of immunoregulatory receptors that inhibit effector responses. The precise balance between activating and inhibiting signals allows innate immune cells to provide protection from invaders while also sparing host tissues. Immunoregulatory receptors bind a variety of different ligands; therefore they are highly diversified within a species or between species. As a result, there are many different types or families of immunoregulatory receptors identified to date. However, they all share in common the ability to detect their extracellular ligands and translate binding into potent effector responses.

Leukocyte-immune type receptors (LITRs) were discovered in channel catfish (*Ictalurus punctatus*) immune cells and belong to a large family of receptors that are related to many mammalian immunoregulatory receptors. Like many teleost immunoregulatory receptor families, the ligands to these receptors remain unknown; therefore characterization of these receptors has relied largely on heterologous expression in mammalian cells. The serendipitous discovery that certain LITR types have the ability to translate target binding into engulfment (i.e. phagocytosis) has provided a useful platform to examine aspects of LITR-mediated signal transduction (Cortes et al, 2012, and 2014). Target engulfment requires cytoskeletal rearrangement, which is a process that relies on highly conserved signaling networks. Therefore by studying the ability of LITR-expressing cells to internalize targets, we can gain insight into the mechanisms by which these receptors promote cytoskeletal rearrangements.

1.2 Objectives of this thesis

The main objective of my research was to develop imaging-based bioassays to examine signal transduction that is induced by two IpLITR-types during the process of internalization of targets. The specific aims of my thesis are:

- 1) To establish procedures for the discrimination of surface-bound, partially engulfed and fully internalized IpLITR targets
- 2) To develop co-transfection and imaging-based strategies to monitor cytoskeletal changes that are induced by IpLITRs

- 3) To use confocal microscopy to examine the distribution of phosphoinositide species during the IpLITR-mediated phagocytic process

1.3 Outline of the thesis

In the second chapter of my thesis, I review innate immunity in bony fish with specific emphasis on innate immune cellular functions and immunoregulatory receptor families that have been identified in fish. In the same chapter, I will discuss channel catfish as an alternative model organism for immunological studies and review literature on IpLITRs. The methods and materials I used in my studies are detailed in Chapter 3. In Chapter 4, I will discuss the development of imaging flow cytometry and confocal microscopy – based techniques to examine the cellular cytoskeleton during IpLITR-induced phagocytosis. I show that the ability of IpLITR 1.1b/ITAM and IpLITR 1.1b/WT to capture and engulf targets in rat mast cells (RBL-2H3) is conserved when these proteins are expressed in a human epithelioid cell (AD293) (Lillico et al, 2015). I show that AD293 cells stably expressing IpLITRs can be subsequently co-transfected with fluorophore-tagged probes without significantly altering the surface expression of the receptors or the ability of these receptors to induce the phagocytic process. Lastly, I found that two IpLITR-types induced very distinct patterns of cytoskeleton remodelling which is likely due to the differential nature of the signaling pathways the two receptors activate. In Chapter 5, I apply the experimental strategy used in Chapter 4 to monitor the phosphoinositide distribution during the IpLITR-induced phagocytic process. I found the re-distribution of phosphatidylinositol 4,5-bisphosphate that is induced by the two

IpLITR-types mirrored the distinct patterns of cytoskeletal remodelling that each receptor induced in Chapter 4. In addition, I show that IpLITR targets are internalized into a phagosome that gains phosphatidylinositol 3-phosphate identity during the early stages of phagosome maturation. In Chapter 6, I will summarize my results and discuss the value of using a combination of phagocytosis and confocal microscopy to monitor IpLITR-mediated phagocytosis and to test the IpLITR-induced recruitment of candidate signaling molecules to the site of phagocytosis.

CHAPTER 2

Literature Review

2.1 Overview

In all animals (vertebrates and invertebrates alike), innate immunity represents a vital first line of protection from invading pathogens, and promotes the destruction of foreign invaders while mitigating substantial damage to host cells and tissues.

Anatomical barriers such as skin, tegument, scales and mucous membranes shield animals from invading pathogens. However, when these innate barriers are breached, cells of the innate immune system are the first to encounter pathogens in the periphery.

Cells of the innate immune system use potent antimicrobial strategies to effectively recognize and destroy pathogens within minutes to hours after pathogen encounter.

This recognition is mediated in part by germline-encoded surface-expressed, immunoregulatory receptor proteins that translate extracellular binding into cellular effector functions. In addition to activating immune responses against pathogens, immunoregulatory receptors also control the suppression of immunity to promote resolution of innate immune cell actions. The balance between stimulatory and inhibitory responses is critical for the initiation, control, and termination of immune responses to prevent host damage and promote tissue repair. In addition to the germline-encoded innate responses, vertebrates also have adaptive immune responses that provide more specificity and long-lasting immunity when 'instructed' to do so by the innate response. However, adaptive immunity takes time to generate and relies on

activation that is mediated by the innate immune response. As a result, all animals primarily rely on innate immunity as a first line of defense.

Teleosts (bony fish) are a large group of ectothermic vertebrates (>20,000 species) that diverged from mammals ~450 million years ago. Aquatic habitats place teleosts in intimate contact with potential pathogens, therefore, teleosts have developed robust innate immune defense strategies to rapidly detect invaders. Many of the fundamental components known to be important for immunity in mammals are also present in teleost fishes including effector cells that mediate immunity, as well as dedicated tissues and organs for the organization of immune cells. However, the receptors that control innate immunity (i.e. immunoregulatory receptors) in teleosts and their associated mechanisms of inducing function are not well understood. In recent years, the increased the availability of fish genomic databases as well as expressed tag sequence (EST) libraries developed from the cDNA of teleost immune tissues and cells has made it possible to identify teleost immunoregulatory receptor families. Potential functions for these receptor families have been predicted based on indirect examinations such as phylogenetic resemblance with mammalian receptor families (Rodriguez-Nunez 2014; Yoder Litman 2011; Montgomery 2011; Fei et al 2016); nevertheless, the exact roles these proteins play in the innate immune response of fish remains to be determined.

In this review, I will provide a brief overview of mammalian innate immunity to establish a comparative framework for my studies regarding teleost immunity. Subsequently, I will introduce teleost immunoreceptor families that are thought to be

involved in regulating teleost innate immune functions. Next I will discuss the channel catfish (*Ictalurus punctatus*) as a valuable model for studying teleost immunity and review the channel catfish leukocyte immune-type receptor family (IpLITRs).

2.2 Innate immunity

In mammals, two major groups of cells mediate immunity: myeloid and lymphoid cells. Representative myeloid cells include macrophages, dendritic cells and granulocytes (neutrophils, eosinophils/mast cells, and basophils). Myeloid cells generally differentiate into innate immune cells that have the ability to engulf and destroy pathogens (phagocytosis), release granules containing potent microbicidal activities (degranulation), and release cellular communication molecules to promote and coordinate immune responses (cytokine secretion) (Reviewed by Beutler, 2004). Myeloid cells include macrophages, dendritic cells, basophils, eosinophils and neutrophils. Although lymphoid cells generally differentiate into cells of the adaptive immune response, natural killer (NK) cells are a specialized lymphocyte with the innate ability to recognize and destroy infected (eg. viruses), damaged/stressed, or transformed (eg. cancer) cells. NK cells also secrete many immunoregulatory cytokines and they are generally considered to be a part of the innate immune response. Innate immune cells generally rely on sophisticated, germline-encoded sensing systems to recognize specific molecules that are often exclusive to pathogens. This recognition can be direct (i.e. direct binding to pathogen) or indirect through recognition of host-

produced molecules that specifically bind to pathogens (opsonisation) to promote their recognition.

Cells that mediate innate immune-like functions observed in mammals have also been described in other vertebrates including birds, frogs, and fish (examples: Gobel et al, 1994; Goyos & Robert, 2009; Horton et al, 1996; Rogers et al, 2008; Shen et al, 2004; Stuge et al, 1997; Yoder, 2004). However, limited information is known about the immunoregulatory receptors and mechanisms that control these cellular functions in non-mammalian vertebrates. In this section, I will introduce genomic regions that encode many human immunoregulatory receptor families and their appearance through evolution to set a comparative stage for studying innate immunity in bony fish.

2.2.1 Immunoregulatory receptors in mammals and other vertebrates

Immunoregulatory receptors are proteins that control many aspects of innate immune cell effector responses. Structurally, immunoregulatory receptors are composed of an extracellular target-binding region, a transmembrane anchoring domain, followed by a cytoplasmic tail. Target-binding regions allow for the specific recognition of a very diverse array of ligands present on pathogens, and dead, dying, or transformed host cells. Structurally, the extracellular binding regions of most immunoregulatory receptors belong to either the C-type lectin-like family or the immunoglobulin superfamily (IgSF); here, I will focus on immunoregulatory receptor-types that contain one or more Ig-like folds in their extracellular regions.

Transmembrane anchoring regions facilitate the expression of immunoregulatory

receptors on the surface of cells while cytoplasmic domains translate extracellular binding into intracellular signaling cascades that result in innate immune effector responses. The cytoplasmic region of immunoregulatory receptors controls their ability to stimulate or inhibit responses. A variety of immunoregulatory receptor families have been described and well characterized in mammals. In humans, two major genomic regions encode receptor families with extracellular Ig-like domains. Human chromosome 1q21-23 encodes the fragment crystallisable receptors (FcRs; reviewed by Takai, 2005; Nimmerjahn & Ravetch, 2008) and Fc receptor-like (FcRLs; reviewed by Davis, 2007). The human leukocyte receptor complex (LRC) on chromosome 19q13 encodes polymorphic receptor families such as the human killer cell Ig-like receptors (KIRs; reviewed by Carrillo-Bustamante et al, 2016; Trowsdale, 2001). One common feature amongst these immunoregulatory receptor families is that they are composed of both stimulatory and inhibitory types that are co-expressed on various innate immune cell-types. Stimulatory and inhibitory signaling networks precisely control the activation, execution, and suppression of innate immune responses to promote pathogen destruction while sparing the host. Although understanding of immunoregulatory receptors and their associated mechanisms in non-mammalian vertebrates is sparse, it is clear that immunoregulatory receptors transcend through vertebrate evolution with expansion and contraction of distinct receptor families based on the immunological needs of the organisms (reviewed by Yoder & Litman, 2011).

In mammals, FcRs are immunoregulatory receptors that bind the fragment crystallisable (Fc) portion of immunoglobulins (antibodies). FcRs are expressed on innate

immune cells where they support the bridging between the specificity of antibodies with the potent antimicrobial activities of innate immune responses (i.e. phagocytosis, degranulation of cytotoxic granules, cytokine secretion). Stimulatory FcRs provide innate immune cells with the ability to become 'armed' with exogenously derived antibodies conferring the cells with specific antigen recognition, which is mediated by the fragment antigen-binding (Fab) portion of the antibody protein. In mice, NK cells express the activating FcγRIII (reviewed by Nimmerjahn & Ravetch, 2008); when IgG3-armed FcγRIII becomes cross-linked by its ligand (specified by the antibody), intracellular signaling induces the NK cell to kill its target via antibody dependent cellular cytotoxicity (ADCC). FcRs on phagocytic cells can also facilitate internalization of immune complexes and antibody-opsonized pathogens (Reviewed Nimmerjahn & Ravetch, 2006; 2007; 2008). In addition to stimulatory FcRs, there are inhibitory FcRs that have integral roles in preventing aberrant immune cell activity (Nimmerjahn & Ravetch, 2006; 2007; 2008).

Cataloguing of immunoregulatory gene families in various mammalian and non-mammalian species has demonstrated that immunoregulatory gene families have appeared, expanded and contracted, or disappeared over evolutionary time as a result of species-specific host/pathogen interactions (Akula et al, 2014; Fayngerts et al, 2007; Yoder & Litman, 2011). In order to study the appearance of FcRs specific for the expanding Ig-isotypes over evolutionary time, Akula et al (2014) used a bioinformatics approach to analyze the presence of FcR-related genes in various vertebrate genomes ranging from mammals, reptiles and birds, amphibians, bony fish, cartilaginous fish and lamprey. These studies demonstrated that within the FcR locus, poly Ig receptors

(PIGRs), FcRLs and the FcR γ chain (a signalling adaptor molecule) were the first genes to appear (Akula et al, 2014). This conclusion was based on the observation that these genes were present in all animals tested from mammals to bony fishes but not in cartilaginous fish and lamprey. In bony fish and chicken, relatively few FcRs and FcRLs were identified but LRC genes were extensively expanded in these organisms suggesting that receptors with the ability to bind the Fc portion of antibodies may be encoded in the LRC (Akula et al, 2014). In fish, there is functional evidence for FcR-mediated control of innate immune activities: FcR-mediated control of NK cell cytotoxicity, FcR-mediated degranulation of mast cells, and FcR-mediated phagocytosis (Shen et al, 2003; Chen et al, 2009; Li et al, 2006). The identity of these FcRs remains to be determined and candidate FcRs in bony fish will be expanded on later in this section. Chicken homologues of FcR/FcRLs are represented by chicken Ig-like receptors (CHIRs): a family of polygenic and polymorphic immunoregulatory receptors encoded on chicken microchromosome 31 (reviewed by Straub et al, 2013). CHIR-AB1 is a receptor for chicken IgY but FcRs for chicken IgM or IgA have not yet been identified (Viertlboeck et al, 2007). Akula et al (2014) proposed that FcR and LRC encoded genes arose from a common ancestor since genes homologous to human FcRs and LRC-encoded proteins appeared with bony fish and are present in birds, frogs, and mammals.

Humans and mice have FcRs that bind specifically to the various antibody isotypes; for example, human chromosome 1q21-23 encodes four classical FcRs that bind the four isotypes of IgG (i.e. Fc γ RI, Fc γ RII, Fc γ RIII, Fc γ RIV) (reviewed by Nimmerjahn & Ravetch, 2008). Also encoded at this location is an FcR for IgE (Fc ϵ RI), an Fc α μ R that

binds both IgM and IgA, and an IgM-binding Fc μ R (Shibuya et al, 2000). All of these receptors are structurally related suggesting that these genetic loci arose from gene duplication events that permitted the expansion of multiple Fc-specific receptors (Davis et al, 2005). In addition to classical FcRs, this chromosomal region encodes Fc receptor-like genes (*fcr11-6*) that are extensively expressed on by various immune cell-types (reviewed by Davis, 2007). Like FcRs, FcRLs are type I transmembrane proteins with extracellular Ig-like domains that relay signaling via cytoplasmic tyrosine-based signaling motifs (Davis, 2007). FcRL4 and FcRL5 are expressed on B cells and regulate B cell antigen receptor signaling by binding IgA and IgG respectively (Wilson et al, 2012), while FcRL6 is expressed on NK cells and binds MHC class II (Schreeder et al, 2010).

NK cells detect host cell health and are capable of killing infected or transformed host cells without requiring sensitization. In mammals, NK cell receptors are encoded in two major genomic regions: the LRC and the natural killer cell complex (NKC) (Trowsdale, 2001). Killer Ig-like receptors (KIRs) are type I transmembrane proteins that are expressed on human NK cells where they regulate the ability of these cells to detect and kill infected or transformed host cells. The extracellular Ig domains of KIRs mediate NK cell recognition of major histocompatibility complex (MHC) class I molecules expressed on the surface healthy, nucleated host cells (Trowsdale, 2001). MHC class I is polygenic and polymorphic, therefore the receptor families that detect these molecules must also have these properties. Host-pathogen interactions drive the evolution of MHC class I in an 'evolutionary arms race'. Therefore, unlike FcRs, distinct NK cell receptor families are expanded in different vertebrate species based on species-specific MHC

repertoires (reviewed by Yoder & Litman, 2011). As such, LRC-encoded NK cell receptor families are not necessarily expanded in all vertebrates. For example, in humans LRC-encoded KIRs mediate control of NK cell activities while mice use the natural killer complex (NKC)–encoded, Ly49 receptor family. Comparison of the NKC and LRC between mice and primates (lemur, orangutan, chimpanzee and human) demonstrates the following: mice have expanded the Ly49 family but contain no KIR genes within the LRC; lemurs lost most Ly49 genes and have a KIR pseudogene; the Ly49 gene found in lemurs became a pseudogene in orangutan, chimpanzee and humans while KIRs are diversified from orangutan to chimpanzee and humans (reviewed by Carrillo-Bustamante et al, 2016). This demonstrates that over evolutionary time, distinct subsets of NK receptor gene families have been expanded or lost within the short period of time that primates have radiated (Yoder & Litman, 2011; Carrillo-Bustamante, 2016).

KIRs have not been identified outside of mammalian species; however, Ig-type receptors with similar characteristics to mammalian LRC-encoded proteins have been described in non-mammalian vertebrates (i.e. bird, frog and fish species) (Yoder & Litman, 2011). As described above, CHIRs are LRC-encoded proteins in chickens that are represented by stimulatory (CHIR-A) and inhibitory (CHIR-B) types (Straub et al, 2013). CHIR-AB contains both stimulatory and inhibitory potential since it encodes cytoplasmic inhibitory and switch signaling motif as well as a positively charged, transmembrane residue to associate with activating adaptors (Straub et al, 2013). In addition to binding IgY, CHIRs are believed to be functional homologs of KIRs due to their polygenicity, polymorphism and paired stimulatory/inhibitory co-expression on immune cells (Straub

et al, 2013). *Xenopus* MHC-linked Ig superfamily V genes (XMIV) are candidate Ig-type NK cell receptors in amphibians; XMIVs are encoded by six genes and are represented by both putative stimulatory and inhibitory types (Ohta et al, 2006). *Xenopus* Ig-like receptors (xILRs) have also been identified in amphibians and are mostly predicted to encode activating receptors that share similarities in protein structure with KIRs and CHIRs (Guselnikov et al, 2010). Bony fish have many Ig-like, polygenic and polymorphic receptor families that will be further discussed in section 2.3. There are many unanswered questions regarding the precise roles that these non-mammalian immunoregulatory receptors play in the innate immunity of their hosts. However, it is evident that, like mammals, non-mammalian vertebrates have access to complex immunoregulatory systems to control innate immune functions. In addition, the presence of canonical tyrosine-based signaling motifs throughout all of the receptor families described here demonstrates the conserved nature of this signal transduction mechanism and thus will be briefly described below.

2.2.2 Tyrosine-based stimulatory signaling

Stimulatory immunoregulatory receptors relay extracellular binding into intracellular signaling networks through cytoplasmic, immunoreceptor tyrosine-based activation motifs (ITAMs, reviewed in Underhill & Goodridge, 2006). ITAMs can be found either directly on the cytoplasmic tail of receptors (e.g. cytoplasmic tail of FcγRIIA) or on an intracellular adaptor molecule that associates with surface-expressed receptors upon ligand binding (e.g. Fc receptor γ-chain, FcRγ) (Hamerman et al, 2009). When an ITAM-

containing receptor binds its ligand, tyrosine residues within the ITAM consensus sequence (YxxI/Lx₍₆₋₁₂₎YxxI/L) become phosphorylated by Src family kinases. Src family kinases are proteins with N-terminal acylations that localize them to the plasma membrane and are responsible for phosphorylating tyrosines within ITAM sequences. ITAM tyrosine phosphorylation promotes recruitment of spleen tyrosine kinase (Syk) family kinases, which rely on dual Src homology 2 (SH2) domains to recognize the phospho-tyrosines within ITAMs. Syk family kinases relay phosphorylation and activate a variety of downstream adaptor molecules to facilitate signaling through many pathways. ITAM-mediated signaling is known to trigger cellular activation and proliferation (e.g. T and B cell receptors in mammals; Pitcher & van Oers, 2003; Dal Porto et al, 2004), phagocytosis and degranulation (FcRs; Nimmerjahn & Ravetch, 2006; Rivera and Gilfillan, 2006), and NK cell-mediated cytotoxicity (e.g. NKG2D, Moretta et al, 2001).

Many stimulatory immunoregulatory receptors have short cytoplasmic tails that cannot transduce signaling autonomously. These receptors rely on associations with signal transduction adaptor molecules containing ITAMs within their cytoplasmic domain (Reviewed in Hamerman et al, 2009). Charged amino acids within the transmembrane domains of the immunoregulatory receptor and adaptor molecule mediate their association. These electrostatic interactions are commonly mediated by a negatively charged aspartic acid in the ITAM-containing adaptor and positively charged lysine or arginine within the transmembrane domain of the ligand-binding immunoregulatory receptor. In myeloid cells, FcRγ and DAP12 are the most commonly identified adaptors (reviewed in Nimmerjahn & Ravetch, 2008; Hamerman et al, 2009).

These adaptors have short extracellular regions with a cysteine residue to facilitate dimerization through disulphide bonds. The cytoplasmic region of these adaptors encodes an ITAM-consensus sequence known to facilitate receptor signaling. DAP12 is known to mediate NK cell-mediated cytotoxicity upon ligation of NK cell-expressed Ly49D, Ly49H (mouse) and CD94/NKG2C (humans). Through its association with Fcγ and Fcε receptors, FcRγ mediates macrophage, neutrophil, and mast cell activation (Hamerman et al, 2009).

2.2.3 Tyrosine-based inhibitory signaling

Inhibitory signaling establishes thresholds for the regulation of cellular activation to prevent overstimulation or aberrant effector functions (Takai, 2005). Inhibitory receptors primarily depend on immunoreceptor tyrosine-based motif (ITIM; motif sequence: S/I/V/LxYxxI/V/L) encoded within the cytoplasmic region of receptors to promote inhibitory signaling cascades (Reviewed by Daëron et al, 2008). Receptor triggering results in tyrosine phosphorylation within the ITIM regions that promote the recruitment of SH2-domain containing phosphatases. Four SH2-domain containing phosphatases have been identified: (1) SH2-containing inositol-5'-phosphatase (SHIP)-1, (2) SHIP-2, (3) SH2 domain-containing phosphatase (SHP)-1 and (4) SHP-2. SHIP-1 and SHP-2 are lipid phosphatases while SHP-1 and SHP-2 are protein tyrosine kinases. Phosphatases inhibit cellular activation by removing activating phosphate groups on signaling molecules to block (or attenuate) kinase-mediated signal transduction.

Immunoreceptor tyrosine-based motifs (ITSM) are another known tyrosine-based signaling motif, however, they are less understood. Evidence suggests that these

motifs can influence both activating and inhibitory signaling. For example, an ITSM motif (TxYxxI/V) within the cytoplasmic tail of CD150 recruits the small SH2-containing adaptor protein 1A (SH2D1A) that regulates the binding of SHIP versus SHP-2 binding at this site (Shlapatska et al, 2001). Additionally, SH2D1A has been demonstrated to recruit and activate class IA phosphatidylinositol 3-kinase (PI3K) and the adaptor protein Nck1; a potential mechanism for stimulatory signaling (Mikhalap et al, 1999; Aoukaty and Tan, 2002; Li et al, 2009).

2.3 Innate immunity in teleost (bony fishes)

Teleosts have adapted to a range of environmental conditions that are imposed by the aquatic nature of their habitats. Teleosts are bathed in their environment and are constantly exposed to bacterial, parasitic and viral pathogens. As a result, teleost rely on strong, instantaneous innate immune defense mechanisms to maintain homeostatic regulation of commensal organisms while avoiding infection. Like mammals, teleost rely on cell-types that execute the same innate effector responses: phagocytosis, cell-mediated cytotoxicity, degranulation and cytokine secretion. In this section, I will review teleost innate immune cell effector functions and select immunoregulatory receptor families that have been described in teleost.

2.3.1 Innate immune cell effector functions in teleost

Phagocytosis is an ancient cellular process that is vital for clearance of pathogens and effete (dead or dying) cells to maintain homeostasis (Reviewed by Barreda et al, 2016).

Phagocytosis is defined as the receptor-mediated capture, actin-dependent engulfment of targets larger than 0.5 μm in size. The process of phagocytosis has been described and continues to be studied in teleost as well as other lower vertebrates and in invertebrates (Buchmann, 2014). For example, in sea bass (*Dicentrarchus labrax* L), macrophages derived from peritoneal exudate demonstrated potent antimicrobial (*Escherichia coli*) phagocytosis (Esteban & Meseguer, 1997). doVale et al (2002) demonstrated that macrophages and neutrophils were the main phagocytic peritoneal leukocytes in sea bass by assessing their ability to phagocytose *Photobacterium damsela* subspecies piscicida. Alfonso et al (1998) showed that, prior to infection with *Yersinia ruckeri*, neutrophils and macrophages resided in different compartments: macrophages were the resident phagocytes in the peritoneal cavity of trout (*Onchorhynchus mykiss*) while neutrophils only infiltrated the peritoneal cavity upon infection. Interestingly, Alfonso et al (1998) also found that, once at the site of infection (i.e. the peritoneal cavity), teleost neutrophils internalized more bacteria than macrophages. Pronephric (kidney) macrophages from Atlantic salmon (*Salmo salar* L) were shown to recognize and phagocytose yeast glucan particles in a manner reminiscent of mammalian macrophage-recognition of β -glucan (Engstad & Robertsen, 1993). Neumann et al demonstrated that goldfish macrophages were a source of autocrine growth factors that promote macrophage proliferation and differentiation from hematopoietic tissues, which promoted the establishment of a self-propagating macrophage cell line (1998). This was the first demonstration that hematopoietic pathways of fish macrophages were distinct from mammalian macrophages, which rely

on stromal cells for growth factors (Neumann et al, 1998). In addition, these cells were demonstrated to mediate phagocytosis of *Aeromonas salmonicida*, a bacterial pathogen of fish (Neumann et al, 1998). More recently, the discovery that B lymphocytes from rainbow trout (and *Xenopus laevis*) had potent phagocytic activity (Li et al, 2006) resulted in a paradigm shift away from the traditional acceptance that professional phagocytosis was limited to myeloid cells (i.e. macrophages, monocytes, and neutrophils). In addition, Nagasawa et al (2014) demonstrated that thrombocytes (nucleated functional equivalent of mammalian platelets present in non-mammalian vertebrates) from multiple teleost species as well as amphibian models displayed phagocytic activity against live bacteria as well as latex beads; in addition, these cells were capable of actively killing phagocytosed bacteria. Collectively, these unique observations demonstrate the value of studying cellular immune functions in teleost such as phagocytosis.

Granulocytes, cells containing intracellular granules with inflammatory mediators (e.g. mast cells, basophils and neutrophils), have also been reported to play a predominant role in teleost immunity. Eosinophilic granular cells, cells that are structurally and functionally similar to mammalian mast cells, are found in many tissues including the teleost mucosal barriers: gastrointestinal tract, skin and gills of teleost (Reviewed by Reite & Evensen, 2006). For example, infection of the gills of bream (*Abramis brama*) with a copepod induced mast cell migration and degranulation in the gills (Dezfuli & Giari, 2008). The same observation was made when brown trout (*Salmo trutta*) were infected with the acanthocephalan *Echinorhynchus truttae* (Dezfuli & Giari,

2008). Neutrophils have been isolated and cultured from goldfish (*Carassius auratus* L.) kidneys and, like mammalian neutrophils, these teleost cells degranulated and produced a robust respiratory burst in response to infection with *Aeromonas salmonicida* (Katzenback & Belosevic, 2009). Recently, Hodgkinson et al (2015) developed an *in vitro* model to further characterize the goldfish neutrophil response to mycobacterial infection. Chen et al (2009) identified an IgD⁺ granulocyte-like population of cells from channel catfish that was capable of degranulation in response to IgD cross-linking. These IgD⁺ granulocyte-like cells did not express Ig δ mRNA but were armed with exogenous IgD through an unknown, IgD-binding receptor. Although teleost granulocytes appear to be functionally similar to their mammalian counterparts, for some teleost species granulocytes do not fit into the acidophilic versus basophilic or neutrophilic classification since staining characteristics of granulocytes differ between teleost species (Zinkl et al, 1991). Nevertheless, teleost possess cells capable of releasing granules containing potent antimicrobial activities when stimulated.

Like mammals, bony fish also have cells with the ability to directly induce apoptosis of targets without prior sensitization (Reviewed by Shen et al, 2002). Nonspecific cytotoxic cells (NCCs) are small, agranular lymphocytes commonly found in teleost lymphoid tissues (i.e. pronephros and spleen) that are able to kill xenogeneic targets, such as fish parasites, as well as allogeneic targets, such as irradiated B cells (Shen et al, 2002). NCCs have been shown to express the granule components perforin, granulysin, and serglycin supporting the hypothesis that effector components of cell-mediated cytotoxicity are conserved through evolution (Praveen et al, 2006). In addition

to NCCs, another subset of cells with NK-like properties from channel catfish has been identified and characterized (Shen et al, 2003 & 2004); these cells will be further discussed in Section 2.4 below.

Although teleost innate immune cells perform effector responses similar to those observed in mammals, the receptors that regulate these responses remain largely unknown. With the advent of genome sequencing, and establishment of EST and cDNA libraries, many teleost immunoregulatory receptor families have been identified. The teleost innate immune response, like its mammalian counterpart, relies on large repertoires of innate immunoregulatory recognition systems. Within the immunoglobulin superfamily (IgSF) alone, five different teleost IgSF gene families have been identified that share structural features with mammalian immunoregulatory receptor families (Reviewed by Rodriguez-Nunez, 2012; Wcisel & Yoder, 2016). Recent examples of immunoregulatory receptor families identified in teleost species include novel immune-type receptors (NITRs), leukocyte immune-type receptors (LITRs), novel immunoglobulin-like transcripts (NILTs), and polymeric immunoglobulin receptor-like proteins (PIGRLs). Here I will briefly introduce NITRs, NILTs and PIGRLs. My thesis research involved functional characterization of LITRs; therefore I will revisit this family of receptors in section 3.1 to formally review what we know of this receptor family.

2.3.2 Novel immune-type receptors

NITRs are a large family of activating and inhibitory IgSF receptors originally discovered in pufferfish in the search for teleost receptors with variable (V) Ig (Strong et al, 1999).

NITRs were identified as having 1 or 2 extracellular Ig domains composed of V or V-like C2 domains (Strong et al, 1999; Yoder et al, 2001). After their identification in pufferfish, NITRs were also identified in zebrafish (Yoder et al, 2001). In zebrafish, at least 12 subfamilies of NITRs have been discovered (Yoder et al, 2008); amongst these subfamilies are receptors encoding ITIM and/or ITIM-related (itim) sequences within their cytoplasmic tail, receptors with transmembrane regions containing a positively charged residue but no identifiable signaling sequence, and secreted proteins (Rodriguez-Nunez, 2014; Yoder, 2008). Using heterologous expression systems, some aspects of NITR function have been explored. For example, Nitr9 is a putative activating receptor that, when heterologously expressed in human NK cells, induced cytotoxicity (Yoder et al, 2004). When co-transfected into human NK cells with the adaptor protein DAP12, Nitr9L complexed with DAP12 to induce phosphorylation of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) (Wei et al, 2007). In the context of NK-92 cells (a human NK cell line), Nitr3.1 inhibited MAPK signaling in an ITIM-dependent manner (Yoder et al, 2001). Functional studies of channel catfish (*Ictalurus punctatus*) NITR11 demonstrated that this putative stimulatory NITR-type induced allorecognition of catfish B cells (Cannon et al, 2008). The highly related NITR10 (93% amino acid identity) was unable to mediate recognition due to a single amino acid substitution within the ectodomain of this receptor that ablated electrostatic interactions required for the recognition of an unknown molecule on the B cells (Cannon et al, 2008). As with many teleost immunoregulatory receptors, the ligands of NITRs have not yet been

identified but NITRs are proposed to be functional orthologs to mammalian killer Ig-like receptors (KIRs) (Yoder & Litman, 2011).

2.3.3 Novel immunoglobulin-like transcripts

Novel immunoglobulin-like transcripts (NILTs) have also been identified in many teleost fishes: rainbow trout (Østergaard et al, 2009; Kock & Fischer, 2008), carp (Stet et al, 2005) and salmon (Østergaard et al, 2010). Like NITRs, NILTs are a polymorphic and polygenic family of receptors with stimulatory and inhibitory types: as determined by the presence of ITIMs, ITAMs, or receptors with short cytoplasmic tails and a positively charged amino acid residue in the transmembrane (Østergaard et al, 2009). The extracellular region of NILTs contains V-type immunoglobulin domains that are structurally related to human natural cytotoxicity receptor NKp44 and triggering receptor expressed on myeloid cells (TREM) molecules (Stet et al, 2005). In carp, NILT messenger RNA was predominantly found in immune tissues such as the head-kidney, spleen and thymus (Stet et al, 2005). In trout, NILT expression was also predominantly expressed in immunological organs (Østergaard et al, 2009). Ligands for NILTs remain unknown; however, the fact that these receptors are polymorphic, polygenic, related to mammalian immunoregulatory receptors, expressed in immune cells/tissues and are represented by stimulatory and inhibitory-types suggests that these teleost receptors play a role in regulating teleost innate immune functions.

2.3.4 Teleost receptors that bind immunoglobulins

In mammals, polymeric immunoglobulin receptors (pIgRs) play an important role in establishing mucosal immunity by facilitating the transport of polymeric immunoglobulins (i.e. IgA and IgM) across mucosal epithelia. For example, in mammalian intestinal epithelia, pIgA (polymeric immunoglobulin receptor for IgA) is expressed on the basolateral surface of epithelial cells where it binds IgA to mediate its transport through the epithelial cells into the intestinal lumen. In addition to mammals, pIgRs have been identified in chicken, *Xenopus*, and multiple fish species (Braathen et al, 2007; Wieland et al, 2004; Feng et al, 2009; Hamuro et al, 2007; Zhang et al, 2010). To date, three immunoglobulin classes have been described in teleost: IgD, IgM, and IgT/IgZ (reviewed by Gomez et al, 2013). Teleost IgM is the predominant Ig isotype in teleost serum. Teleost IgT/IgZ is predominantly found in mucosal barriers (i.e. fish skin, gills, and gastrointestinal tract) where it plays an integral role in maintaining homeostatic balance with commensal organisms while providing protection from pathogens (Zhang et al 2010; Gomez et al, 2013). The role of IgD in teleost immunity remains elusive. In fish, pIgRs have two extracellular Ig domains: the teleost D1 is related to D1 from mammals, birds, and amphibians while the teleost D2 is related to D5 of these other vertebrates suggesting that the D1-D5 configuration is sufficient to mediate binding to pIg (Hamuro et al, 2007; Rombout et al, 2008; Feng et al, 2009). However, further studies are still required to confirm the mode of teleost pIg binding to its receptor. Teleost pIgR been shown to bind and facilitate the transport of IgM and IgT at mucosal sites, an important barrier for defense against constant exposure to

pathogens in an aquatic environment (Feng et al, 2009; Hamuro et al, 2007; Zhang et al, 2010).

In mammals, Fc receptors are expressed on a variety of immune cells giving them the ability to 'arm' themselves with exogenously acquired Ig (Nimmerjahn & Ravetch, 2007). In teleost, there is functional evidence suggesting that Fc receptors are expressed on immune cells that facilitate binding of exogenously derived Ig (e.g. Chen et al, 2009; Shen et al, 2003). For example, NK-like cells in channel catfish bound serum-derived IgM via a putative Fc μ R that promoted ADCC (Shen et al, 2003). Likewise, Chen et al (2009) demonstrated that channel catfish granulocyte-like cells bound IgD via a putative Fc δ R that induced degranulation. The finding that trout IgM⁺ B cell subsets could phagocytose and induce a potent killing response of IgM-opsonized targets also suggests a role for an FcR (Li et al, 2006). In the search for teleost FcRs, Stafford et al (2006b) screened channel catfish EST libraries for mammalian FcR sequences and identified a single copy FcR gene in channel catfish (i.e. IpFcRI) that lacked GPI-linkage motifs as well as transmembrane and cytoplasmic regions. IpFcRI message was detected primarily in lymphoid tissues by Northern blot whereas lower levels were seen in non-lymphoid tissues such as heart, gill, and liver (Stafford et al, 2006b). Since the IpFcRI sequence lacked a transmembrane domain, Stafford et al (2006b) tested whether the protein was secreted. Soluble IpFcRI was detected by Western blot in the supernatants of cells transiently transfected with native IpFcRI cDNA demonstrating IpFcRI was a secreted protein. Lastly, an Ig-binding assay demonstrated that IpFcR bound to catfish IgM suggesting IpFcR is a 'bona fide' teleost Fc μ R (Stafford et al, 2006b). The role that

IpFcRI plays in catfish immunity is unknown, however, its identification provided support for understanding the evolution of FcRs.

2.4 Channel catfish as a model organism for understanding teleost immunity

Channel catfish (*Ictalurus punctatus*) are one of the few teleost species from which long-term leukocytes with distinct functionality have been isolated and maintained in culture without the need for transformation. Clonal cell lines include innate immune cells (e.g. macrophages and NK-like cells) as well as adaptive immune cells (i.e. B cells, T cells). These long-term clonal cells are isolated from catfish peripheral blood leukocytes using different approaches and are phenotypically characterized based on function and expression profiles (reviewed by Shen et al, 2002). For example, when catfish are pre-immunized with irradiated catfish B cells, T-cell receptor (TCR) $\alpha\beta$ -positive cytotoxic cells capable of killing the allogeneic B cells can be extracted and cloned from mixed leukocyte cultures incubated with allogeneic B cells (Stuge et al, 2000). These cells are specifically cytotoxic for an allogeneic antigen on catfish B cells. Contrastingly, when peripheral blood leukocytes from non-immunized catfish are incubated with allogeneic B cells, cells with NK-like activity expand (Shen et al, 2003). Like clonal cytotoxic lymphocytes, cells with NK-like activity are cytotoxic against irradiated B cells; however, NK-like cytotoxicity does not require pre-exposure to antigen (i.e. through immunization). In addition, cells with NK-like activity do not express mRNA for T cell receptor and spontaneously kill allogeneic targets using calcium-independent and γ -dependent killing mechanisms (Shen et al, 2003; Shen et al,

2004). Catfish 3B11 and 1G8 cell lines are B cells generated by stimulating peripheral blood leukocytes with lipopolysaccharide (Miller et al, 1994a; Miller et al, 1994b). These B cells have the ability to express and rearrange their immunoglobulin μ -chain as well as secrete IgM (Miller et al, 1994). When catfish are immunized with Freund's adjuvant, a subset of peripheral blood leukocytes that demonstrate phagocytic activity against latex beads, produce interleukin-1, and present antigen can be extracted (Vallejo et al, 1990). An example of these monocyte/macrophage-like cells is the 42TA line (Vallejo et al, 1990).

In addition to immortalized clonal catfish cell lines, many immune relevant genes for catfish innate immunity have been identified including pattern recognition receptors, antimicrobial peptide, complement, lectins and cytokines that are important for providing immediate defense against invaders (Zhou et al, 2012; Zhang et al, 2012; Pridgeon et al, 2012; Rajendran et al, 2012). The use of channel catfish cell lines to functionally characterize teleost leukocytes is a powerful tool to examine teleost immune responses and has broadened our understanding of the roles and mechanisms by which teleost immune cells protect their host. However, the receptors that regulate teleost leukocyte responses and their associated signaling networks are largely unknown.

2.5 Channel catfish leukocyte immune-type receptors

2.5.1 Discovery of a polymorphic and polygenic receptor family in teleost

Channel catfish leukocyte immune-type receptors (IpLITRs) were originally discovered from EST libraries generated from alloantigen stimulated peripheral blood leukocytes (Stafford, 2006). IpLITRs are type I transmembrane proteins that belonged to the IgSF with varied numbers of extracellular C2-like Ig domains and either short or long cytoplasmic tails (Stafford, 2006). IpLITR1 and IpLITR3 prototypes were isolated from the cDNA of NK-like cells that were enriched from mixed lymphocyte cultures. IpLITR2 was obtained from the cDNA library of a catfish macrophage cell line. IpLITR1 contains four predicted extracellular Ig-like domains, a transmembrane segment, and long cytoplasmic region with two ITIMs, one ITIM-like motif and an overlapping ITSM suggesting that this receptor mediates inhibitory signal transduction. The short cytoplasmic tail-containing IpLITR 2 (contains three Ig domains) and IpLITR 3 (contains six Ig domains) were predicted to be stimulatory via association with immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptors due to the lack of signaling motifs within their cytoplasmic tails and presence of a charged lysine residue in their transmembrane segments.

Putative stimulatory and inhibitory IpLITR-types were co-expressed in catfish clonal macrophages, B, T, and NK-like cells as well as a mixed lymphocyte culture but not in a catfish fibroblast cell line indicating that IpLITR expression was restricted to immune cell-types. Additionally, IpLITRs were highly polymorphic amongst sibling catfish and multiple independently assorting loci encoded IpLITR genes. LITR-encoding

sequences were also identified in the zebrafish (*Danio rerio*) genome and EST databases demonstrating that LITRs are not exclusive to catfish (Stafford et al, 2006). In addition, other teleost species have sequences within their genome with high similarity to IpLITR sequences (e.g. salmon and herring sialoadhesin-like protein) as demonstrated by BLASTp analysis (Fei et al, 2016). Taken together, these observations support the hypothesis that IpLITRs play an important role in regulating various teleost immune cell function(s) (Stafford et al 2006).

Further supporting a role for IpLITRs in teleost immunity was the finding that IpLITRs are distantly related to two distinct mammalian immunoregulatory receptor families as determined by protein basic local alignment search tool (BLASTp) and phylogenetic analyses (Stafford et al, 2006). IpLITR 2 shared phylogenetic homology with mammalian FcRs and Fc receptor-like molecules (FcRLs) while IpLITR 1 and IpLITR 3 were related to FcR/FcRLs and leukocyte receptor complex (LRC)-encoded receptors. The IpLITR D1 and D2 Ig domains were phylogenetically related to mammalian FcR/FcRL receptor families while the D3 and D4 domains of IpLITR1 and IpLITR3 showed a closer phylogenetic relationship (relative to the D1 and D2 Ig domains) with human LRC-encoded proteins. This demonstrated that IpLITRs are composed of Ig-like domains that are reminiscent of immunoregulatory receptor-types from two major genomic regions in mammals (i.e. FcR/FcRL cluster and LRC)(Stafford et al, 2006).

Fei et al (2016) recently published updated (BLASTp) database searches for IpLITR-like proteins in other vertebrates providing support for the hypothesis that certain IgSF members may have evolved from a common ancestor that has since

undergone diversification (Stet et al, 2005; Stafford et al, 2006; Nikolaidis et al, 2005). IpLITRs are distantly related to human IgSF members: FcRLs, FcRs, LILRs, KIRs, sialic acid-binding Ig-type lectins (Siglecs), carcinoembryonic antigen-related cell adhesion molecules (CEACAMs). In addition, BLASTp searches suggest that IpLITRs are related to IgSF members in other teleost species such as Asian arowana FcRL5 (KPP56756), Salmon KIR3DL1-like (XP_014042396), cichlid IgSF 1-like Rc (XP_014267965), salmon KIR3DS1-like (XP_014043811), and catfish LITR3 (NP_001187136) (Fei et al, 2016). However, IpLITRs are not phylogenetically related to teleost NITRs, NILTs, pIgR, and IpFcRI (Stafford, 2006). Fei et al (2016) proposed that an ancestral IgSF gave rise to IpLITRs, which have since diversified through vertebrate evolution into immunoregulatory receptors responsible for mediating a broad range of immune functions including antibody binding, self- and non-self recognition, adhesion, and pathogen detection. However, whether or not members of the IpLITR family control any of these immune responses in catfish remains to be functionally determined.

To gain insight into their potential immunoregulatory roles, our lab has focused on biochemically and functionally characterizing IpLITRs. IpLITR ligands are currently unknown and, until recently (Taylor et al, 2016), IpLITR-specific antibodies had not been identified. In the absence of known IpLITR ligands, our characterization of these receptors has relied largely on a reductionist and heterologous expression approach using epitope-tagged IpLITRs in a variety of mammalian cell-lines including rat basophilic leukemia (RBL)-2H3, lymphokine-activated killer cells (derived from splenocytes of C57BL/6 mice), HEK-293 cells, and AD293 cells (Mewes et al, 2009; Montgomery et al,

2009; Montgomery et al, 2012; Cortes et al, 2012; Cortes et al, 2014; Lillico et al, 2015; Zwozdesky et al, 2016). Many teleost immunologists rely on heterologous expression systems to functionally characterize teleost immunoregulatory receptors (egs. Yoder et al, 2004; Wei et al, 2007; Cannon et al, 2008). Epitope tag technology, in conjunction with heterologous expression, has facilitated our ability to specifically engage IpLITRs using commercially available antibodies to trigger receptor-mediated signaling and functional events. Additionally, we have used receptor chimeric constructs that linked the cytoplasmic region of IpLITRs with the extracellular domains of mammalian receptors (i.e. KIR-2DL3) for which ligands are known (i.e. HLA-Cw3 and anti-KIR2D mAb DX27) to further facilitate examination of IpLITR-mediated functional responses in mouse NK cells (Montgomery et al, 2012). These studies have provided valuable new information regarding the potential of IpLITRs to regulate various cellular innate immune responses including NK cell-mediated killing, phagocytosis, degranulation, and cytokine secretion.

2.6 Examination of stimulatory IpLITR-types

2.6.1 Stimulatory IpLITRs associate with intracellular signaling adaptors

To determine whether putative stimulatory IpLITR receptor-types associated with adaptor molecules, IpLITR (2.6b) was hemagglutinin (HA) epitope-tagged and transfected alone or in the presence of the FLAG-tagged catfish adaptor IpFcR γ , IpFcR γ -like (L), IpCD3 ζ -L, and IpDAP12. Using this strategy, Mewes et al (2009) demonstrated that IpFcR γ , IpFcR γ -L and IpCD3 ζ -L (but not IpDAP12) co-immunoprecipitated with IpLITR2.6b while only IpFcR γ and IpFcR γ -L facilitated IpLITR 2.6b cell surface expression

in HEK-293T cells. Interestingly, in mammals, receptors with a charged lysine in the transmembrane domain commonly associate with DAP12 but not FcR γ (Feng et al, 2006); yet IpLITR 2.6b contains a lysine within its transmembrane (Stafford et al, 2007). Therefore, to test whether the type of charged residue influenced the specificity of IpLITR 2.6b-adaptor molecule association, Mewes et al (2009) mutated the lysine (K₁₉₉) within the transmembrane of IpLITR 2.6b to an arginine (R₁₉₉) or an uncharged alanine (A₁₉₉). These studies demonstrated that the stimulatory IpLITRs do not require a charged transmembrane to associate with adaptor molecules (Mewes et al, 2009). However, neutralizing the charged transmembrane of IpFcR γ -L (D₃₀ to A₃₀) abrogated its association with IpLITR 2.6b thus suggesting an important role for this charged residue in facilitating the receptor-adaptor interaction (Mewes et al, 2009). Overall, these studies demonstrated that a putative stimulatory IpLITR-type associated with channel catfish adaptor molecules is armed with ITAM-signaling potential. Further studies were then devoted to examining the functional capacity of the IpLITR 2.6b-IpFcR γ -L complex to mediate innate immune activities in well-characterized mammalian innate immune cell lines.

To directly examine IpLITR 2.6b/IpFcR γ -L mediated effector responses, a chimeric construct consisting of the extracellular domains of IpLITR 2.6b fused to the transmembrane (TM) and cytoplasmic domains of IpFcR γ -L was generated. To specifically examine the activity of this complex, an HA-epitope tag was added to the N-terminus of the receptor chimera and its charged TM residue D₃₀ was neutralized to alanine to avoid potential associations with endogenous adaptor molecules expressed in

the cell lines used (Cortes et al, 2012). When this IpLITR 2.6b/IpFcR γ -L (now referred to as IpLITR 2.6b) chimeric complex, expressed in rat basophilic leukemia (RBL)-2H3 cells, was cross-linked using anti (α)-HA monoclonal antibody (mAb), it triggered mast cell degranulation (release of granules containing potent antimicrobial products). This response was abrogated when the tyrosines within the ITAM were mutated to phenylalanine demonstrating a dependence on ITAM-mediated signal transduction. IpLITR2.6b cross-linking in RBL-2H3 cells also rapidly activated ERK/MAPK signaling as demonstrated by Western blotting and phospho-specific antibodies against extracellular signal-regulated kinase (ERK)1/2 and protein kinase B (Akt; Cortes et al, 2012); this activation was sustained up to 32 minutes after receptor induction (Cortes et al, 2014). Furthermore, when the activity of signaling proteins was targeted using pharmacological blockers, Src family protein tyrosine kinase, phosphatidylinositol 3-kinase (PI3K), mitogen activated protein kinase (MAPK) kinase (MEK1 and MEK2), and protein kinase C (PKC) activities appeared to be required for IpLITR 2.6b-mediated stimulatory signal transduction. IpLITR 2.6b induced the secretion of pro-inflammatory cytokines (Interleukin (IL)-3, IL-4, IL-6, and TNF- α) in RBL-2H3 cells at levels comparable to cells chemically stimulated with phorbol myristate acetate, calcium ionophore, or cells triggered through endogenously expressed Fc ϵ RI (Cortes et al, 2014). In addition, IpLITR 2.6b induced phosphorylation of many proteins (including Akt and ERK1/2) known to participate in mitogen-activated protein kinases (MAPK) signaling as measured using a MAPK array. MAPK signaling is known to control cell survival and proliferation via a series of tyrosine-kinase activities (Asati et al, 2016). This result coincided with the

previous result that suggested that IpLITR 2.6b cross-linking induced and sustained activation of ERK1/2 and Akt (Cortes et al, 2012). Lastly, when incubated with α HA-coated, 4.5 μ m polystyrene microspheres, IpLITR 2.6b induced teleost ITAM-dependent but Fc ϵ RI-independent phagocytosis that was calcium- and actin-dependent (Cortes et al, 2012; Cortes et al, 2014). Collectively, these studies demonstrated that IpLITR 2.6b was a potent inducer of degranulation, cytokine secretion, and phagocytosis in a mammalian immune cell line. This was the first functional and biochemical information for a putative stimulatory IpLITR that set the stage for further studies focused on characterizing the mechanism by which a teleost ITAM promotes signal transduction and functional responses in mammalian innate immune cells (Cortes et al, 2012). The ability of a teleost ITAM containing-receptor to network a series of signaling and effector responses in a transfected mammalian immune cell indicated that the ITAM-mediated signaling network is likely conserved between fish and mammals.

Flow cytometry-based characterization of IpLITR 2.6b-mediated phagocytosis demonstrated this process was reliant on the activity of signal transduction molecules known to be involved in mammalian Fc receptor ITAM-mediated phagocytosis (Lillico et al, 2015). Specifically, IpLITR 2.6b-mediated phagocytosis was sensitive to pharmacological blockers of actin polymerization (Latrunculin B), PKC (Go6976), Syk kinase (ER 27391), Akt (Akt inhibitor VIII), PI3K (Wortmannin), c-Src tyrosine kinases (KB SRC4 and PP2), Cdc42 (ML 141), Rac1/2/3 (EHT 1864), phosphoinositide-dependent kinase 1 (PDK1; GSK 2334470), and MEK1/2 (U0126). In addition, when the IpLITR 2.6b-mediated phagocytic process was visualized using confocal microscopy, it was shown

that IpLITR 2.6b-mediated internalization followed a linear progression of target binding, pseudopod extension, and internalization that is characteristic of a mammalian FcR-mediated phagocytic 'phenotype' (Lillico et al, 2015; Levin et al, 2015). Collectively, these results strongly supported the hypothesis that a teleost ITAM can mediate canonical ITAM-induced signaling for target engulfment.

2.7 Examination of putative inhibitory IpLITR-types

2.7.1 IpLITR-mediated abrogation of NK cell-mediated killing

To test whether a teleost receptor containing ITIM motifs promoted inhibitory actions reminiscent of mammalian ITIM-containing receptor systems (i.e. ITIM-mediated recruitment of phosphatases to inhibit function), the biochemical recruitment and functionality of a putative inhibitory IpLITR was examined (Montgomery et al, 2009; Montgomery et al, 2012). For these studies, the cytoplasmic region of IpLITR 1.1b or IpLITR 1.2a was fused to the ectodomain and transmembrane segments of the human NK cell receptor, KIR2DL3, for which a natural ligand has been identified (i.e. HLA-Cw3) as well as the commercial availability of anti-KIR2D monoclonal antibody DX27. The cytoplasmic region of IpLITR 1.1b contains a membrane proximal and distal domain; each cassette encodes three tyrosine (Y) residues (Proximal: Y₄₃₃, Y₄₅₃ and Y₄₆₃; Distal: Y₄₇₇, Y₄₉₉ and Y₅₀₃). The distal cytoplasmic region of IpLITR 1.1b encodes two ITIMs (Y₄₇₇, Y₄₉₉) and one ITSM (Y₅₀₃). IpLITR 1.2a is closely related to IpLITR 1.1b (>88% identical) and differs in that IpLITR 1.2a does not contain the membrane proximal cytoplasmic region of IpLITR 1.1b (Stafford et al, 2007). In addition, IpLITR 1.2a encodes a serine

residue (S₄₂₉) instead of the corresponding tyrosine within the distal cytoplasmic region of IpLITR 1.1b (Y₄₉₉). Therefore, this putative inhibitory IpLITR variant served to specifically examine the role of an ITIM (Y₄₇₇) and the ITSM (Y₅₀₃) within the distal cytoplasmic region of IpLITR 1.1b during IpLITR 1.1b-mediated signal transduction events. These studies revealed that IpLITR 1.1b and IpLITR 1.2a triggering resulted in the ITIM-dependent attenuation of mammalian NK cell-mediated cytotoxicity mediated by the recruitment of SHP (Montgomery et al, 2009; Montgomery et al, 2012). Interestingly, the IpLITR 1.1b-mediated functional response was not affected when this receptor was co-expressed with a catalytically inactive SHP-1 (Montgomery et al, 2012). This suggested that IpLITR 1.1b-induced inhibitory actions depended on two independent mechanisms that were differentially mediated by its membrane proximal and distal cytoplasmic regions. Specifically, the proximal cytoplasmic region of IpLITR 1.1b contains a C-terminal Src kinase (Csk) binding motif: **Y**[T/**A**/S][K/R/**Q**/N][M/I/**V**/R]. In mammals, Csk attenuates signaling by phosphorylating a regulatory tyrosine within the C-terminus of Src family kinases (i.e. Lyn Fyn, Hck, etc.), which inhibits kinase activity (Okada et al, 1991; Chong YP et al, 2005). Csk recruitment to the IpLITR 1.1b proximal cytoplasmic region (at Y₄₅₃) was demonstrated using site-directed mutagenesis and co-immunoprecipitation techniques (Montgomery et al, 2012). This revealed that IpLITR 1.1b-mediated abrogation of mouse NK cell-mediated cytotoxicity was facilitated by a SHP-dependent (distal cytoplasmic domain) and SHP-independent (proximal cytoplasmic domain) mechanism. This was the first demonstration that a teleost receptor had the

unique ability to independently and differentially mediate the same functional response through its proximal and distal cytoplasmic regions.

2.7.2 IpLITR 1.1b-mediated induction of phagocytosis

IpLITR 1.1b-mediated activities were further characterized in RBL-2H3 cells to explore other innate immunological responses that this receptor could mediate. To achieve this, IpLITR 1.1b cDNA was cloned into a mammalian expression vector to confer it with a HA-epitope tag, then stably expressed RBL-2H3 cells (Cortes et al, 2014). These studies demonstrated that when IpLITR 1.1b was cross-linked, it induced transient phosphosphorylation of ERK1/2 and Akt, suggesting that IpLITR 1.1b could also promote stimulatory kinase-driven activity (Cortes et al, 2014). ERK1/2 and Akt are both downstream effectors of signaling pathways known to stimulate cellular activities such as cell proliferation, growth, and survival (Asati et al, 2016). Surprisingly, IpLITR 1.1b also promoted phagocytosis of 4.5 μ m beads as measured by flow cytometry. However, in contrast to IpLITR 2.6b, this IpLITR 1.1b-mediated response was refractory to calcium chelation (EDTA). Additionally, IpLITR 1.1b-mediated phagocytosis required actin polymerization since treatment with Cytochalasin D inhibited IpLITR 1.1b (and 2.6b)-mediated phagocytic response. Removal of the IpLITR 1.1b cytoplasmic region also abrogated the phagocytic response even though the truncated receptor was expressed on the surface of cells suggesting this phagocytic response was mediated by the receptor's cytoplasmic region. To determine the role of Y₄₇₇ and Y₅₀₃ in the IpLITR 1.1b-mediated phagocytic response, the phagocytic activity of the closely related IpLITR 1.2a

was assessed. IpLITR 1.2a also induced the phagocytosis of 4.5 μm beads in RBL-2H3 cells suggesting a possible role for Y₄₇₇ and Y₅₀₃ in IpLITR 1.1b-mediated phagocytosis. These results supported the notion that IpLITR 1.1b exhibited functional plasticity (context-dependent signaling) and transduced a novel phagocytic pathway, using potentially unique proximal and distal cytoplasmic regions to differentially induce phagocytosis in RBL-2H3 cells (Cortes et al, 2014).

The examination of IpLITR 1.1b-mediated phagocytosis in RBL-2H3 cells did not assess the role of the proximal cytoplasmic domain of this receptor. However, Montgomery et al (2012) demonstrated that the proximal cytoplasmic region of IpLITR 1.1b could also contribute to IpLITR 1.1b-mediated signal transduction in a mechanism independent of the distal cytoplasmic region. Therefore, it was possible that the proximal region also contributed to IpLITR 1.1b-mediated phagocytosis. This hypothesis was recently tested by Zwozdesky et al (2016) and will be discussed in section 3.3.5.

2.7.3 Pharmacological and phenotypic profiling of IpLITR 1.1b-mediated phagocytosis

Phagocytosis is defined as the receptor-mediated, actin-dependent, recognition and engulfment of targets that are larger than 0.5 μm in size (e.g. pathogens or dead/dying host cells). During classical mammalian ITAM-mediated phagocytosis, receptor triggering results in the ITAM-dependent recruitment and activation of spleen tyrosine kinase (Syk) to the location of activated receptors (Reviewed by Flannagan et al, 2012). Activated Syk kinase recruits and activates adaptor molecules such as the transmembrane protein linker of activated T cells (LAT) which brings many of the

signaling molecules required for ITAM-mediated signal transduction into close proximity with the engaged receptors and plasma membrane to facilitate rapid activation of these molecules. Phosphorylation of LAT promotes the recruitment of Grb2, an adaptor molecule that recruits Grb2-associated binder (Gab2). Additionally, phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃], a lipid signaling molecule, is generated by the activity of phosphatidylinositol 3-kinase (PI3K). PI3K activity is dependent on its interaction with Gab2 and Syk. Concurrently, Syk-dependent recruitment and activation of guanine exchange factors (e.g. Vav) results in the activation of small RhoGTPases (e.g. Cdc42, Rac1 and Rac2). Small RhoGTPases promote the recruitment and activation of Wiskott-Aldrich syndrome protein (WASP) and WASP-family verpolin-homologous protein (WAVE). During ITAM-mediated phagocytosis, WAVE and WASP activate the Arp2/3 complex, which nucleates F-actin polymerization to promote the acquisition and engulfment of targets.

To demonstrate that the IpLITR 1.1b-mediated signalling pathway was unique to ITAM-mediated phagocytosis (i.e. FcR), the sensitivity of IpLITR 1.1b-mediated phagocytosis to various inhibitors of the ITAM pathway was assessed (Lillico et al, 2015). IpLITR 1.1b did not show sensitivity to inhibitors of many of the signalling molecules known to mediate mammalian ITAM-induced signal transduction (i.e. Cdc42, Rac family GTPases, Akt, MEK1/2, PKC, and PI3K). However, IpLITR 1.1b-mediated phagocytosis did require F-actin polymerization, as well as the activities of Src-family tyrosine kinase, Syk (Lillico et al, 2015). This was distinct from the ITAM-containing IpLITR 2.6b, which did require the activities of PI3K, Cdc42, Src, PI3K, Rac, Akt, PKC, Syk, and MEK1/2. The

dependence of IpLITR 1.1b on the activities of Src family kinases and Syk kinase suggested that this receptor likely mediates phagocytic signaling events using tyrosine phosphorylation-mediated recruitment of intracellular mediators. These results were supportive of the hypothesis that the teleost receptor IpLITR 1.1b is capable of networking to the mammalian cellular cytoskeletal machinery (i.e. F-actin polymerization machinery) through a unique ITAM-independent signaling pathway.

In summary, IpLITR 1.1b is an ITIM-containing receptor that recruits inhibitory phosphatases (i.e. SHP-1/2) to abrogate function in the context of mammalian NK cells (Montgomery et al, 2009; Montgomery et al, 2012). However, this same receptor is able to promote phagocytosis in the context of RBL-2H3 cells (Cortes et al, 2014) using a pathway distinct from the prototypical ITAM-mediated pathway (Lillico et al, 2015). IpLITR 1.1b demonstrated the unique ability to mediate both inhibitory and stimulatory responses in a context-dependent manner, a concept described as functional plasticity (egs. Álvarez-Errico et al, 2007; Izawa et al, 2009; Wang et al, 2011). Collectively, these observations suggested that IpLITR 1.1b mediates a unique or novel mode to network receptor triggering into F-actin-dependent phagocytosis.

During ITAM-mediated phagocytosis, there is a linear progression from target binding, extension of pseudopodia around targets, and scission of the target-containing vacuole (i.e. phagosome) from the plasma membrane to facilitate target entry into the cell (Reviewed by Flannagan et al, 2012). Since IpLITR 1.1b appeared to network extracellular binding to F-actin polymerization distinctly from traditional ITAM-dependent receptors, it was hypothesized that this receptor may also induce distinct

patterns of target acquisition and engulfment. To test this hypothesis, the IpLITR 1.1b-mediated phagocytic process was visualized using confocal microscopy (Lillico et al, 2015). For these studies, RBL-2H3 cells were grown on glass coverslips then incubated with beads for one hour. Cells were fixed before visualizing. This visual examination of the IpLITR –mediated phagocytic process demonstrated that the two receptors (i.e. IpLITR 1.1b and IpLITR 2.6b) mediated distinct modes of target acquisition and engulfment ('phagocytic phenotypes'). IpLITR 2.6b-expressing cells exhibited a classic engulfment phenotype reminiscent of FcR phagocytosis in mammals (Lillico et al, 2015). For example, IpLITR 2.6b-mediated phagocytosis followed a linear progression of binding and extension of pseudopodia around targets to facilitate entry. On the other hand, IpLITR 1.1b-expressing cells displayed a broad range of observable 'phenotypes' featuring many beads being tethered to membranous protrusions (targets bound by individual or multiple cells), within stalled phagocytic cup-like structures, and in some cases the targets were completely engulfed. All together, these studies further reinforced the unique nature of IpLITR 1.1b-mediated target capture and engulfment phenotype suggesting that this receptor used a unique signal transduction mechanism to translate extracellular binding into a stimulatory response that induced cytoskeletal rearrangements required for the capture and engulfment process (i.e. phagocytosis).

2.7.4 Models for potential IpLITR 1.1b-induced signaling

Fei et al (2016) recently proposed potential models by which IpLITR 1.1b might mediate signal transduction based on bioinformatics and literature review with the overall

hypothesis that the two cytoplasmic regions (proximal and distal) independently regulated IpLITR 1.1b-mediated phagocytosis.

One possible mechanism was for IpLITR 1.1b to induce stimulatory signaling through its immunoreceptor tyrosine-based switch motif (ITSM) in the distal cytoplasmic region. Switch motifs have been shown to mediate inhibitory and stimulatory activities based on the type of signaling molecule they bind: inhibitory molecules such as SHP or stimulatory proteins such as SH2D1A and EAT-2 (Chemnitz et al, 2004; Shlapatska et al, 2001). SH2D1A-dependent recruitment and activation of p85 of class IA PI3Ks (Mikhalap et al, 1999; Aoukaty et al, 2002) can promote phagocytosis via Akt phosphorylation and the generation of phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃). There is also evidence of SHP-2 –mediated stimulatory signaling which involves the recruitment of SHP-2 to ITIMs and/or ITSMs where it serves as a scaffold for the recruitment and activation of Grb2 and subsequent engagement of class I PI3K (Brummer et al, 2010; Belov & Mohammadi, 2012). Grb2 is an adaptor molecule that facilitates the recruitment of PI3K, a lipid-modifying enzyme that is important for phospholipid-based intracellular signaling (reviewed by Levin et al, 2015).

This first model predicted that IpLITR 1.1b mediated stimulatory signaling occurred by recruiting SHP-2 and/or SH2D1A to Y₄₇₇ and Y₅₀₃, respectively. These events would then result in the recruitment and activation of PI3K and PI3K-dependent activation of Akt-mediated intracellular signaling to induce IpLITR 1.1b-mediated degranulation and phagocytosis. The examination of IpLITR 1.1b-mediated phagocytosis in RBL-2H3 cells did not assess the role of the proximal cytoplasmic domain of this

receptor. However, Montgomery et al (2012) demonstrated that the proximal cytoplasmic region of IpLITR 1.1b could also contribute to IpLITR 1.1b-mediated signal transduction in a mechanism independent of the distal cytoplasmic region. Therefore, it was possible that the proximal region also contributed to IpLITR 1.1b-mediated phagocytosis.

The ability of IpLITR 1.1b to rapidly capture targets and in some cases accommodate their complete engulfment in an actin polymerization-dependent manner suggested that IpLITR 1.1b might exist in a primed state that facilitates a basal level of receptor activation for complete engulfment of targets. A novel, short-circuited phagocytic pathway utilized by the human carcinoembryonic antigen-related cell adhesion molecule (CEACAM)3 was recently identified by Pils et al (2012) in which CEACAM3 was shown to recruit and activate WAVE2 in an Nck-dependent manner. The WAVE2 complex is a multi-protein complex consisting of molecular mediators that promote actin polymerization (Pils et al, 2012; Takenawa and Suetsugu, 2007). The adaptor Nck is recruited to the consensus sequence HIYDEV (Frese *et al*, 2006): a sequence that is also present within the proximal cytoplasmic domain (Y₄₃₃) of the wild type IpLITR 1.1b receptor (Stafford et al, 2007; Montgomery et al, 2009). Upon receptor ligation, IpLITR 1.1b may bind Nck at the proximal region of the cytoplasmic tail and facilitate rapid engulfment of targets by localizing and activating the WAVE2 complex to IpLITR 1.1b. This would bring molecules capable of activating the actin polymerization machinery in close proximity to the phagocytic cup and facilitate rapid IpLITR 1.1b-mediated capture and internalization.

In addition, Syk kinase has also been shown to be recruited to phosphorylated ITIM motifs within the cytoplasmic tail of the human platelet endothelial cell adhesion molecule (PECAM-1) that are spaced twenty-two amino acids apart (Wang et al, 2011): the exact separation between ITIM motifs (Y₄₇₇ and Y₄₉₉) within the distal region of the IpLITR 1.1b cytoplasmic tail (Fei et al, 2016). Therefore, Src kinase-mediated phosphorylation of Y₄₇₇ and Y₄₉₉ may promote the recruitment of Syk kinase to IpLITR 1.1b. In turn, Syk would facilitate the activation of a guanine exchange factor, such as Vav or RhoA, which would promote the activity of RhoGTPases and thus activate the polymerization of actin.

2.7.5 Testing proposed models for IpLITR 1.1b-induced signaling networks

To test the previously proposed models, the ability of the IpLITR 1.1b cytoplasmic regions (proximal and distal regions compared to full length) to associate with SH2-domain containing proteins (SHP-2, Csk, Syk, Grb2, Nck1, PI3K p85 α , Vav1 and Vav3) was examined using biochemical techniques (Zwozdesky et al, 2016). For these studies, cytoplasmic regions of IpLITR 1.1b were fused to glutathione S-transferase (GST) to generate 'bait' proteins to which signaling molecules would adhere and precipitate with. Tyrosines within the bait protein were artificially phosphorylated using *Escherichia coli* TKB1 cells, which have an inducible tyrosine kinase, to examine the difference in protein recruitment upon phosphorylation. Purified GST fusion proteins were incubated with lysates from the human epithelioid AD293 cell line expressing FLAG-tagged SH2-domain containing proteins listed above. Since Vav is known to be involved in signaling that

culminates in F-actin polymerization (Reviewed by Katzav, 2009), the recruitment of Vav1 and Vav3 were also tested. The cytoplasmic tail of IpLITR 1.1b formed a complex with SHP-2, Csk, Syk, Grb2, Nck1, PI3K p85 α , and Vav1 (Zwozdesky et al, 2016). The proximal cytoplasmic region recruited Csk, Grb2, Nck1, PI3K p85 α and Vav1 but not SHP-2, Syk, or Vav3. The distal cytoplasmic domain recruited SHP-2, Syk, PI3K p85 α but not Vav1 or Vav3. This demonstrated that the molecules that were hypothesized to be involved in IpLITR 1.1b mediated phagocytosis, were recruited to the cytoplasmic region of the receptor. The ability for the proximal and distal regions to recruit Csk and SHP-2 respectively further confirmed that this technical approach reproduced results previously demonstrated by Montgomery et al (2009 and 2012).

The original characterization of IpLITR 1.1b abrogation of cytotoxicity in NK cells demonstrated that the proximal and distal cytoplasmic regions could independently and differentially control the same functional outcome (Montgomery et al, 2009, 2012). As such, it was hypothesized that the proximal and distal cytoplasmic regions of IpLITR 1.1b could also independently and differentially control the induction of dynamic F-actin reorganization required for IpLITR 1.1b-mediated phagocytosis. This hypothesis was tested using imaging flow cytometry in conjunction with a counterstaining strategy to detect beads that remained surface bound (Zwozdesky et al, 2016). This technique allowed for a population screen of the phagocytic activity of cells and counterstaining provided resolution between cells that had engulfed at least one bead (i.e. 'phagocytic cell') versus those that only had surface-bound beads. For these studies, the distal or proximal signaling regions were individually fused to the transmembrane and

extracellular domains of IpLITR 1.1b to functionally assess the individual cytoplasmic domains. These constructs were stably expressed in a human epithelioid cell line (i.e. AD293 cells) to demonstrate that the ability of IpLITR 1.1b to mediate phagocytosis was not limited to the context of RBL-2H3 cells. Specifically, by demonstrating that IpLITR 1.1b-mediated phagocytosis could be induced in non-immune, non-phagocytic cells, we could infer that the IpLITR 1.1b-mediated functional activities were conserved between different mammalian cell types. When the full-length IpLITR 1.1b receptor was engaged for phagocytosis, it predominantly displayed a surface-bound phenotype (with some engulfment) that was previously observed for this receptor in RBL-2H3 cells. Like cells expressing full length IpLITR 1.1b, cells expressing the proximal or distal IpLITR 1.1b constructs displayed a predominant tethering phenotype. This tethering was rapid and within 15 minutes, the proportion of full-length IpLITR 1.1b-expressing cells with tethered beads had reached 62%.

Collectively, these results supported the hypothesis that the IpLITR 1.1b proximal and distal cytoplasmic regions regulate intracellular signaling independently to control the same overall effector response (i.e. extracellular target capture and engulfment). Additionally, this study revealed that IpLITR 1.1b-mediated tethering is a very rapid process. IpLITR 1.1b-mediated functional plasticity was once again exhibited as this ITIM-containing receptor (with ITIM-mediated inhibitory capacity in the context of NK cells) induced a stimulatory response in the context of a non-immune cell. In addition, these observations demonstrated differential recruitment of signaling molecules to the proximal and distal cytoplasmic regions that are predicted to be involved in IpLITR 1.1b-

induced signal transduction upon receptor engagement and phosphorylation. Further studies will be required to validate a role for these signaling molecules as well as to track their temporal and spatial distribution during IpLITR 1.1b-mediated signal transduction to understand how signaling is relayed upon the activation of this receptor.

2.8 Conclusions

The functional characterization of teleost innate immune cells and their effector functions has been largely facilitated by the availability of channel catfish immune cell lines with the unique ability to proliferate without transformation. Although teleost immune cell-types can perform the same functional responses as mammalian immune cells, the immunoregulatory receptors that control these functions have yet to be identified. The growing database of genomic information regarding teleost species has provided new opportunities to identify potential gene families that control teleost immunity. However, the precise roles that these different receptor families play in teleost immunity remain largely unknown.

Teleost leukocyte immune-type receptors (LITRs) are structurally and phylogenetically related to multiple mammalian immunoregulatory receptor families such as human FcRLs, FcRs, LILRs, KIRs, CEACAMs, and others (Fei et al, 2016). Many of these mammalian receptors are exclusively known to control immune functions (e.g. KIRs) while others (e.g. CEACAMs) also have roles in adhesion and immunoregulation. The ability of two IpLITRs to promote a phagocytic response in AD293 cells is highly indicative of the ability of these receptors to translate extracellular binding into F-actin

reorganization (a requirement for phagocytosis). Cytoskeletal rearrangements are required for many immune (and non-immune) cellular processes such as cell-cell adhesion (e.g. in epithelial layers or immune synapses), cell-matrix adhesion, cell migration, and phagocytosis (Cougoule et al, 2004). The types of signaling molecules that facilitate F-actin polymerization are highly conserved (reviewed by Cougoule et al, 2004; Barreda et al, 2016). Therefore it is tempting to propose that IpLITRs are functional equivalents to many of the receptor families above; IpLITRs may contribute to the control of innate immunity by participating in F-actin polymerization-dependent processes. Teleost immune cells can perform antibody-dependent cellular functions such as ADCC by catfish NK-like cells (Shen et al, 2003) and IgD-induced degranulation of catfish granulocyte-like cells (Edholm et al, 2008; Chen et al, 2009) but these activities have yet to be attributed to any teleost FcRs. In addition, a putative FcR presumably facilitated IgM-mediated phagocytosis by trout B cells, however, the identity of this receptor remains unknown (Li et al, 2006). More recently, IgT+ B cells (rainbow trout) were demonstrated to have phagocytic activity comparable to IgM+ B cells (Zhang et al, 2010). It is conceivable that IpLITRs may be the functional equivalents to FcRs in teleost; however, further experimentation is required to formally demonstrate a role for IpLITRs in teleost immunity. Although many questions regarding teleost immunity remain unanswered, the increasing availability of research tools and techniques, such as genomic data and heterologous expression systems, have broadened our understanding of teleost immunity and by extension, the evolution and complexity of the vertebrate immune system.

CHAPTER 3

Materials and Methods

3.1 Cell Lines

AD293 cells are a derivative of human embryonic kidney (HEK) 293 cells with enhanced adherence to tissue culture dishes, flattened morphology and larger surface area resulting in higher transfection efficiency. AD293 cells were purchased from ATCC.

3.2 Cell Culture

AD293 cells stably expressing IpLITR 1.1b/WT or IpLITR 1.1b/ITAM were grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle medium (DMEM; Gibco®) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; GE Healthcare) and 400 µg/mL G418 disulfate salt solution (Sigma-Aldrich). Cells were passaged every three to four days when they had reached ~90% confluence per well (approx. 3×10^5 cell/well) of a 6-well tissue-culture plate (Aaka Scientific). Once cells reached confluence, media was aspirated, and cells carefully washed with 1 mL Dulbecco's phosphate buffered saline (D-PBS; Sigma-Aldrich) to remove residual FBS and debris. Rinsed cells were trypsinized with trypsin/EDTA diluted to 0.05% (v/v) in D-PBS. Four hundred microliters of trypsin solution was gently layered onto cell monolayer then excess immediately removed. Cells were incubated in trypsin solution for 2 minutes at 37°C and 5% CO₂ then cells re-suspended in 1 mL DMEM/FBS by gentle pipetting. Seventy-five microliters of this cell suspension was

transferred to a new well of a 6-well plate containing 3 mL DMEM/FBS. AD293 cells stably expressing IpLITR 1.1b/WT or IpLITR 1.1b/ITAM were maintained in DMEM/FBS containing 400 µg/mL G418 disulfate salt solution (Sigma-Aldrich; DMEM/FBS/G418-400) instead of the G418-free counterpart.

3.3 Freezing and thawing of cells

AD293 cells stably expressing IpLITR 1.1b/WT or IpLITR 1.1b/ITAM were harvested from one well of a 6-well plate as indicated above and washed twice in 10 mL 1X D-PBS (pre-warmed to 37°C). In between washes, cells were collected by centrifugation at 600 x *g* for 5 minutes at room temperature and subsequent aspiration of PBS. Cell pellet was resuspended in 1 mL DMEM/FBS/G418-400 and 1 mL 20% DMSO/FBS. One milliliter cell suspension was aliquoted per 2-mL cryovial and cells frozen gradually in a Styrofoam® container at –80°C overnight.

Cells were thawed in a 37°C water bath within 40-60 seconds then cryovial decontaminated in 70% (v/v) ethanol at room temperature. The cell suspension was transferred to 10 mL pre-warmed DMEM/FBS and cells collected by centrifugation at 400 × *g* for 5 minutes at room temperature. Growth medium was removed by aspiration and cells resuspended in 1 mL fresh DMEM/FBS/G418-400. The cell suspension was transferred to one well of a 6-well plate (per cryovial) containing 2 mL fresh DMEM/FBS/G418-400.

3.4 Antibodies

The antibodies used for flow cytometry were the following: mouse anti-hemagglutinin (HA) tag, clone HA.C5, immunoglobulin (Ig) G3 isotype monoclonal antibody (mAb) (abm®); Mouse IgG3 lambda isotype control purified clone B10 (eBioscience); Phycoerythrin (PE)-conjugated goat anti-mouse IgG (H+L) polyclonal antibody (pAb; Jackson ImmunoResearch); Alexa Fluor® 647-conjugated rabbit anti-mouse IgG (H+L) (Invitrogen)

The antibodies used to coat protein A-preadsorbed phagocytic targets were: mouse anti-hemagglutinin (HA) tag, clone HA.C5, immunoglobulin (Ig) G3 isotype monoclonal antibody (mAb) (abm®); Mouse IgG3 lambda isotype control purified clone B10 (eBioscience)

The antibody used for visualizing externally bound beads by confocal microscopy was Cy5® goat anti-mouse IgG (H+L) pAb (Invitrogen)

3.5 Generation of AD293 cell lines with stable surface expression N-terminal HA-tagged IpLITR 1.1b/ITAM and IpLITR 1.1b/WT receptors

The generation of AD293 cells stably expressing IpLITRs was recently published by Zwozdesky et al (2016). To generate IpLITR 1.1b/ITAM, the ectodomain and transmembrane segment of IpLITR 1.1b (GenBank Accession: ABI16050) was fused to the cytoplasmic region of IpFcRγ-L (GenBank accession AF543420) by splice overlap extension polymerase chain reaction then subcloned into the mammalian expression vector pDisplay (Invitrogen Life Technologies) using SmaI and SalI. Full-length IpLITR 1.1b/WT (TS32.17 L1.1b) was obtained from GenScript® in pUC57 and sub-cloned into pDisplay using SmaI and SalI. One microgram of IpLITR 1.1b/ITAM or 1.1b/WT –pDisplay was transfected into AD293 cells in a 24-well

plate using Turbofect *in vitro* transfection reagent (Thermo Scientific) according to the manufacturer's instructions. Cells were cultured in DMEM/FBS for 2 days after which transfectants were selected with 800 µg/mL of G418 disulfate salt solution for one week. Serial limiting dilution of viable cells was used to isolate clones expressing IpLITR-pDisplay. The expression level of IpLITRs was monitored by flow cytometry (Beckman Coulter) as described previously (Cortes et al, 2014). Briefly, the HA-tagged receptors were detected on the cell surface with anti-HA tag monoclonal antibody (αHA mAb) and illuminated with goat anti-mouse phycoerythrin (GαM PE). The fluorescence intensity of αHA mAb and GαM PE complexes on cells expressing HA-tagged IpLITRs was compared to that of cells treated with IgG3 isotype control. Positive clones were maintained in DMEM/FBS/G418-400 as indicated in section 3.2 of this thesis. A schematic representation of the IpLITR constructs used in this thesis is shown in Figure 3.1.

3.6 Green fluorescent protein (GFP)-tagged constructs used in this thesis

Lifect-GFP, PH-PLCδ₁-GFP, 2xFYVE-GFP, and Syk-GFP were all a kind gift from Dr. Nicolas Touret (University of Alberta). Lifect-GFP is a filamentous actin marker composed of the first 17 amino acids of the *Saccharomyces cerevisiae* actin binding protein (Abp)140 fused to GFP (Riedl et al., 2008). PH-PLCδ₁-GFP is the pleckstrin homology domain of human phospholipase C (PLC) δ₁ that binds to phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂]. The development of this construct was documented by Várnai and Balla (1998). pEGFP-2xFYVE encodes enhanced (E)GFP fused to two tandem FYVE domains (residues 147-223 of mouse Hrs) and has been demonstrated to bind phosphatidylinositol 3-phosphate (PI3P) with high affinity (Gillooly, D. et al, 2000).

3.7 Cellular transfections with GFP-tagged chimeric constructs

AD293 cells stably expressing IpLITRs were seeded in tissue-culture plates at cell densities indicated in Table 3.1 twenty-four hours prior to transfection in DMEM/FBS/G418 to allow for adhesion. Prior to transfection, media was replaced with antibiotic-free DMEM/FBS to minimize the formation of cytotoxic antibiotic/transfection reagent complexes. Cells were transfected using Turbofect™ transfection reagent (Fermentas) as follows:

plasmid/Turbofect™ mixture was prepared in sterile OptiMEM® (Gibco) as indicated in Table 3.1 to maintain a ratio of 1 µg plasmid per two microliters of Turbofect™. For example, when cells were transfected in a 12-well tissue culture plate, 1 µg plasmid was diluted in 200 µl of OptiMEM® then 2 µl of Turbofect™ was added.

Plasmid/Turbofect™/OptiMEM® transfection mixture was gently vortexed for 5 seconds then allowed to incubate at room temperature for 20 minutes. Subsequently, the appropriate volume of transfection mixture per well was added drop-wise onto cells so that each well received 1 µg plasmid, 200 µl of OptiMEM®, and 2 µl of Turbofect™. Cells were incubated overnight in DMEM/FBS/transfection mixture at 37°C and 5% CO₂ prior to assays below. Cells transfected for confocal microscopy-based phagocytic assays were transfected in 12-well plates at confluence indicated in Table 3.1 in order to visualize isolated cells.

3.8 Imaging flow cytometry-based analysis of IpLITR and GFP co-expression

Three wells of AD293 cells stably expressing IpLITR 1.1b/ITAM were co-transfected in a 6-well plate as indicated above. Twenty-four hours post-transfection, the cells were harvested and pooled then stained for IpLITR and Lifeact-GFP co-expression. Three hundred thousand

cells were washed in 1 mL antibody staining buffer then pelleted at 200 rcf for two minutes at 4°C. Supernatant was removed by aspiration and cells were gently raked to loosen cell pellet. Cells were then incubated on ice in a fifty-microliter solution containing 0.1 µg of mouse anti-HA primary for 30 minutes with a gentle rake after 15 minutes of incubation. Cells were washed in 1 mL antibody staining buffer (0.05% sodium azide, 1% BSA, PBS), pelleted, and gently raked after removing excess antibody-containing buffer. IpLITR-bound primary antibody was similarly stained in a fifty-microliter solution containing 0.1 µg of rabbit anti-mouse AlexaFluor® 647 conjugated secondary antibody. After washing and removal of excess secondary antibody, cells were fixed in a 1% paraformaldehyde (v/v) diluted in PBS. Cells were immediately analyzed for LifeactGFP and HA-IPLITR expression by imaging flow cytometry (ImageStream®^X Mark II).

3.9 Preparation of phagocytic targets

Polybead® carboxylate or Fluoresbrite® carboxy Bright Blue (BB) 4.5 µm microspheres (Polysciences) were coated with Protein A (from *Staphylococcus aureus*; Sigma-Aldrich) then mouse αHA mAb or mouse IgG3 as follows. Two hundred fifty microliters containing 4.99×10^8 particles/mL were sedimented by centrifugation at 161000 rcf for 3 minutes then beads washed three times in one milliliter of 0.1 M boric acid. After the third wash, the beads were resuspended in 500 µL of 0.1 M boric acid and pre-coated with 200 µg of Protein A overnight by rotating at room temperature. Fluoresbrite® beads were protected from light. Excess protein A solution was removed by centrifugation (161000 rcf for 10 minutes) and beads washed for 30 minutes with one milliliter of 10 mg/mL BSA diluted in 0.1M boric acid

on a rotator at room temperature. After three consecutive washes, the beads were resuspended in one milliliter of 10 mg/mL BSA then divided into two 500- μ L aliquots. Five micrograms of mouse α HA mAb or mouse IgG3 were absorbed onto the Protein A-coated beads overnight at 4°C on a rotator. Excess antibody was removed by centrifugation then beads washed three times in one milliliter of 10 mg/mL BSA diluted in D-PBS. Beads were vortexed for 10 seconds in wash buffer between washes to remove loosely bound antibody. After washing, bead pellets were resuspended in 500 μ L of 10 mg/mL BSA diluted in D-PBS to a final concentration of 1×10^5 beads per microliter. Antibody-coated beads were stored at 4°C and used within one and a half weeks after preparation. Immediately before using, desired amount of beads was washed in 1 mL phagocytosis buffer (1 mg BSA in 50% OptiMEM/PBS v/v) then resuspended in this buffer to 1×10^5 beads per microliter.

3.10 Imaging flow cytometry-based phagocytosis assay

The imaging flow cytometry-based phagocytosis assay and corresponding data analysis used in this thesis is a variation of the procedure described by Fei et al (2016b). AD293 cells stably expressing IpLITR 1.1b/ITAM or IpLITR 1.1b/WT were seeded into four wells of a 6-well plate at 3×10^5 cell per well. Twenty-four hours later, cells from two wells were transfected with 1 μ g of Lifeact-GFP and 2 μ L of Turbofect™ per well for twenty-four hours. After harvesting, all transfected cells were pooled and counted using a hemacytometer. Similarly, non-transfected IpLITR-stable AD293 cells from remaining wells were pooled and counted. Three hundred thousand cells were incubated with either isotype (mouse IgG3) or mouse α HA –coated Fluoresbrite® Bright Blue Carboxylate Microspheres (9×10^5 beads per

sample) for 1 hour at 37°C in 300 µL of phagocytosis buffer. Every twenty minutes, the cells and beads were re-suspended by raking sample tubes along a tube rack. One hour after incubation of cells with targets, excess media was removed and extracellular beads stained with rabbit anti-mouse AF647®-conjugated secondary antibody for thirty minutes on ice. Excess antibody was washed and removed. Loosely associated beads were cleaved with 200 µL of a 0.5% trypsin/EDTA/PBS solution for fifteen minutes on ice. Trypsin was inactivated with a 5% BSA/PBS (w/v), 2mM EDTA buffer. Lastly, cells were re-suspended and fixed in 60 µL of 1% PFA/PBS and phagocytic capacity of cells was assessed by imaging flow cytometry (ImageStream®X Mark II; Flow Cytometry Core, Faculty of Medicine and Dentistry). The flow rate was set on low and a 40x objective was used.

Using the Imaris analysis software, single cells were identified by plotting aspect ratio versus area (size versus circularity, Figure 3.2). The aspect ratio of AD293 cells was between 0.6-1 and area between 300-800 (gated in R1, Figure 3.2A). Single-focused cells in region (R)2 (Figure 3.2B) were used for downstream analysis. GFP fluoresced in channel (Ch) 02, Fluoresbrite® carboxy BB beads fluoresced in Ch 07 (blue), and AF647 fluoresced in Ch 11 (red). Bead masks were used to identify beads based on their fluorescent properties. To identify BB beads in Ch 7, (Range(Dilate(Peak(M07, Ch07, Bright, 10), 1), 15-5000, 0.3-1)) was the mask used. To identify beads in Ch 11, (Range(Dilate(Peak(M11, Ch11, Bright, 10), 1), 15-5000, 0.3-1)) was used. The Peak function was used to identify beads in the blue or red channels with pixel fluorescence values of 10 or higher relative to the background. The Dilate function was used to dilate the Peak mask by one pixel. The Range function was used to select beads (components) that were between 15-5000 pixels in size with an aspect ratio

of 0.3 to 1. Collectively, these masks were used to create a spot count feature plotted in Figure 3.2C. Gated in R3 are cells associated with three beads or less since the mask does not retain accuracy when more beads per cell are analyzed. Connected component masks were then used in Figure 3.2F to subdivide region 3 into cells with only internalized beads (R4), cells with one or more internalized bead (R7), and only surface bound beads (R5 & R6). Cells co-expressing Lifeact-GFP (or GFP construct of interest) were plotted in Figure 3.2D. Lastly, bead-cell association is shown in Figure 3.2E by plotting fluorescence intensity in Ch 11 versus Ch 07. Region '0' represents cells not associated with beads and R9 represents cells associated with beads.

3.11 Immunofluorescence assay to discriminate bead position

AD293 cells stably expressing IpLITR 1.1b/ITAM were seeded onto poly-D-lysine –coated coverslips (Neuvitro) then co-transfected with Lifeact-GFP for twenty-four hours (1 µg/plasmid). Cells were then washed with PBS and anti-HA coated beads (effector to target ratio of 1:2) in a 1 mg/mL BSA/PBS/OptiMEM solution and beads centrifuged onto cells (1500 rpm for 1 min). The cells were incubated with beads for 4, 8, 16, and 32 minutes at 37°C to capture beads in early stages of internalization. Next, the cells were washed twice with PBS and fixed with a 4% paraformaldehyde/PBS (w/v) solution for 10 minutes at room temperature. The PFA solution was washed twice with PBS and non-specific protein-protein associations blocked with 3% BSA/PBS for 30 minutes at room temperature. The cells were then inverted onto a 100-µL drop of Cy5-conjugated GαM secondary antibody (0.5 µg of antibody). The secondary antibody solution was prepared in antibody staining buffer. After

staining exposed α HA mAb on extracellular microspheres with the antibody for 30 minutes at 4°C, the coverslips were mounted onto glass slides and imaged via confocal microscopy (Zeiss 710 laser-scanning microscope, Cell Imaging Facility, Department of Oncology) with the following parameters: Plan-Apochromat 40x/1.3 Oil DIC M27; Average line 2, one pixel = 0.09 μ m, 16-bit using filters for DAPI (410-484 nm), FITC (493-581 nm) and Cy5 (633-758 nm).

3.12 Confocal microscopy-based phagocytosis assay

AD293 cells stably expressing IpLITR 1.1b/ITAM or 1.1b/WT were seeded at 6×10^4 cell/well in five wells of a 12-well plate, four of which contain an 18mm diameter, #1.5 thickness poly-D-lysine coated coverslip. After allowing the cells adhere overnight, the cells were transfected with 0.5 μ g Lifeact-GFP (or desired construct)¹ and 1 μ l Turbofect™ per well for twenty-four hours. Cells were harvested from the well that did not contain a coverslip and counted using a hemacytometer in order to maintain an effector to target ratio of two beads per cell. Cells in the remaining wells were washed twice with 1 mL D-PBS then 625- μ L phagocytosis buffer containing desired concentration of α HA coated beads was added per well. Beads were centrifuged onto IpLITR 1.1b/ITAM -expressing cells at 1500rpm for 1 minute and immediately incubated for 4, 8, 16, or 32 minutes at 37°C then fixed with 4% paraformaldehyde (v/v) in D-PBS at room temperature for 10 minutes. Cells expressing IpLITR 1.1b/WT were incubated with beads for 8, 16, 32, and 60 minutes prior to fixing due to the lower efficiency of internalization of this receptor. After rinsing excess paraformaldehyde three times with 1 mL D-PBS, samples were blocked in 3% BSA/PBS (v/v)

¹ Other constructs used were PH-PLC δ ₁-GFP, 2xFYVE-GFP, and SykGFP.

for 30 minutes at room temperature to reduce background protein-protein associations. Extracellular beads were stained with a Cy5-conjugated GαM secondary antibody by inverting cells onto a 100 µL drop of antibody at 2.5 µg/mL antibody staining buffer for 30 minutes at 4°C. Excess antibody was removed by washing three times with 1 mL antibody staining buffer then cells mounted onto slides with ProLong® Gold anti-fade reagent. Mounting media was allowed to cure overnight and the cells imaged on a Zeiss 710 laser-scanning microscope (Cell Imaging Facility, Department of Oncology) with the same parameters indicated in section 3.11.

3.13 Generation of EGFP-tagged signaling molecules

Src homology domain-containing Csk, Grb2, Nck1, SH2D1A, p85α, Syk and SHP2 were amplified from FLAG-tagged recombinant constructs and subcloned into pEGFP-N1 (kindly provided by Dr. Deborah Burshtyn, University of Alberta) to generate EGFP chimeric constructs. The generation of FLAG-tagged recombinant constructs has been reported by Zwozdesky, et al (2016). Briefly, mRNA for the signaling molecules above was extracted from AD293 cells and converted to cDNA (SuperScript VILO cDNA synthesis kit; Invitrogen). Amplified cDNA products (i.e. insert fragments) were sub-cloned into p ET1.2/blunt (Clone ET, Thermo Scientific), a plasmid that is permissive to sub-cloning blunt ended DNA. Lastly, insert fragments were restriction digested (enzyme combinations in Table 3.2) from p ET to sub-clone inserts into p3×FLAG-CMV14 expression vector (Sigma-Aldrich).

To generate N-terminal EGFP-tagged signalling molecules, cDNA was amplified using polymerase chain reaction (PCR) from their p3×FLAG-CMV14 counterparts as follows.

Briefly, 10 ng of template were amplified with primers detailed in Table 3.2 using 0.5 U Phusion High-fidelity DNA polymerase (Thermo Scientific) according to manufacturers recommendations. The cycling conditions were: 98°C for 5 min, 35×[95°C for 20 seconds, 62°C for 30 seconds, and 72°C for 30 seconds], and a final extension step at 72°C for 5 minutes. PCR reaction products were separated and visualized on 1% TAE-agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen). PCR products were excised, gel purified (Qiagen Gel Extraction Kit) then cloned into p ET1.2/blunt using the blunt-end protocol (Clone ET, Thermo Scientific). p ET plasmids (1 µg/plasmid) were double digested using 1 µL per Fast Digest restriction enzyme (Thermo Scientific) indicated in Table 3.2 for two hours at 37°C. Double digested products were separated on 1% agarose gels, insert fragment excised, gel purified (as above), then ligated into pEGFP-N1. Ligation into pEGFP-N1 was performed by incubating 100 ng of insert fragment and 10 ng of pEGFP-N1 fragment with 1 µL T4 DNA ligase (Fermentas) for 1.5 hours at room temperature. Ligation products were transformed into competent DH5α *E. coli* and transformed cells spread onto LB agar supplemented with kanamycin (50 µg/µL; LB-kan). Positive clones (transformed with pEGFP-signaling molecule) were identified by double digesting plasmid extracted using the QIAprep® Spin Miniprep Kit (Qiagen) according to manufacturer's instructions then visualizing fragments on a 1% TAE-agarose gel. Purified plasmids were sequenced to ensure appropriate cDNA sequence. All Sanger sequencing was performed by the Molecular Biology Services Unit in the Department of Biological Sciences, University of Alberta, on a 3730 Genetic Analyzer.

IpLITR 1.1b/WT

IpLITR 1.1b/ITAM

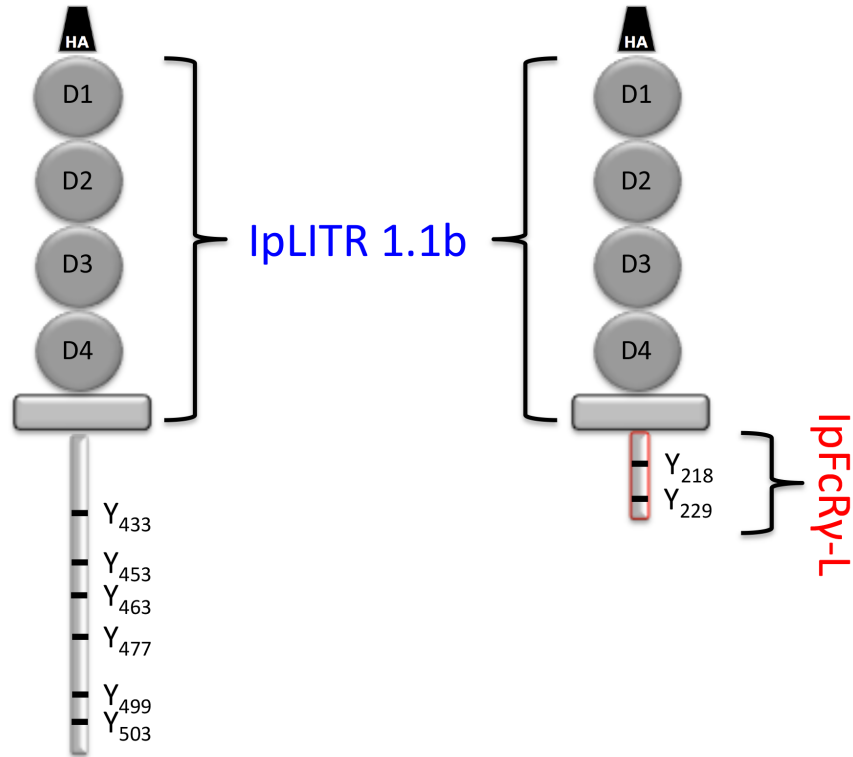


Figure 3. 1 Structural schematic of IpLITR constructs used in this thesis. Full-length IpLITR 1.1b/WT receptor (TS32.17 L1.1b DQ858241.1) obtained from GenScript® in pUC57 was sub-cloned into the pDisplay mammalian expression vector. Using splice overlap extension polymerase chain reaction, the ecto- and transmembrane domains of IpLITR 1.1b was fused to the cytoplasmic region of IpFcRγ-L (GenBank accession AF543420) to generate IpLITR 1.1b/ITAM.

Table 3. 1 Transient co-transfection conditions of AD293 cells with GFP-chimeric constructs. AD293 cells stably expressing IpLITR 1.1b/ITAM or IpLITR 1.1b/WT were seeded at cell densities indicated 24 hours prior to transfection. Cells to be used for confocal microscopy were seeded on 18 mm diameter poly-D-lysine coated coverslips in 12-well plates. Cells used for ImageStream-based analysis were seeded in 6-well plates. Cells were transfected in antibiotic-free DMEM/FBS for 24 hours prior to analysis.

Wells/plate	Vol culture media/well	Cell Seed Density	Confluence at transfection	OptiMEM	Plasmid	Turbofect™
12-well	2 mL	6×10^4 cell/well	50-60%	200 μ l	0.5 μ g	1 μ L
6-well	3 mL	3×10^5 cell/well	~70%	400 μ l	1 μ g	2 μ L

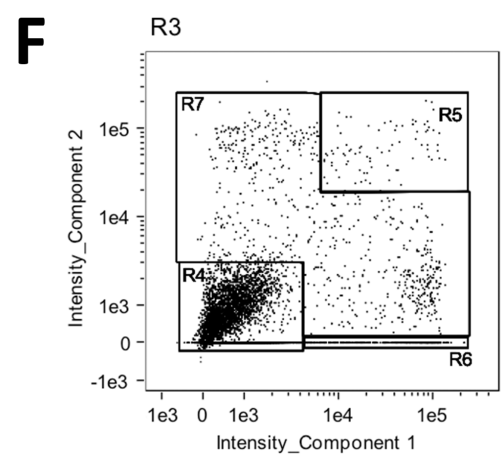
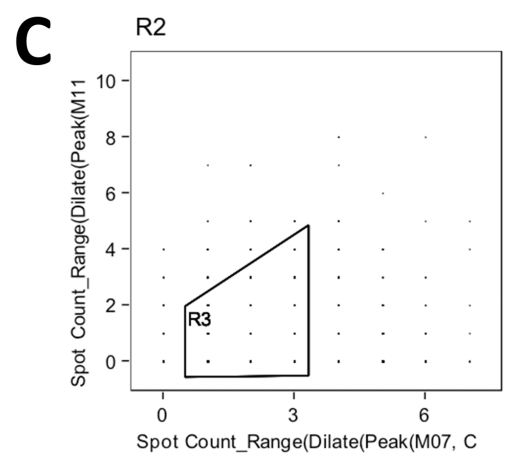
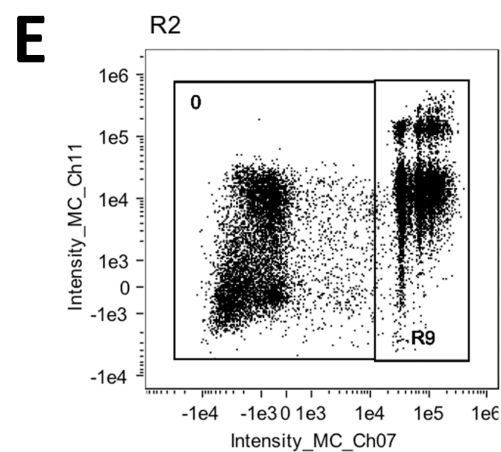
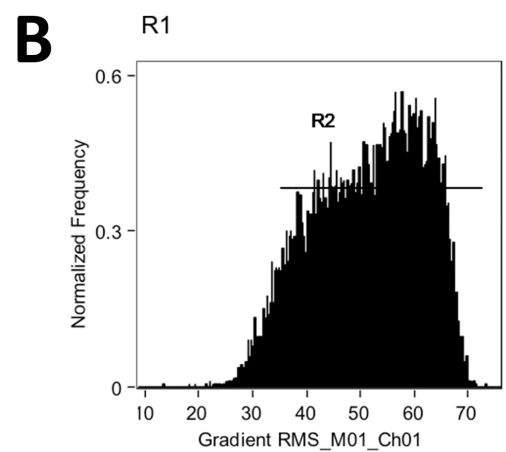
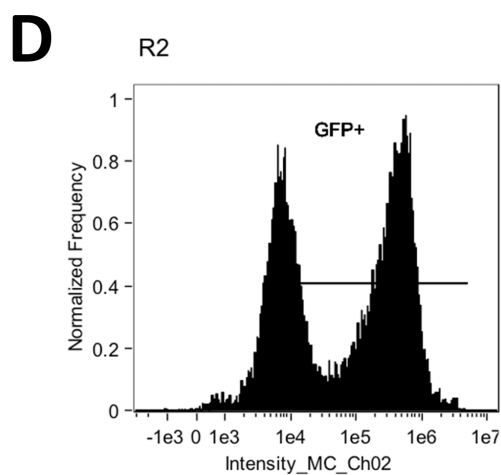
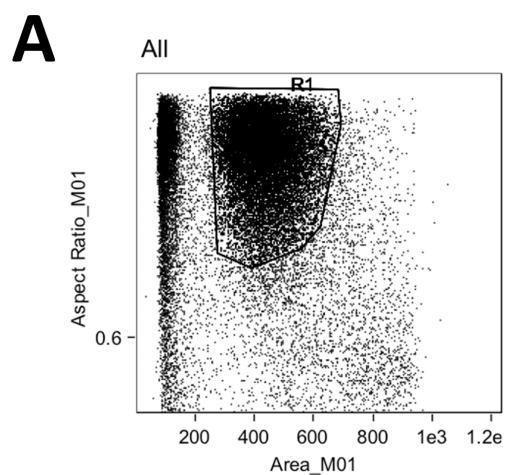


Figure 3. 2 Gating strategy used to analyze IpLITR-mediated phagocytosis using the Amnis ImageStream and Imaris software. Cells co-expressing IpLITR 1.1b/ITAM or 1.1b/WT and Lifeact-GFP, PH-PLC-GFP, 2xFYVE-GFP, or SykGFP were incubated with α HA coated, 4.5 μ m Fluoresbrite® carboxy BB targets. Exposed α HA was counterstained with an AF647-conjugated secondary antibody to illuminate surface bound beads. GFP fluoresced in channel (Ch) 02, Fluoresbrite® carboxy BB beads fluoresced in Ch 07, and AF647 fluoresced in Ch 11. Size versus circularity shown in (A) was used to identify single cells. Single cells in focus of the camera were in region 2 (B). Bead spot count masks were used in (C) to identify and count beads. GFP-expressing cells (GFP+) were gated in (D) to ensure expression. Bead-cell associations were determined by plotting the intensity in Ch 11 versus Ch07 (E). Only cells in gate R9 were associated with beads. Cells associated with three beads or fewer were further subdivided based in (F) based on only having internalized beads (R4), having one or more internalized bead (R7), or having surface bound beads only (R5 & R6).

Table 3. 2 List of primers and restriction enzyme combinations used in this thesis to generate EGFP-tagged signaling proteins. Human signaling proteins indicated in table were amplified from p3×FLAG-CMV14 constructs encoding the cDNA (accession number provided) for the molecule. PCR fragments were subcloned into p ET1.2/blunt then double digested using the combination of restriction enzymes indicated in Table. Insert fragments were ligated into pEGFP-N1 double digested with the same enzymes then sequenced after plasmid purification and sequencing. All primers were obtained from Integrated DNA Technologies (IDT) and restriction enzymes are FastDigest (Thermo Scientific).

Signaling Protein	GenBank Accession#	Forward Primer 5'-3'	Reverse Primer 5'-3'	Restriction Enzymes
Csk	NM_004383.2	AAGCTTATGTCAGCAATACAGGCCG CC	GGATCCGACAGGTGCAGCTCGTG	HindIII/ BamHI
Grb2	CR541942	AAGCTTATGGAAGCCATCGCCAAAT AT	GGATCCGAGACGTTCCGGTTC	HindIII/ BamHI
Nck1	NM_0012919 9.1	AAGCTTATGGCAGAAGAAGTGGTG GTA	GGATCCGATGATAAATGCTTGACA AG	HindIII/ BamHI
SH2D1A (SAP)	NM_0011149 37.2	AAGCTTATGGACGCAGTGGCTGTGT AT	GGATCCGATGGGGCTTTCAGGCAG AC	HindIII/ BamHI
SHP2	D13540.1	AAGCTTATGACATCGCGGAGATGGT TT	GGATCCGATCTGAACTTTCTGCT G	HindIII/ BamHI
Syk	Z29630.1	AAGCTTATGGCCAGCAGCGGCATG GCT	GGATCCGAGTTCACCACGTCATAGT AG	HindIII/ BamHI
P85α (PI3K)	NM_181523.2	GAATTCATGAGTGCTGAGGGGTAC	GGATCCCCTCGCCTCTGCTG	EcoRI/Bam HI

CHAPTER 4

Development of an imaging-based assay to monitor teleost leukocyte immune-type receptor-mediated phagocytosis

4.1 Introduction

Phagocytosis, the receptor-mediated and actin-dependent ingestion of extracellular targets greater than 0.5 μm in size, is a fundamental, evolutionarily conserved cellular process important for protection against invading pathogens as well as clearance of dead or dying cells (Flannagan, 2012; Freeman & Grinstein, 2014; Gordon, 2016).

Phagocytosis is performed by specialized immune cell-types called phagocytes and these cells are armed with an array of surface-expressed phagocytic receptors. Structurally, phagocytic receptors are composed of an extracellular binding region and a transmembrane anchoring domain. To translate extracellular ligand binding into a cellular response (i.e. phagocytosis), phagocytic receptors either associate with signaling adaptor proteins or they contain a cytoplasmic region capable of mediating intracellular signal transduction. The extracellular binding region of phagocytic receptors enables phagocytes to recognize various targets (e.g. pathogens or dying cells) either through direct binding or indirectly via opsonins (e.g. antibodies and complement proteins). Despite the diversity of phagocytic receptor-types and their broad array of ligands, these receptors generally promote phagocytosis through tightly regulated tyrosine phosphorylation events that lead to the induction of localized phospholipid metabolism,

activation of small GTPases that recruit the actin nucleation (Arp2/3) complex, culminating in the stimulation of cytoskeletal rearrangements that directs the dynamic plasma membrane structures required for target engulfment (Flannagan, 2012, Freeman & Grinstein, 2014; Mao & Finnemann, 2015).

Our present understanding of the phagocytic process has been gained primarily from a few well-studied phagocytic receptor-types in mammals (Flannagan, et al 2012). These include the fragment crystallisable gamma receptors (FcγR), Dectin-1, and complement receptor 3 (CR3). These different immune receptors are all capable of recognizing specific extracellular ligands and stimulating phagocytes to promote actin polymerization required for the engulfment of the targets into a membrane bound vacuole known as the phagosome. Although these different receptors all stimulate the phagocytic machinery, the mechanisms by which they trigger this process are functionally and molecularly distinct resulting in morphologically unique engulfment phenotypes based on dynamic remodelling of the cellular cytoskeleton (Mao & Finnemann, 2015; Lee et al, 2011). For example, Lee et al (2011) found that engagement of Dectin-1 by its fungal ligand, zymosan, promoted the formation of a distinctive, actin-dependent pedestal prior to target engulfment that appeared to move the target away from the cell just prior to the formation of extended pseudopods that surrounded the target. On the other hand, Fc receptor-mediated phagocytosis of antibody-opsonized targets has been shown to mediate the extension of actin-dependent pseudopodia around targets without the formation of a protrusive pedestal to facilitate engulfment (Swanson, .A. and Hoppe, 2004; Swanson, 2008). Finally, during CR3-mediated

phagocytosis, receptor engagement induces invagination of the plasma membrane during target engulfment, which is uniquely different from the behaviours observed during Dectin-1 and FcR-mediated phagocytosis (reviewed in Flannagan et al, 2012; Mao & Finnemann, 2015). Despite the diversity in the modes of target acquisition and engulfment (i.e. phagocytic phenotypes) induced by various phagocytic receptors, common to all phagocytic processes is the observation that actin-driven protrusions facilitate the initial capture of phagocytic targets, and the requirement for actin depolymerisation at the base of the phagocytic cup to permit entry of targets into phagocytes (Flannagan et al., 2012; Freeman et al 2014).

Phagocytosis is an ancient cellular process thought to have evolved from primarily nutrient acquisition and homeostatic roles into an effector function vital for immunity (reviewed by Barreda et al, 2016). Phagocytes have also been described in various animal groups, including invertebrates and vertebrates (reviewed by Buchmann, 2014). Despite the knowledge that cells capable of engulfing targets (i.e. phagocytes) exist in vertebrate species ranging from teleost to mammals (Engstad and Robertsen, 1993; Frøystad et al, 1998; Li et al, 2006), the process of phagocytosis in fish is understudied; in part because the receptors that mediate this process in non-mammalian animals have not been identified. Channel catfish (*Ictalurus punctatus*) leukocyte immune-type receptors (IpLITRs) are immunoregulatory receptors that share distant phylogenetic relationships and structural features with mammalian receptor families, including the FcR proteins (Stafford et al, 2006; Fei et al, 2016). Our overall appreciation of the precise roles that IpLITRs play in teleost immunity is impaired due to

the unknown identity of their ligands and lack of IpLITR-specific antibody reagents. Our lab has employed heterologous expression systems to explore potential functions of these receptors in various mammalian cell lines, a common strategy for deciphering the functional capabilities of non-mammalian immunoregulatory receptor-types (examples: Wei et al, 2007; Cortes et al, 2014; Lillico et al, 2015). For example, by fusing the cytoplasmic tail of select IpLITRs with the transmembrane and ectodomains of the human NK cell receptor KIR2DL3, Montgomery et al (2009) demonstrated that IpLITR 1.1b, a putative inhibitory IpLITR-type, abrogated mouse NK cell mediated cytotoxicity by recruiting Src homology 2 domain-containing protein tyrosine phosphatases (SHP)-1 and SHP-2. Additionally, Cortes, et al (2012) used a mammalian expression vector that supplies IpLITRs with an N-terminal hemagglutinin (HA) tag permitting specific engagement of the surface-expressed receptor with commercially available mouse α HA tag mAb. Using this approach, Cortes et al (2012) demonstrated that HA tag-mediated triggering of IpLITRs could regulate degranulation, cytokine secretion, and the activation of intracellular signaling events. Additionally, two representative receptors, IpLITR 2.6b/IpFcR γ -L and IpLITR 1.1b were shown to stimulate phagocytosis of α HA mAb-coated, 4.5 μ m beads using standard flow cytometric-based phagocytosis assays (Cortes et al, 2012; Cortes et al, 2014; Lillico et al., 2015).

IpLITR 2.6b/IpFcR γ -L is a chimeric fusion construct containing the extracellular immunoglobulin domains (D1 and D2) of IpLITR 2.6b fused with the transmembrane and cytoplasmic tail of the channel catfish Fc receptor gamma-like protein (Fc γ R-L): an ITAM-containing adaptor molecule recruited to the charged transmembrane domain of IpLITR

2.6b (Mewes et al, 2009). Mutation of the ITAM-containing tyrosines to phenylalanine abrogated IpLITR 2.6b/IpFcR γ -L- (now referred to as IpLITR 2.6b) mediated phagocytosis (Cortes et al, 2012). Flow cytometry-based pharmacological profiling of IpLITR 2.6b-mediated phagocytosis also demonstrated that this receptor required the activities of PKC, Syk, Akt, PI3K, Rac family GTPases (e.g. Cdc42), and c-Src; all common signalling proteins used by the mammalian Fc γ receptor (Fc γ R) (Lillico et al, 2015). Most importantly, IpLITR 2.6b-mediated phagocytosis was actin-dependent as inhibition of actin polymerization with Latrunculin B (an inhibitor of F-actin polymerization) completely abrogated its phagocytic activity (Lillico et al, 2015). When examined by confocal microscopy, it was also shown that IpLITR 2.6b-expressing cells completely engulfed multiple α HA-coated 4.5 μ m target beads per cell, behaviour reminiscent of the mammalian Fc receptor-mediated phagocytic phenotype (Lillico et al, 2015). Collectively, these observations suggest that a canonical ITAM-mediated phagocytic pathway appears to be regulated by a teleost immunoregulatory receptor-type.

In comparison, IpLITR 1.1b is an ITIM-containing receptor with a long cytoplasmic tail containing six tyrosines divided between a proximal and distal signaling cassette both of which have been shown to independently mediate inhibition of cytotoxicity in the context of mouse NK cells (Montgomery et al, 2012). Interestingly, when IpLITR 1.1b was expressed in rat basophil leukemia (RBL-2H3) cells, it unexpectedly also promoted the phagocytosis of 4.5 μ m targets (Cortes et al, 2014). Additionally, flow cytometry-based pharmacological profiling of IpLITR 1.1b-mediated phagocytosis demonstrated that inhibition of signalling molecules pivotal to ITAM-mediated signalling did not affect

IpLITR 1.1b-mediated phagocytosis (Lillico et al, 2015). However, when polymerization of F-actin was blocked with Latrunculin B, IpLITR 1.1b-mediated phagocytosis was abrogated. Confocal microscopic analysis further revealed that IpLITR 1.1b-expressing cells generated extended membranous protrusions, engulfed fewer beads overall (than IpLITR 2.6b-expressing cells), and had a higher degree of partially engulfed beads. These observations demonstrated the unique ability of a non-mammalian receptor to engage an ITAM-independent signaling pathway that translated extracellular binding with F-actin polymerization. In turn, F-actin reorganization facilitated remodelling of the plasma membrane and the generation of extended membranous protrusions that actively participated in the capture and engulfment of extracellular targets.

Imaging-based studies have demonstrated the importance of spatial and temporal coordination of signaling events to promote the dynamic cytoskeletal (F-actin) changes that facilitate plasma membrane distortion around targets (reviewed by Levin et al, 2015). To date, the majority of our studies regarding IpLITR-mediated phagocytosis have been end-point assays, which does not permit spatial and temporal resolution of the dynamic nature of IpLITR-mediated signal transduction that leads to actin remodelling during the IpLITR-mediated phagocytic process. The objective of this thesis chapter was to develop imaging-based bioassays to further monitor the IpLITR-mediated phagocytic process to spatially and temporally define IpLITR-mediated cytoskeletal changes that promote the distinct phagocytic phenotypes of IpLITRs. Specifically, in this chapter, my research aims were: i) to establish procedures for the discrimination of surface bound, partially engulfed and fully internalized targets; ii) to develop a co-

transfection strategy to monitor the F-actin distribution during the IpLITR-mediated phagocytic process, and; iii) to use confocal microscopy to examine actin distribution in AD293 cells stably expressing IpLITRs. Overall, completion of my research aims provides a new imaging-based platform that can be further applied to monitor the recruitment and distribution of specific signaling molecules during IpLITR-mediated phagocytosis.

4.2 Results

4.2.1 Confocal microscopy examination of surface bound, partially engulfed and fully internalized targets using IpLITR and Lifeact-GFP co-expressing AD293 cells

To test the ITAM-independent and ITAM-dependent IpLITR-mediated phagocytic pathways, I used AD293 cells stably expressing one of two constructs: IpLITR 1.1b/ITAM and 1.1b/WT (Fig 4.1 A). IpLITR 1.1b/WT is the full-length IpLITR 1.1b protein (i.e. naturally devoid of ITAM motifs). IpLITR 1.1b/ITAM is composed of the IpLITR 1.1b ecto- and transmembrane domains fused to the ITAM-containing, cytoplasmic region of IpFcR γ -L. Both constructs were cloned into the mammalian expression vector pDisplay, which conferred the proteins with a hemagglutinin (HA)-epitope tag to specifically engage the receptors with α HA monoclonal antibody (mAb)-coated 4.5 μ m beads and monitor the IpLITR phagocytic process in AD293 transfectants (Fig 4.1 A). In order to provide a reference point for the location of beads relative to cells, the cellular cytoskeleton or endosomal vesicles were detected with Lifeact-GFP and 2xFYVE-GFP respectively (Fig 4.1 B). There are generally three main locations of target beads during the phagocytic process: (1) bound to the surface of cells (2) partially engulfed, or (3)

internalized (Fig 4.2 B). To visually discriminate beads in these locations using confocal microscopy, I used a counterstaining strategy to detect areas of beads that were inaccessible to the antibody used (i.e. due to tight association with plasma membrane). AD293 stably expressing IpLITR 1.1b/ITAM were co-transfected with Lifeact-GFP (Fig 4.1 B). Co-transfected cells were incubated with targets (beads pre-absorbed with protein A and coated with mouse α HA mAb) and then fixed to capture beads in various positions relative to the plasma membrane of the cell. Extracellular exposed areas of beads were stained with a Cy5-conjugated goat anti-mouse (G α M Cy5) secondary antibody (Fig 4.2 A). Shown in Figure 4.2 C is a representative image of an AD293 cell with at least two partially engulfed beads (blue, indicated by arrows), the G α M Cy5 staining around the beads (red), and the corresponding F-actin distribution around the targets (green). Figure 4.2 D shows representative phagocytic events for beads that are surface bound (top panels), partially engulfed (middle panels), or internalized (bottom panels). The entire circumference of surface bound beads (Aii) is stained with G α M Cy5 while internalized beads do not stain (Cii). The bead observed in Bi is bound by the cell but is not internalized. The incompletely engulfed target is only partially stained on areas of the bead opposite the actin-rich phagocytic cup (Bii-iv). To further support that targets without G α M Cy5 staining were internalized, IpLITR 1.1b/ITAM AD293 stables were also co-transfected with 2xFYVE-GFP, a probe for phosphatidylinositol 3-phosphate [PI(3)P], a lipid marker of the early maturing phagosome (Gillooly et al, 2000; Levin et al, 2015) (Fig 4.1 B). These co-transfected cells were then incubated with phagocytic targets and then stained with G α M Cy5 as indicated above. As expected, beads that were in the

early stages of phagosome maturation did not stain with GαM Cy5 (Fig. 4.2 E Bi-Biv). Contrary to these internalized beads, partially engulfed beads were stained by GαM Cy5 but were not surrounded by PI(3)P-rich vesicle (Fig. 4.2 E Ai-Aiv). Taken together, these results confirm that this staining strategy can be used to accurately discriminate targets that are surface bound, partially engulfed, and internalized during the phagocytic process in IpLITR-expressing AD293 stables.

4.2.2 Imaging flow cytometric examination of surface bound, partially engulfed and fully internalized targets using IpLITR and Lifeact-GFP co-expressing AD293 cells

To validate, at a populational level, the retention of IpLITR 1.1b/ITAM or IpLITR 1.1b/WT expression after co-transfection, I used imaging flow cytometry to assess IpLITR co-expression with Lifeact-GFP. I accomplished this by detecting IpLITRs on the cell surface using αHA mAb followed by staining with an AF647-conjugated secondary antibody. The surface expression level of IpLITR 1.1b/ITAM in AD293 cells stably expressing the receptor is shown in Figure 4.3 A. When AD293 cells stably expressing IpLITR 1.1b/ITAM were co-transfected with Lifeact-GFP, approximately 47.4% of the population co-expressed the two molecules (Figure 4.3 B). Likewise, Lifeact-GFP and IpLITR 1.1b/WT co-expression is shown in Figure 4.4b relative to the basal surface IpLITR 1.1b/WT expression when cells were not co-transfected (Figure 4.4 A). This demonstrated that AD293 cells stably expressing IpLITRs could be co-transfected with Lifeact-GFP without affecting the relative surface expression of IpLITRs. Cells were also stained with isotype control IgG3 antibody (Appendix Figures A1, A2, A6, and A7). Although there was some

background staining, this antibody did not detect IpLITRs on the surface of cells (visual examination of images not shown).

To examine whether the co-transfection procedure altered the phagocytic phenotypes of the two IpLITR-receptor types, I used an imaging flow cytometry-based phagocytic assay of Lifeact-GFP co-transfected, IpLITR 1.1b/ITAM or 1.1b/WT – expressing AD293 cells and applied my previously developed counterstaining strategy to discriminate the relative bead positions (surface-bound vs. phagocytosed). Lifeact-GFP co-transfected IpLITR 1.1b/ITAM or 1.1b/WT cells were incubated with α HA mAb or IgG3 –coated, 4.5 μ m Bright Blue fluorescent beads in suspension. After incubating the beads with cells for one hour, exposed α HA mAb on surface-bound beads was stained with an AF647-conjugated rabbit anti-mouse (R α M AF647) secondary antibody; thus cells with one or more surface bound targets would fluoresce at both 647 and 450 nm (Figure 4.5 A & C, blue square). Fully internalized beads were not accessible to R α M AF647; therefore cells with only internalized beads would fluoresce in the AF450/DAPI channel. Using the IDEAS[®] software, masks (set of pixels containing regions of interest) were created to identify beads in both the blue channel (beads) and red channel (counterstain for surface-bound beads). These masks were then used in a spot count feature to identify cells associated with three or fewer beads. Lastly, a connected component mask (set of pixels that are all connected to each other) was used to determine the location of beads as follows; (1) cells with surface bound targets only vs. (2) cells with at least one internalized bead (Figure 4.5 B and D). Cells with at least one completely internalized bead were considered a ‘phagocytic cell’ regardless of how

many beads remained surface-bound. Using the criteria above, I observed that 57.7% of AD293 cells stably expressing IpLITR 1.1b/ITAM had internalized at least one anti-HA mAb –coated bead while only 3.7% of cells had only surface bound beads (Figure 4.6 A). Thirty-six percent of Lifeact-GFP co-transfected, IpLITR 1.1b/ITAM-expressing cells internalized at least one bead while 2.3% of cells only had surface bound beads (Figure 4.6 A). The remaining population of cells (38.6% of IpLITR 1.1b/ITAM and 61.5% of IpLITR 1.1b/ITAM-Lifeact-GFP cells) were not associated with beads (Fig. 4.6). AD293 cells stably expressing IpLITR 1.1b/WT predominantly bound α HA mAb –coated beads on their surface (28% of cells) instead of fully internalizing them (16.3%; Figure 4.6 B). Similarly, 27.5% of Lifeact-GFP co-transfected, IpLITR 1.1b/WT expressing cells only had surface bound beads while 7.1% had fully internalized at least one bead (Figure 4.6 B). Approximately 56% of IpLITR 1.1b/WT cells and 65% of 1.1b/WT-Lifeact-GFP cells were not associated with any beads. Isotype control (IgG3) coated-beads did not promote internalization (Figure 4.5 A and B) as ~2% of each cell-type only associated with these control beads. Furthermore, this consisted mainly of beads that were detected at the same time as cells in the flow chamber but did not actually appear bound to cells (Fig 4.5 A and B). This demonstrated that bead-cell associations observed in Figs 4.5-4.6 were specifically IpLITR-mediated events (i.e. induced by the ligation of the N-terminal HA tag with α HA mAb-coated beads). Overall, these experiments also demonstrated that co-transfection with Lifeact-GFP diminished the ability of cells to phagocytose beads but did not alter the overall phagocytic phenotype of IpLITR-expressing AD293 stables.

4.2.3 Confocal microscopy-based examination of actin distribution in AD293 cells stably expressing IpLITR 1.1b/ITAM during the phagocytic process

After demonstrating at the populational level that Lifeact-IpLITR transfected AD293 cells display no alteration in their phagocytic phenotypes, I next examined their behaviours at higher resolution using confocal microscopy. To visualize the distribution of actin during IpLITR-mediated phagocytosis, I fixed Lifeact-IpLITR 1.1b/ITAM cells in various stages of engulfment by using relatively short bead-cell incubation times (i.e. 4, 8, 16, and 32 minutes). These time points gave me the ability to capture early stages of target acquisition and partial engulfment (i.e. 4-16 minutes) as well as full target internalization (32 minutes). When cells were incubated with beads for 4 minutes, the vast majority of cells were still in the capture stage (i.e. most beads/cell associations were not intimate enough to exclude counterstaining with GαM Cy5). When phagocytosis was fixed after 8 and 16 minutes of incubation, partially and fully engulfed beads were predominantly observed. Lastly, after a 32-minute incubation, the majority of cells had multiple beads inside cells and very few remained partially engulfed. Figure 4.7 (left panels) shows the predicted Lifeact-GFP distribution during the early stages of prototypical ITAM-mediated phagocytosis; i.e. target binding, pseudopod extension, phagosome sealing, and phagosome maturation. Additionally shown are representative IpLITR 1.1b/ITAM and Lifeact-GFP co-expressing cells with surface bound targets (Figure 4.7 Ai-ii), extending pseudopods (Figure 4.7 Bi-ii), sealing (Figure 4.7 Ci-ii) and maturing phagosomes (Figure 4.7 Di-ii). Shown in Figure 4.8 A-E are five representative targets that are classified as surface bound based on the GαM Cy5 staining around the target

beads. In Figure 4.8 A-B are beads appearing to be captured by pseudopodia, while in Figure 4.8 C-E phagocytic cups have begun to form. In Figure 4.8, panels F- show representative GαM Cy5 and Lifeact-GFP distribution around targets that are being partially engulfed. Although the beads in Figure 4.8 F-I appear to have a similar GαM Cy5 staining pattern, the distribution pattern of actin around each differs slightly. However, it is clear that engagement of IpLITR 1.1b/ITAM induces temporal and spatially distinct changes in F-actin distribution (especially in Fig. 4.8 G, H, and I). Furthermore, Figure 4.8 G, H, and demonstrate that as the pseudopodia extend around targets and a phagosome begins to form, actin depolymerizes at the base of the phagocytic cup. Lastly, Figure 4.8 K-O show representative images of internalized beads that are no longer accessible to GαM Cy5 demonstrating that F-actin no longer surrounds the phagosome.

4.2.4 Confocal microscopy-based examination of actin distribution in AD293 cells stably expressing IpLITR 1.1b/WT during the phagocytic process

The assays described above in section 4.2.3 were also performed to examine the actin distribution during IpLITR 1.1b/WT-mediated phagocytosis. Lillico et al, (2015) previously reported that IpLITR 1.1b/WT demonstrated a predominant receptor-mediated target tethering phenotype, which I confirmed in AD293 cells (Fig 4.5 C&D; Fig 4.6B). Therefore, I chose to incubate Lifeact-GFP co-transfected IpLITR 1.1b/WT cells with αHA mAb coated BrightBlue beads for 8, 16, 32, and 60 minutes. I reasoned that giving the cells a longer period of time to capture and engulf beads would increase the

probability of finding phagocytic events in the pseudopod extension, sealing, and maturation steps. Overall, when cells were incubated for 8 and 16 minutes, the majority of cells had surface associated beads (demonstrated by little to no exclusion of GαM Cy5 mAb) thus were probably in the target acquisition phase. Cells incubated with beads for 32 and 60 minutes demonstrated a more broad distribution of phagocytic events (i.e. more cells in various stages of engulfing beads). Complete target engulfment was predominantly observed after 60 minutes of bead/cell incubation; however, many cells still remained in various stages of target engulfment. Compared to the prototypical ITAM-mediated phagocytic process, beads appeared to 'sink' into cells during IpLITR 1.1b WT-mediated phagocytosis (Fig 4.9). Actin-rich pseudopodia surrounding surface bound targets are evident in Figure 4.10 A-E. However, I also observed that, when targets are partially engulfed, actin depolymerizes at the base of the phagocytic cup and the longer, actin-rich pseudopodia are not present (Figure 4.10 F-). Once beads were fully internalized, actin was not highly polymerized around targets as was expected (Fig 4.10 K-O). Overall, these results show that compared with IpLITR 1.1b/ITAM, the construct encoding the full-length CYT region of IpLITR 1.1b mediated a unique pattern of actin reorganization during the IpLITR 1.1b/WT-mediated phagocytic process.

4.3 Discussion and Conclusions

To gain preliminary insight into mechanisms by which IpLITRs promote and coordinate target engulfment, I developed a series of imaging-based assays that can be used to specifically monitor the IpLITR-mediated phagocytic process. First, I demonstrated that we can discriminate surface bound, partially engulfed, and internalized beads using a fluorophore-conjugated antibody that binds exposed antibody on α HA-coated beads. Next, I showed that AD293 cells stably expressing IpLITRs are permissive to co-transfection with Lifeact-GFP; as such, co-transfected cells co-express GFP and IpLITRs while maintaining their phagocytic phenotypes. Lastly, I demonstrated that F-actin polymerization patterns shown for an IpLITR ITAM-dependent and ITAM-independent receptor differ during the phagocytic process.

Previously, we relied on confocal Z-stack reconstructions of plasma membrane-stained cells and brightfield images to approximate the relative location of target beads. However, beads are highly refractile thus making it difficult to appreciate bead position relative to the cell or visualize cellular structures, such as pseudopodia, at the forming phagocytic cups. Additionally, using the AD293 cell line, plasma membrane stains induced blebbing of the cells making it difficult to accurately image them during phagocytosis. To more precisely discriminate internalized beads from those undergoing engulfment, I adjusted previously described methods for target discrimination in our phagocytic assay. Methods for identifying external or partially internalized beads have been previously published using biotinylated targets and fluorescent avidin (Flannagan, et al, 2014) as well as fluorescent dye-conjugated antibodies (Schlam et al, 2015). These

methods relied on the tight association between targets and the plasma membrane at the phagocytic cup that exclude large proteins from accessing ligands within the synapse. Additionally, internalized targets are protected from antibody staining by an intact plasma membrane and phagosomal membrane (Fig 4.2 B). Here, I have shown that an AF647 and Cy5 –conjugated secondary antibody raised against the Fc portion of IgG3 (Fig 4.2 A), the α HA antibody isotype coating HA-tagged IpLITR phagocytic targets, provides an accurate estimation of bead positions during the phagocytic process (i.e. outside/surface-bound, partially engulfed, and fully internalized) using AD293 transfectants.

I also co-transfected AD293 cells stably expressing N-terminal HA-epitope tagged IpLITR constructs with a green fluorescent protein (GFP)-tagged probe that tracks F-actin inside the cell (i.e. Lifeact-GFP; Fig 4.1 B). The non-immune AD293 epithelioid cell line is a derivative of the more commonly known HEK-293 cells that lack phagocytic receptors and thus are not endogenously capable of performing phagocytosis. However, when transfected with bona-fide phagocytic receptors (e.g. FcRs and Dectin-1), AD293 cells have been shown to support receptor-mediated phagocytosis using confocal microscopy techniques (Flannagan et al., 2014; Flannagan et al, 2012; Underhill & Goodridge, 2012). Similar to HEK-293 cells, AD293 cells are easily transfectable; however, AD293 cells have enhanced adherence compared to their parental cell line: a desirable property for my confocal microscopic studies. Previously our IpLITR-mediated phagocytosis studies used RBL-2H3 cells expressing IpLITR 1.1b and IpLITR 2.6b to examine the ability of these receptors to mediate target acquisition and engulfment. Heterologous overexpression

of IpLITRs in a mammalian immune cell line (i.e. RBL-2H3) could have induced miscommunication between the teleost receptors and endogenous RBL-2H3 signaling machinery in order to contribute to the unique IpLITR-mediated activities. Due to the artificial nature of our functional examination of IpLITR-mediated responses, it was important to ensure that IpLITR-mediated phagocytosis was not an RBL-2H3 –specific phenomenon and that the overall IpLITR-mediated phagocytic phenotypes previously demonstrated in RBL-2H3 cells were conserved in other cellular systems. Specifically, RBL-2H3 cells are a rat immune cell line while AD293 cells are a human epithelial cell line; thus demonstration that IpLITRs can mediate the same functional response with overall similar receptor-specific phenotypes in two different cell lines, supports the hypothesis that IpLITR-induced signal transduction pathways couple receptor ligation with F-actin reorganization and dynamic plasma membrane remodelling. In addition, to demonstrate that previously observed IpLITR-mediated phagocytic phenotypes were primarily a result of unique signal transduction events (as opposed to extracellular binding), I compared the full-length IpLITR 1.1b (IpLITR 1.1b/WT) with an IpLITR construct composed of the IpLITR 1.1b ecto- and transmembrane domains fused to the cytoplasmic region of IpFcγR-L (called IpLITR 1.1b/ITAM in this thesis; Fig 4.1 A). These constructs were stably transfected in AD293 cells and expressed at relatively similar levels as measured by flow cytometry (compare Figs 4.3 A and 4.4 A). Using imaging flow cytometry, I demonstrated that IpLITRs retained their phagocytic phenotype in AD293 cells (Fig 4.6 A and B) compared to previously documented observations in RBL-2H3 cells (Lillico et al, 2015) and confirmed that IpLITR-mediated phagocytosis is not just

an RBL-2H3 –specific phenomenon, strongly suggestive that IpLITR 1.1b-mediated activities are similar between different cell types. Specifically, after a one-hour incubation with α HA-coated beads, IpLITR 1.1b/WT –expressing cells predominantly bound beads on their surface but relatively fewer beads were fully internalized. On the other hand, IpLITR 1.1b/ITAM cells predominantly internalized beads, as relatively few beads were still surface bound at the same time point. Importantly, I also showed that AD293 cells stably expressing IpLITRs co-transfected with Lifeact-GFP retain these unique phagocytic phenotypes. The importance of this result was two-fold; during the development of methods to identify bead position (i.e. my first aim), my preliminary microscopy-based observations regarding co-transfection of AD293 cells stably expressing IpLITR 1.1b/WT with Lifeact-GFP appeared to reproduce observations by Lillico et al (2015) regarding the inefficiency of IpLITR 1.1b to fully engulf targets in RBL-2H3 cells. However, I was not certain that this observation was a result of the activity of the receptor or due to negative effects of the co-transfection approach. Specifically, I was not certain that co-transfection with Lifeact-GFP was not altering the expression or activity of IpLITR 1.1b/WT, resulting in the overall lack of bead internalization. Therefore, in order to quantify, at a populational level, the retention of IpLITR expression as well as the phagocytic activities of the two receptors (IpLITR 1.1b/WT and IpLITR 1.1b/ITAM), I employed the imaging flow cytometry-based approach which gave me the ability to assess large populations of cells: a limitation of a microscopy-based analyses. While the overall phagocytic activity of co-transfected cells appeared to be reduced when compared to non-transfected AD293 cells stably expressing receptor, the

cells retained their expected phenotypes; specifically, IpLITR 1.1b/ITAM-expressing cells predominantly engulfed targets while IpLITR 1.1b/WT-expressing cells predominantly tethered targets. Therefore, this result validated my microscopy-based observations.

Finally, I imaged the temporal events of IpLITR 1.1b/ITAM and IpLITR 1.1b/WT - mediated phagocytosis and their corresponding actin distribution when targets were surface bound, partially engulfed, and internalized using confocal microscopy of cells fixed at the various stages of target capture and engulfment. Interestingly, I observed that the F-actin distributions during the IpLITR-mediated phagocytic processes differed between the two receptor-types. For example, IpLITR 1.1b/ITAM-mediated cytoskeletal changes during phagocytosis were reminiscent of those observed during mammalian FcR-mediated phagocytosis. F-actin polymerization was triggered upon target binding and persisted along pseudopodia that extended around beads until the bead-containing phagosome was sealed and internalized. Concurrently, F-actin depolymerized at the base of the phagocytic cup promoting entry of the nascent phagosome into the cell. Changes in the distribution of F-actin during IpLITR 1.1b/WT-mediated phagocytosis were also observed. However, distinct from the IpLITR 1.1b/ITAM-mediated phagocytic cup, F-actin-rich pseudopodia were not always evident during IpLITR 1.1b/WT-mediated engulfment. F-actin depolymerisation at the base of the phagocytic cup permitted the entry of beads into cells, as was the case for ITAM-mediated entry. The striking difference between the teleost ITAM-dependent and –independent engagement of actin polymerization was not surprising since pharmacological studies had previously demonstrated that IpLITR 1.1b/WT operates independently of many of the molecules

involved in the classical ITAM-mediated pathway (Lillico et al, 2015). Furthermore, previous confocal microscopy-based visualization of IpLITR 1.1b-mediated phagocytosis revealed that this receptor promoted the extension of membranous protrusions, with a vast majority of beads remaining surface-bound in what appeared to be stalled phagocytic cup-like structures despite being incubated with the cells for one hour at 37°C (Lillico et al, 2015). Although I was not able to reproduce a role for membranous extensions for IpLITR 1.1b/WT in AD293 cells, I did rely on longer bead-cell incubations to optimize my chance of visualizing the complete IpLITR 1.1b/WT-induced phagocytic process (i.e. fully internalized beads). This supported previous observations regarding the overall stalled phagocytic phenotype previously observed in RBL-2H3 cells as well as in my imaging flow cytometry-based analyses (Lillico et al, 2015). Overall, my observations demonstrate that IpLITR 1.1b/ITAM and IpLITR 1.1b/WT mediate distinct patterns of F-actin polymerization that is likely due to unique activation and recruitment of signaling networks to activate the actin polymerization machinery.

IpLITR 1.1b was previously identified as an inhibitory receptor that abrogated cytotoxicity in the context of mouse NK cells in an ITIM-dependent (via recruitment of SHP2) and –independent (via recruitment of Csk) manner (Montgomery et al, 2009; Montgomery et al, 2012). Additionally, the regulation of this effector response was uniquely mediated through two independent pathways involving the proximal and distal regions of the IpLITR 1.1b cytoplasmic tail (Montgomery et al, 2012): this lead to the hypothesis that the two cytoplasmic regions of IpLITR 1.1b independently mediate effector functions and may work in concert to fine-tune responses. The cytoplasmic

region of IpLITR 1.1b has been broadly divided into a membrane proximal and membrane distal region. The proximal region encodes tyrosine residues at positions 433, 453, and 463 (i.e. Y₄₃₃, Y₄₅₃, Y₄₆₃) that are not contained within recognizable motifs such as ITIMs or ITAMs. The distal region encodes two ITIMs (Y₄₇₇ and Y₄₉₉) as well as a switch motif (ITSM, Y₅₀₃). Interestingly, in the context of RBL-2H3 cells, IpLITR 1.1b induced phagocytosis of 4.5 µm beads, which demonstrated that the presence of canonical signaling motifs (i.e. ITAMs vs. ITIMs) does not always infer the activity of receptor types (Cortes et al, 2014). Specifically, IpLITR 1.1b was predicted to be an inhibitory receptor based on the presence of two ITIM motifs within the cytoplasmic region of the receptors (Montgomery et al, 2009). Although IpLITR 1.1b inhibited cell-mediated cytotoxicity in the context of mouse NK cells in a SHP-2 –dependent manner (Montgomery et al, 2012), this same receptor stimulated target capture and engulfment in RBL-2H3 cells (Cortes et al, 2014). The IpLITR 1.1b-mediated phagocytic response was reliant on the presence of the cytoplasmic tail suggesting that this region was involved in the signal transduction required for phagocytosis. In order to test whether the IpLITR 1.1b-mediated phagocytic activity relied on signal transduction through Y₄₇₇ (ITIM) and/or the ITSM (Y₅₀₃), the phagocytic activity of IpLITR 1.2a was examined. IpLITR 1.2a shares 88% similarity with IpLITR 1.1b with the exception that IpLITR 1.2a lacks the proximal cytoplasmic region encoding Y₄₃₃, Y₄₅₃, and Y₄₆₃. Additionally, the corresponding tyrosine at position 499 (Y₄₉₉ of IpLITR 1.1b) encodes a serine instead of tyrosine (i.e. S₄₂₉); therefore, this ‘naturally mutated’ version of IpLITR 1.1b served as a tool to understand whether Y₄₇₇ and Y₅₀₃ were specifically involved for promoting IpLITR

1.1b-mediated phagocytosis. Indeed, IpLITR 1.2a promoted phagocytosis in RBL-2H3 cells at levels comparable to IpLITR 1.1b suggesting that the ITIM (Y₄₇₇) and/or its ITSM (Y₅₀₃) played a role in IpLITR 1.1b-mediated phagocytosis (Cortes et al, 2014). To further characterize the IpLITR 1.1b-mediated signal transduction pathway, the sensitivity of IpLITR 1.1b-mediated phagocytosis to pharmacological blockers of actin, PI3K, Cdc42, Src family tyrosine kinase, PDK, Rac, Akt, PKC, Syk kinase, MEK1/2, and microtubules were subsequently assessed using a standard flow cytometric phagocytosis assay (Lillico et al, 2015). From these experiments, it was determined that IpLITR 1.1b-mediated phagocytosis required F-actin polymerization and perhaps the activities of Syk and Src family kinases but it was independent of the common signaling components of ITAM-dependent phagocytic signaling. Additionally, morphological assessment of the IpLITR 1.1b-mediated phagocytosis using confocal microscopy showed that this receptor was capable of inducing internalization of beads. However, the overwhelming majority of beads were captured by membranous protrusions or in stalled phagocytic cups: a dramatically unique phenotype when compared to the ITAM-dependent (IpLITR 2.6b) receptor, which fully internalized multiple beads per cell (Lillico et al, 2015).

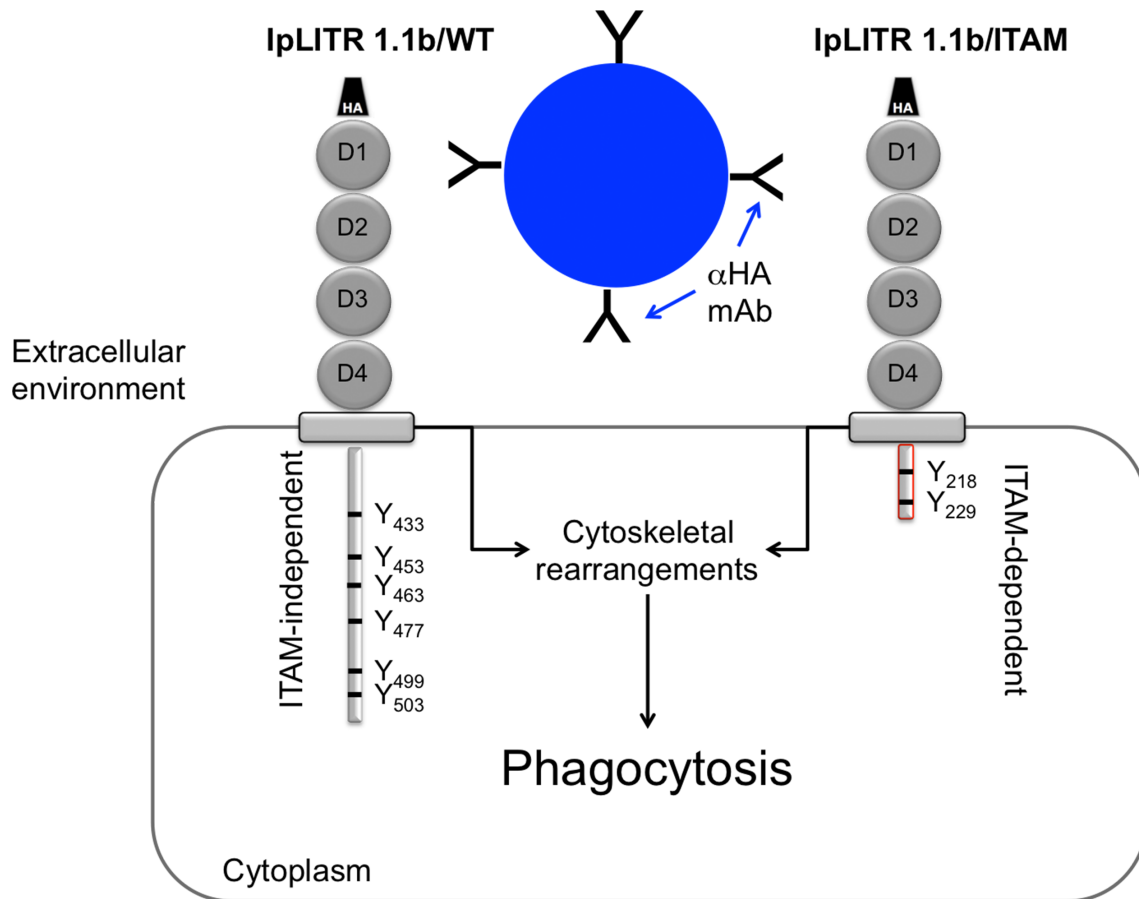
The stalled nature of IpLITR 1.1b-mediated phagocytic cups could be explained by the insensitivity of this receptor to PI3K inhibition (Lillico et al, 2015). PI3K appears to be involved in the later stages of the phagosome closure for targets larger than ~3 μ m (Araki et al, 1996; Cox et al, 1999; Beemiller et al, 2006). However, the ability of IpLITR 1.1b to internalize some targets suggests that this receptor may exist in a primed state that facilitates the rapid activation of the actin polymerization machinery required for

engulfment. This model is based on the recently described phagocytic signaling pathway of CEACAM3 in which receptor ligation results in the rapid recruitment and activation of the WAVE2 complex in an Nck1 and/or Nck2 –dependent manner (Pils et al, 2012). The WAVE2 complex is a small GTPase (i.e. Rac) effector, multi-protein complex known to bind Arp2/3 to promote actin polymerization when activated (Reviewed by Takenawa & Suetsugu, 2007). Nck binds the HIYDEV consensus sequence (Frese et al, 2006), which is also found in the proximal region of IpLITR 1.1b at Y₄₃₃ (Stafford et al, 2007; Montgomery et al, 2009). Therefore, we predict that IpLITR 1.1b engagement promotes rapid internalization of beads in an Nck-dependent fashion. Additionally, Lillico et al (2015) demonstrated a role for Src family kinases and Syk kinase during IpLITR 1.1b-mediated phagocytosis. The cytoplasmic tail of PECAM-1 contains two ITIMs spaced twenty-two amino acids apart which has been shown to accommodate the two SH2 domains of Syk to mediate stimulatory signaling (Wang et al, 2011). This is the exact spacing between the ITIMs within the distal cytoplasmic region of IpLITR 1.1b (Y₄₇₇ and Y₄₉₉); therefore, we predict that, upon ligation, IpLITR 1.1b becomes phosphorylated by Src family kinases at Y₄₇₇ and Y₄₉₉, facilitating the recruitment and activation of Syk kinase. Syk can then recruit and activate guanine exchange factors that promote the activity of small Rho GTPases: small proteins that directly regulate the activation of the actin polymerization machinery via for example the WAVE2 complex (Mao & Finnemann, 2015).

To date, our understanding of IpLITR-mediated signal transduction is based on end-point assays that do not provide temporal and spatial resolution for the recruitment

of proteins during IpLITR 1.1b-mediated phagocytosis. Confocal microscopy is a valuable tool to visualize the localization and dynamic redistribution of specific signaling molecules at the phagocytic cup (Grinstein, 2010). By fixing cells in early stages of target engulfment (binding, phagocytic cup formation, and complete engulfment), I have demonstrated that, once engaged at the plasma membrane, IpLITR 1.1b induces a unique pattern of cytoskeletal changes compared to the ITAM-dependent receptor. However, examination of static images limited my appreciation of the dynamic nature of IpLITR 1.1b/WT –mediated target binding and acquisition. Recent live cell imaging studies of IpLITR 1.1b-mediated phagocytosis in RBL-2H3 cells expressing Lifeact-GFP demonstrated that this receptor often induced the extension of actin-rich protrusions that captured targets and mediated their engulfment (Lillico and Stafford, unpublished observations). Using static images, I was not always able to observe a role for these protrusions thus it will be necessary to carry these experiments out using live AD293 cells. However, my series of imaging-based experiments using static images can provide preliminary framework for testing the localization of specific molecules (described above) to the IpLITR 1.1b-mediated phagocytic cup that can be further verified with the development of a live-cell imaging procedure.

A



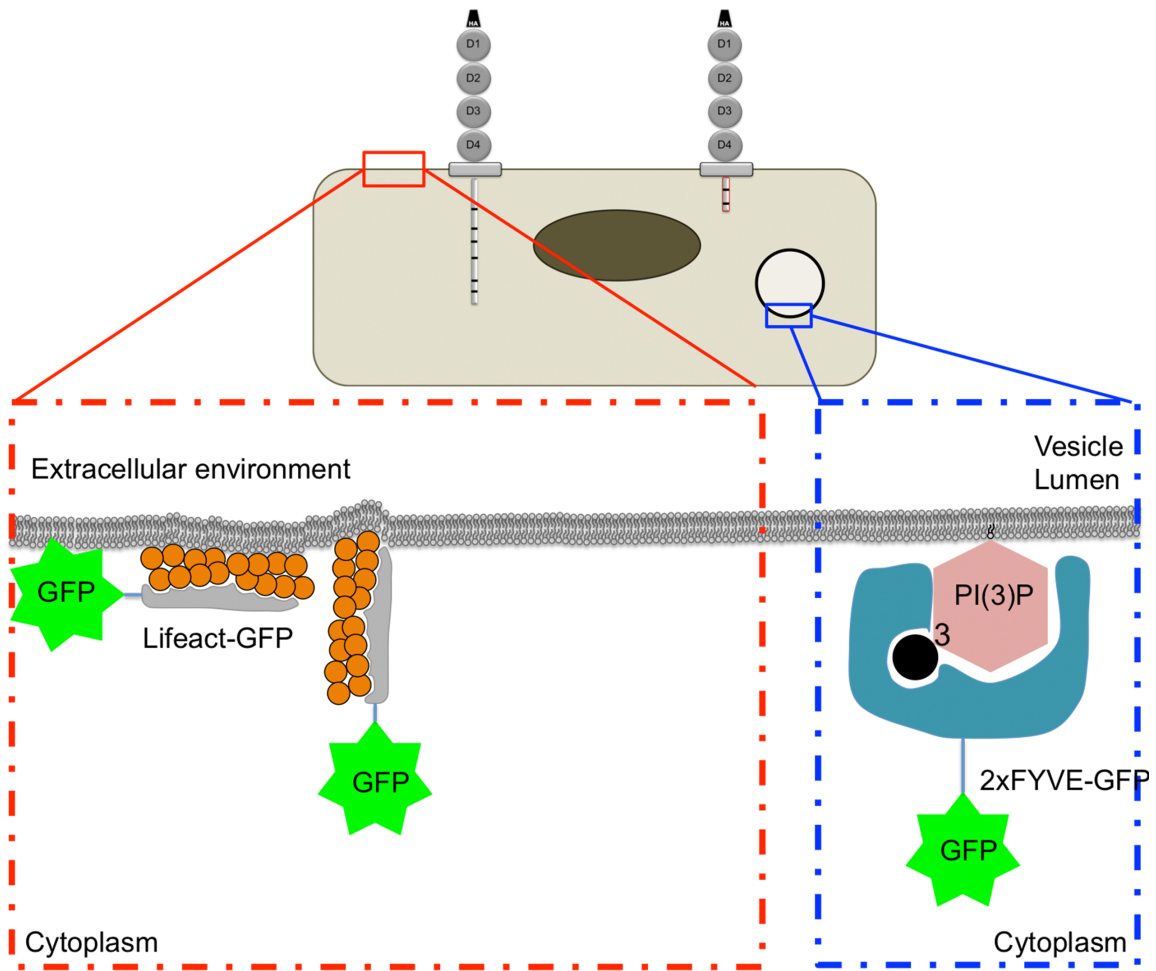
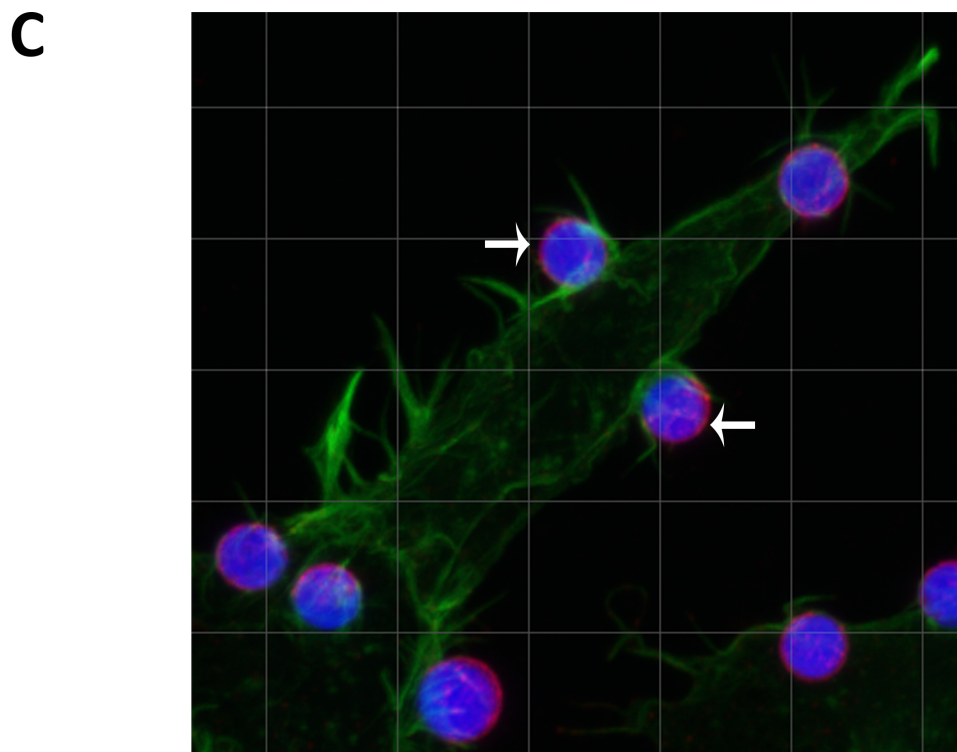
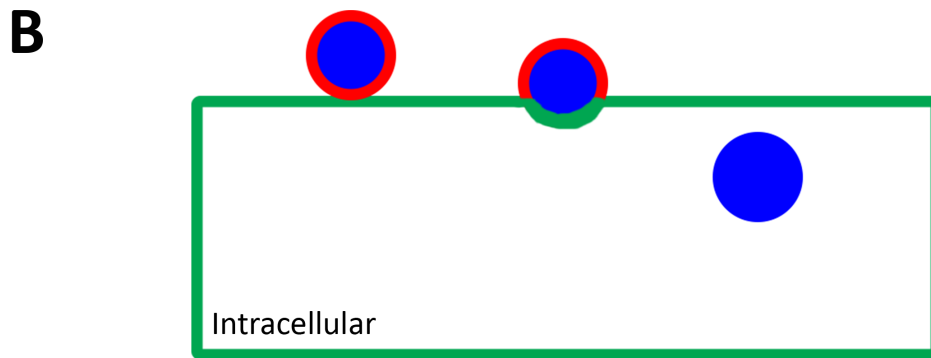
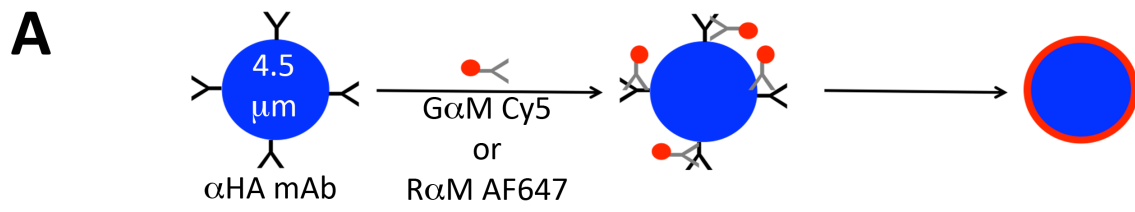
B

Figure 4. 1 IpLITR constructs and probes used in this chapter. (A) The four immunoglobulin domain (D)-containing IpLITR 1.1b/WT and 1.1b/ITAM are fused to a hemagglutinin (HA) epitope tag used to facilitate artificial engagement of the receptors using anti-HA (α HA) monoclonal antibody (mAb). Engagement of IpLITRs by α HA-coated 4.5 μ m beads (blue microsphere) results in cytoskeletal rearrangements required for capture and engulfment of beads. IpLITR 1.1b/WT is an ITAM-independent receptor while IpLITR 1.1b/ITAM encodes two tandem ITAMs within its cytoplasmic tail. Tyrosines (Y) and their respective locations within the cytoplasmic tail are indicated. (B) Lifeact-GFP is a 17 amino acid probe fused to green fluorescent protein (GFP) that binds filamentous (F) actin. Lifeact-GFP was used to detect F-actin (orange spheres) in cells stably expressing IpLITR 1.1b/ITAM or IpLITR 1.1b/WT. 2xFYVE-GFP was used to detect phosphatidylinositol 3-phosphate (PI[3]P) found in the cytoplasmic leaflet of endosomal vesicles and the early maturing phagosome (Gillooly et al, 2009).



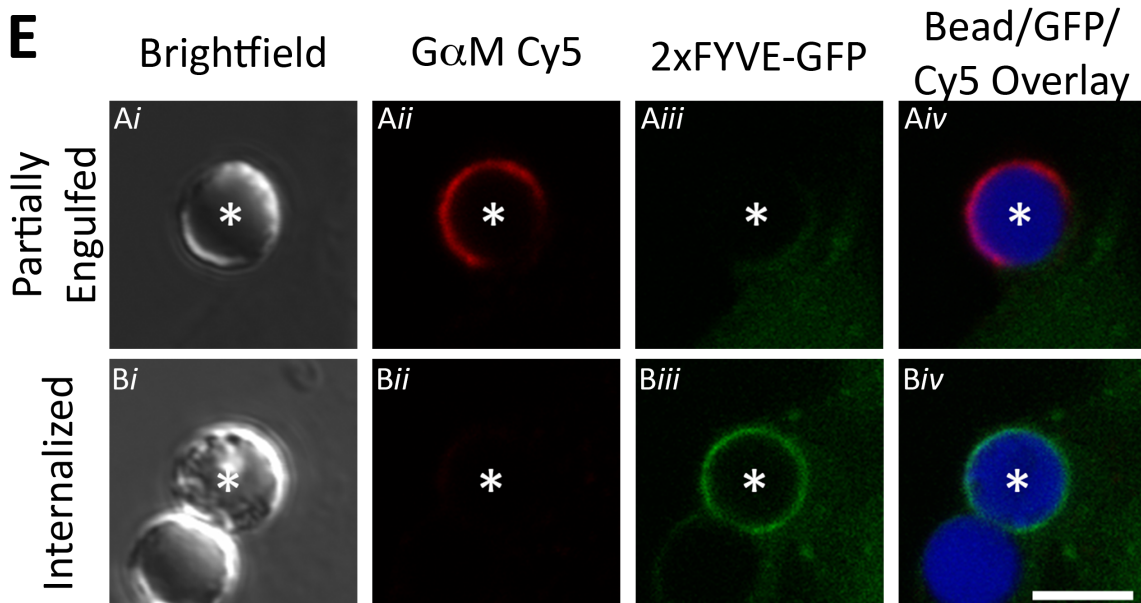
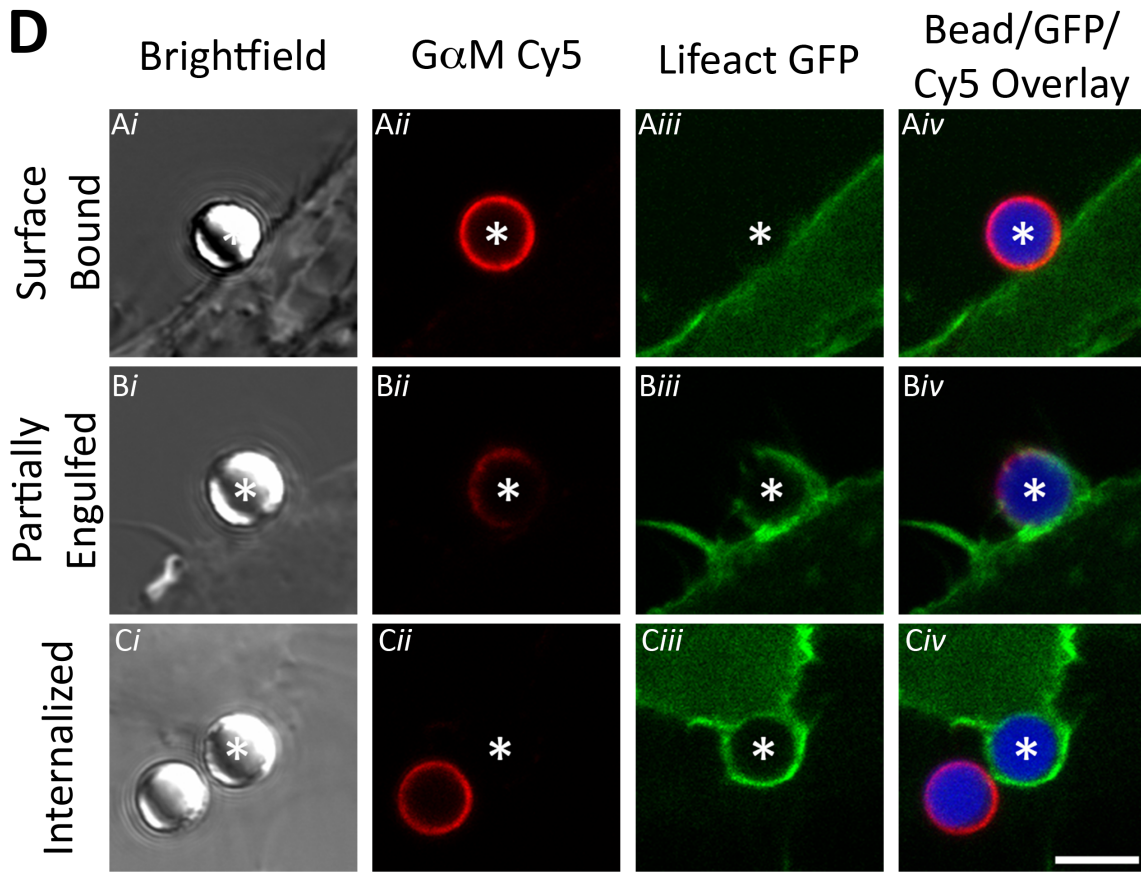


Figure 4. 2 Phagocytic targets can be differentially stained based on their position relative to the plasma membrane. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 1.5 \times 10^5$ cells) co-transfected with Lifeact-GFP (green), an F-actin marker, were incubated with mouse anti-hemagglutinin (α HA) monoclonal antibody (mAb) -coated, $4.5 \mu\text{m}$ Bright Blue[®] (BB) targets (3×10^5 beads) then fixed. (A) Exposed α HA mAb on surface bound or partially engulfed beads was counterstained with $0.25 \mu\text{g}$ Cy5 (red fluorophore)-conjugated goat anti-mouse (G α M Cy5) polyclonal antibody. AlexaFluor[®] 647 –conjugated rabbit anti-mouse (R α M AF647) secondary antibody was used in flow cytometry-based phagocytosis experiments. Both secondary antibodies bind the heavy and light chains of mouse IgG3, the α HA mAb isotype used in this thesis. (B) Representative schematic of G α M Cy5 and/or R α M AF647 accessibility to surface bound, partially engulfed, and internalized beads. (C) Confocal microscopy-based three-dimensional reconstruction of a cell expressing Lifeact-GFP (green) with an anti-HA coated BB bead (blue) tightly associated with the plasma membrane (indicated by arrow). Red fluorescence outlines exposed areas of beads (arrows) due to Cy5 counterstain. (D) Representative confocal microscopy-based images of G α M Cy5 (red) and Lifeact-GFP (green) distribution for beads (blue) that are surface bound, partially engulfed, or internalized and corresponding brightfield view. Lifeact-GFP was used to detect filamentous actin. (E) Representative G α M Cy5 (red) and 2xFYVE-GFP (green) distribution for bead (blue) is internalized and corresponding brightfield view. 2xFYVE-GFP detected a lipid marker of endosomes and early maturing phagosomes (i.e. phosphatidylinositol 3-phosphate). Scale bar, $5 \mu\text{m}$.

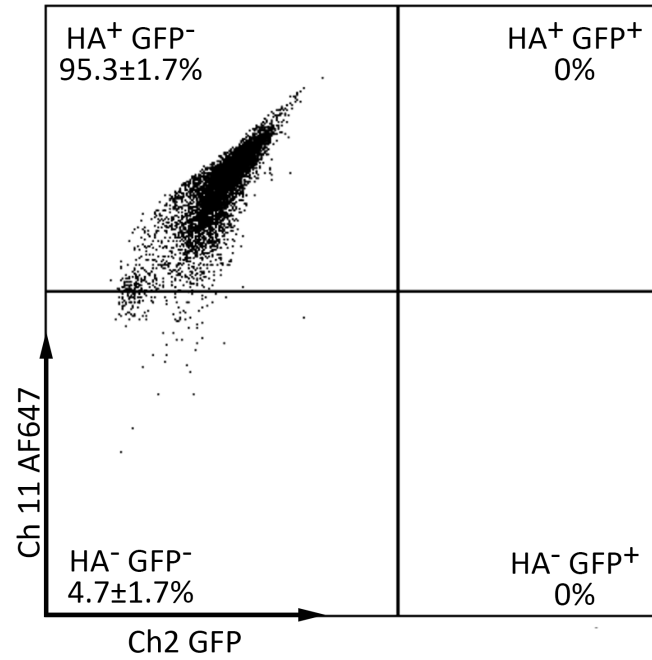
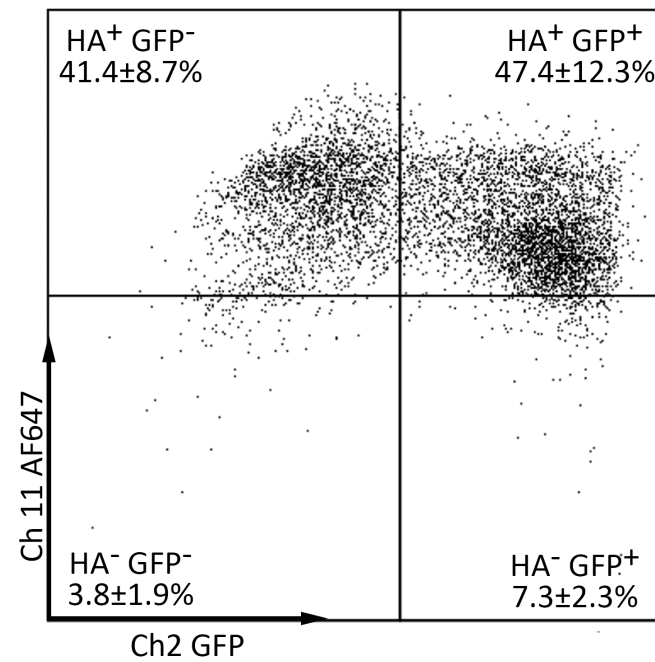
A**IpLITR 1.1b ITAM CYT****B****+ Lifeact-GFP**

Figure 4. 3 Imaging flow cytometry-based co-expression analysis of Lifeact-GFP and IpLITR 1.1b/ITAM in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) were transiently co-transfected with Lifeact-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μg αHA mAb followed by staining with 0.5 μg AF647-conjugated rabbit $\alpha\text{-mouse}$ IgG. Lifeact-GFP and IpLITR co-expression was compared to AD293 cells stably expressing IpLITR 1.1b/ITAM that were not transfected with Lifeact-GFP. IpLITR 1.1b/ITAM expression in non-transfected cells (A) and co-transfected cells (B) shown. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

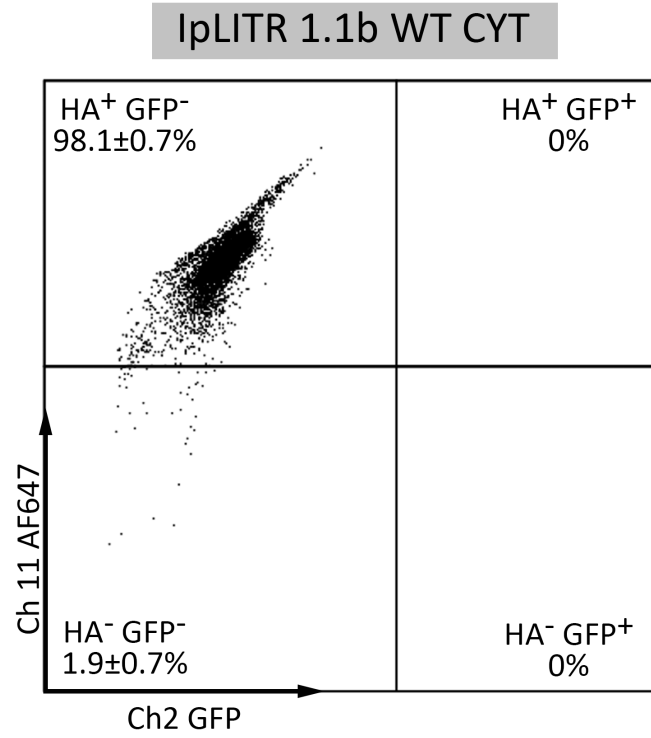
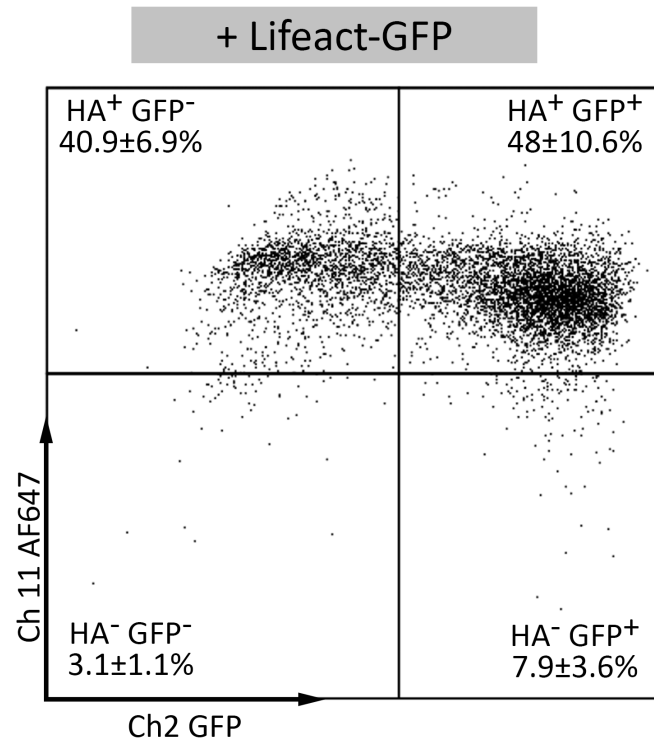
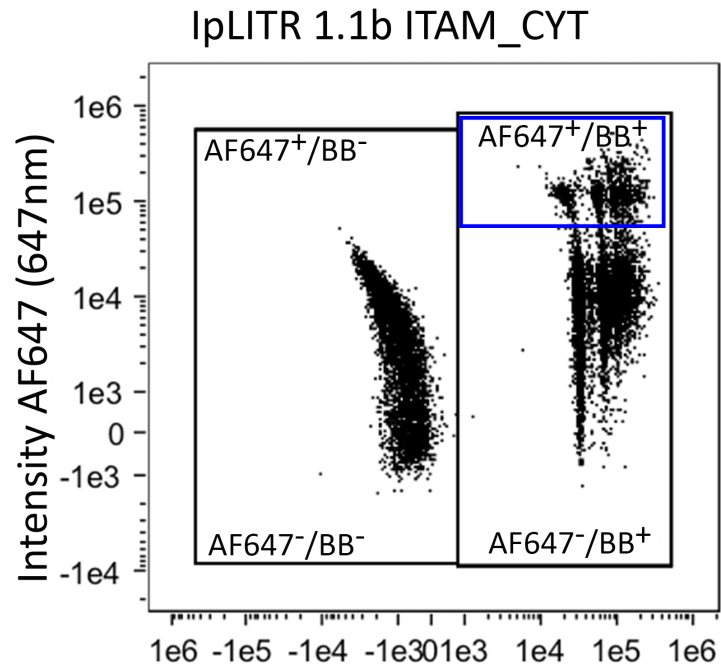
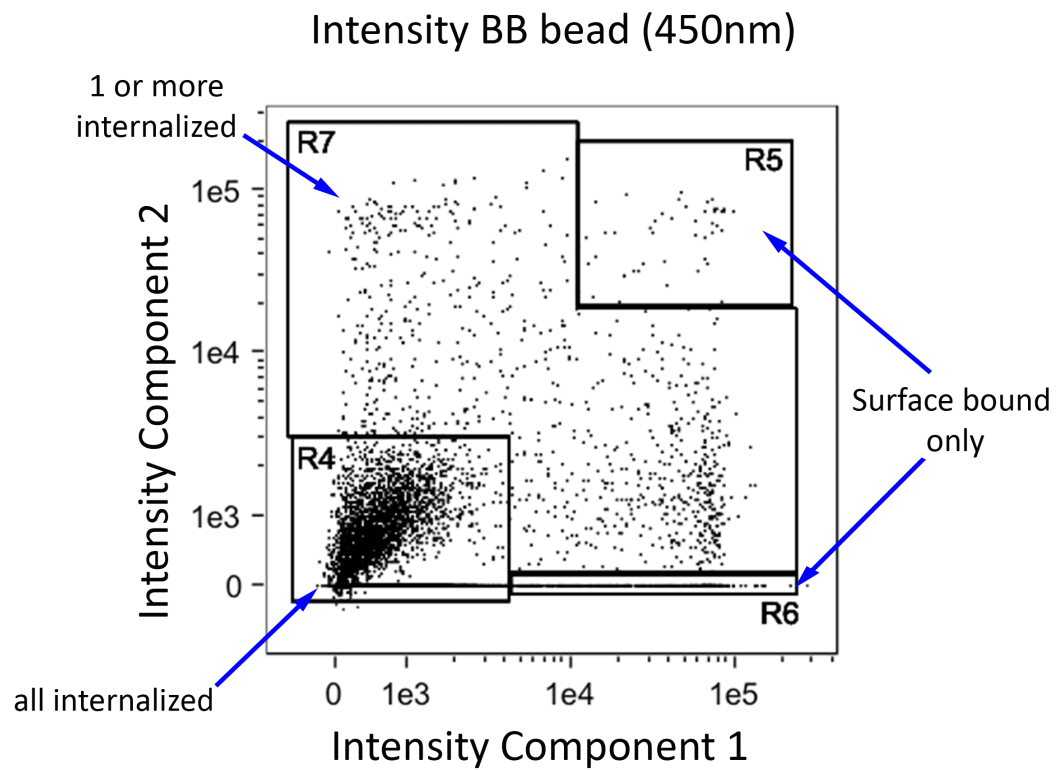
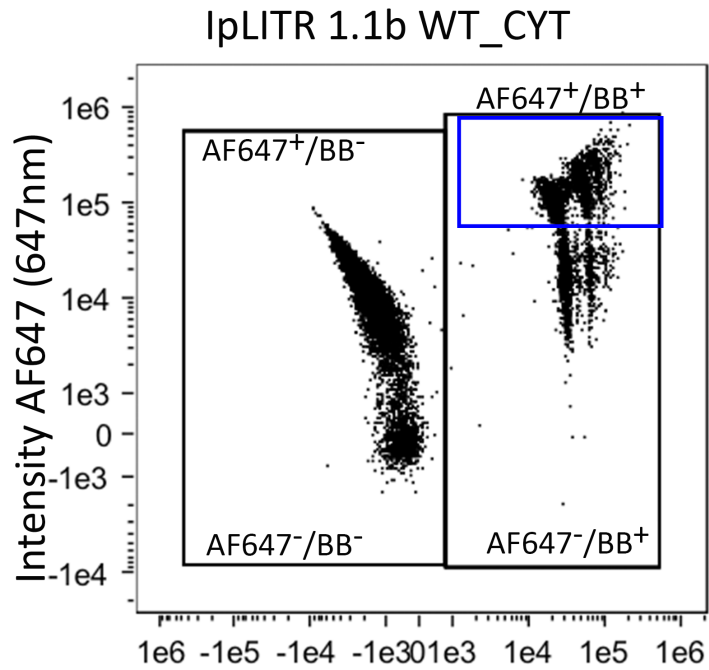
A**B**

Figure 4. 4 Imaging flow cytometry-based co-expression analysis of Lifeact-GFP and IpLITR 1.1b/WT in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/WT ($\sim 3 \times 10^5$ cells) were transiently co-transfected with Lifeact-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μg αHA mAb followed by staining with 0.5 μg AF647-conjugated rabbit α -mouse IgG. Lifeact-GFP and IpLITR co-expression was compared to AD293 cells stably expressing IpLITR 1.1b/WT that were not transfected with Lifeact-GFP. IpLITR 1.1b/WT expression in non-transfected cells (A) and co-transfected cells (B) shown. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

A**B**

C



D

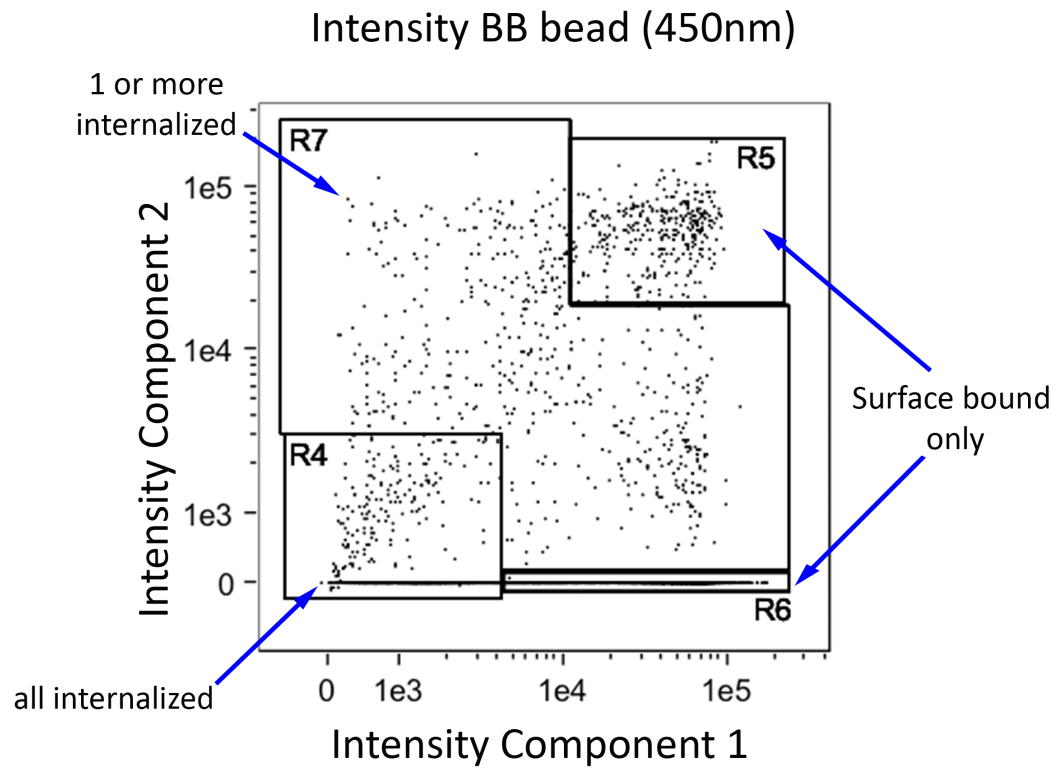
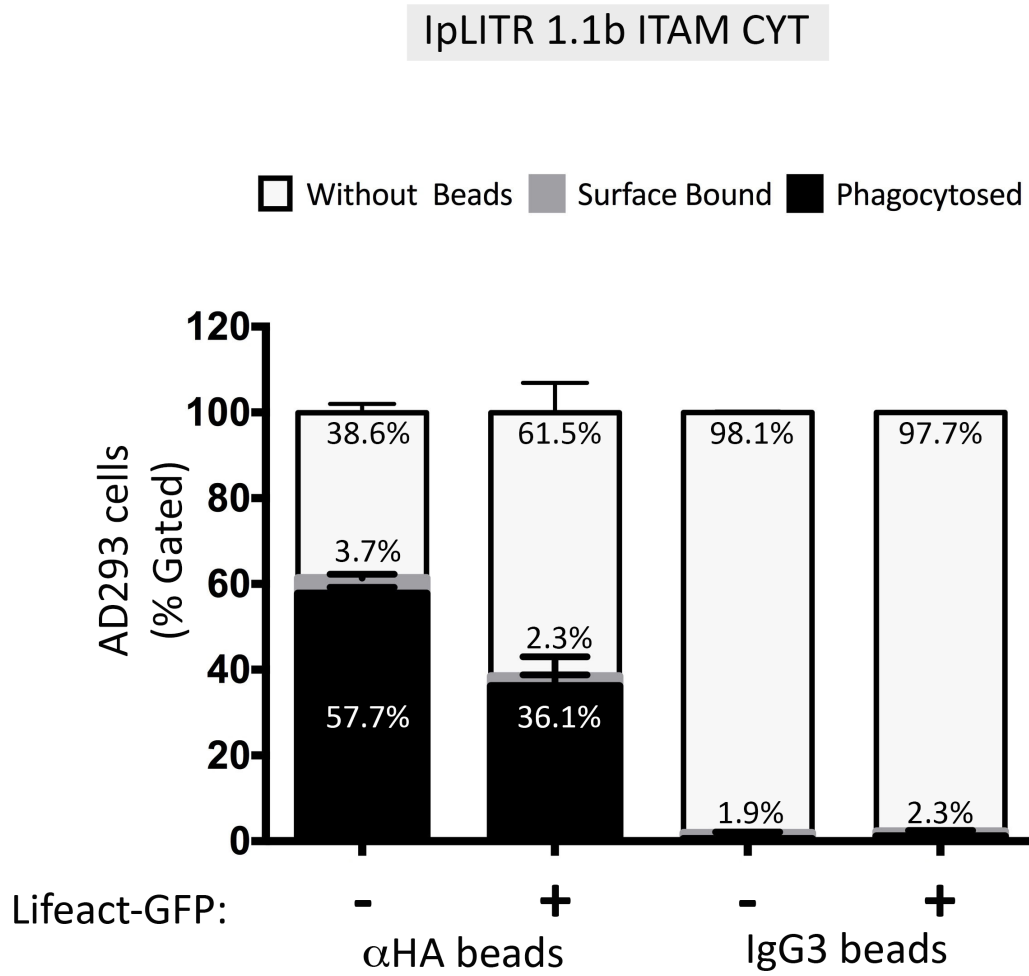


Figure 4. 5 Gating and component masking strategy for imaging flow cytometry-based analysis of IpLITR 1.1b/ITAM and 1.1b/WT –mediated target capture and engulfment.

AD293 cells stably expressing IpLITR 1.1b/ITAM and 1.1b/WT ($\sim 3 \times 10^5$ cells) were incubated with anti-HA mAb-coated BB beads (9×10^5 beads/sample; emission at 450nm) at 37°C for one hour. Surface bound beads were counterstained with AF647-conjugated rabbit anti-mouse IgG. Fluorescence intensity of AF647 and BB beads were plotted against each other showing the association of beads with IpLITR 1.1b/ITAM (A) or 1.1b/WT (C) –expressing cells. (+) represents fluorescence while (–) represents no fluorescence. Events marked by blue box represent cells that are associated with one or more surface bound bead. The population of BB⁺ cells associated with three or fewer beads are sub-analyzed in B (IpLITR 1.1b/ITAM) and D (1.1b/WT): events in region (R)4 only have internalized beads; events in R7 have internalized at least 1 bead; events in R5 and R6 only have surface bound beads. Numerical results graphed in below in Figure 4.6.

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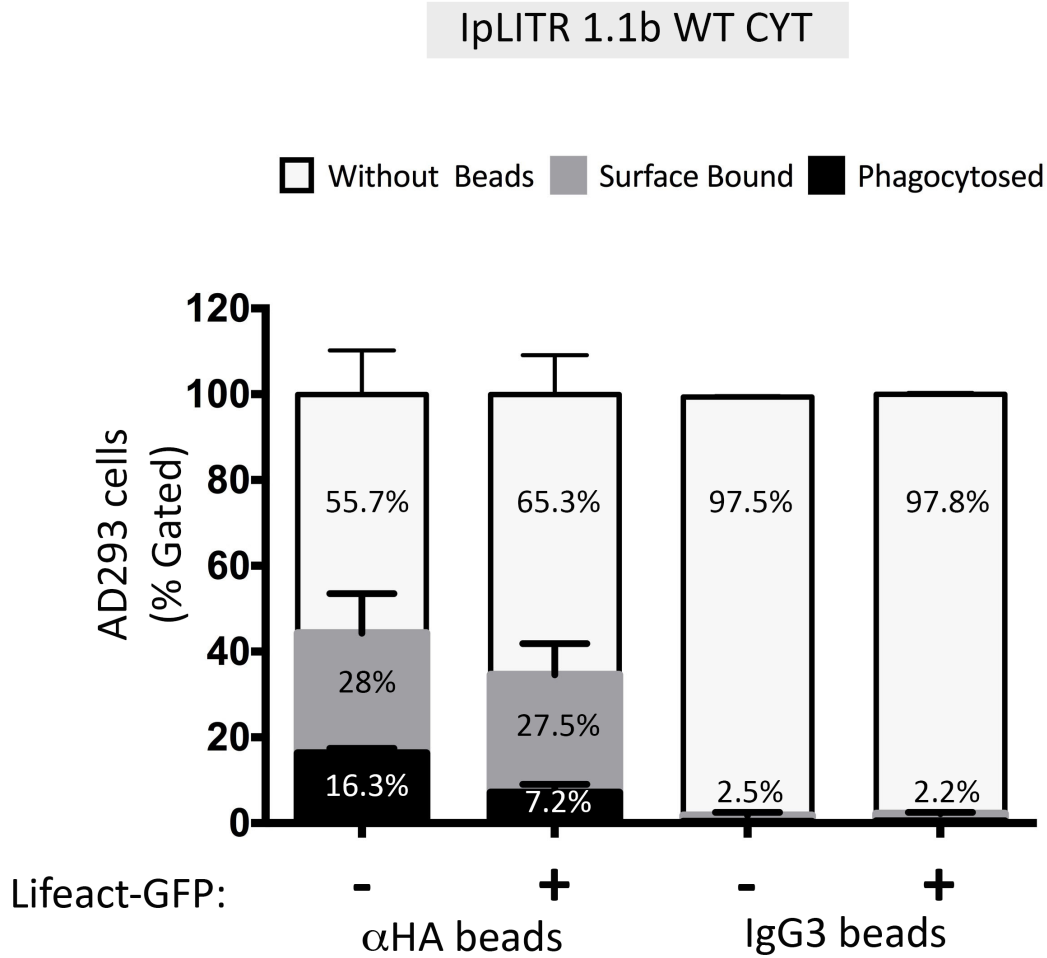
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Figure 4. 6 IpLITR 1.1b/ITAM and 1.1b/WT retain their phagocytic phenotype after co-transfection with Lifeact-GFP. AD293 cells stably expressing IpLITR 1.1b/ITAM (A) and 1.1b/WT (B) were co-transfected with Lifeact-GFP (+) for twenty-four hours. The ability of co-transfected cells (3×10^5 cell/sample) to internalize α HA mAb or IgG3 isotype control –coated beads (9×10^5 beads/sample) at 37°C for one hour was assessed by imaging flow cytometry in comparison to cells without Lifeact-GFP (-). Numbers represent the mean percent of population that had internalized at least one bead (black), only had surface bound beads (gray), or were not associated with beads (white). Mean $\pm$ SEM (error bars) of three independent experiments shown.

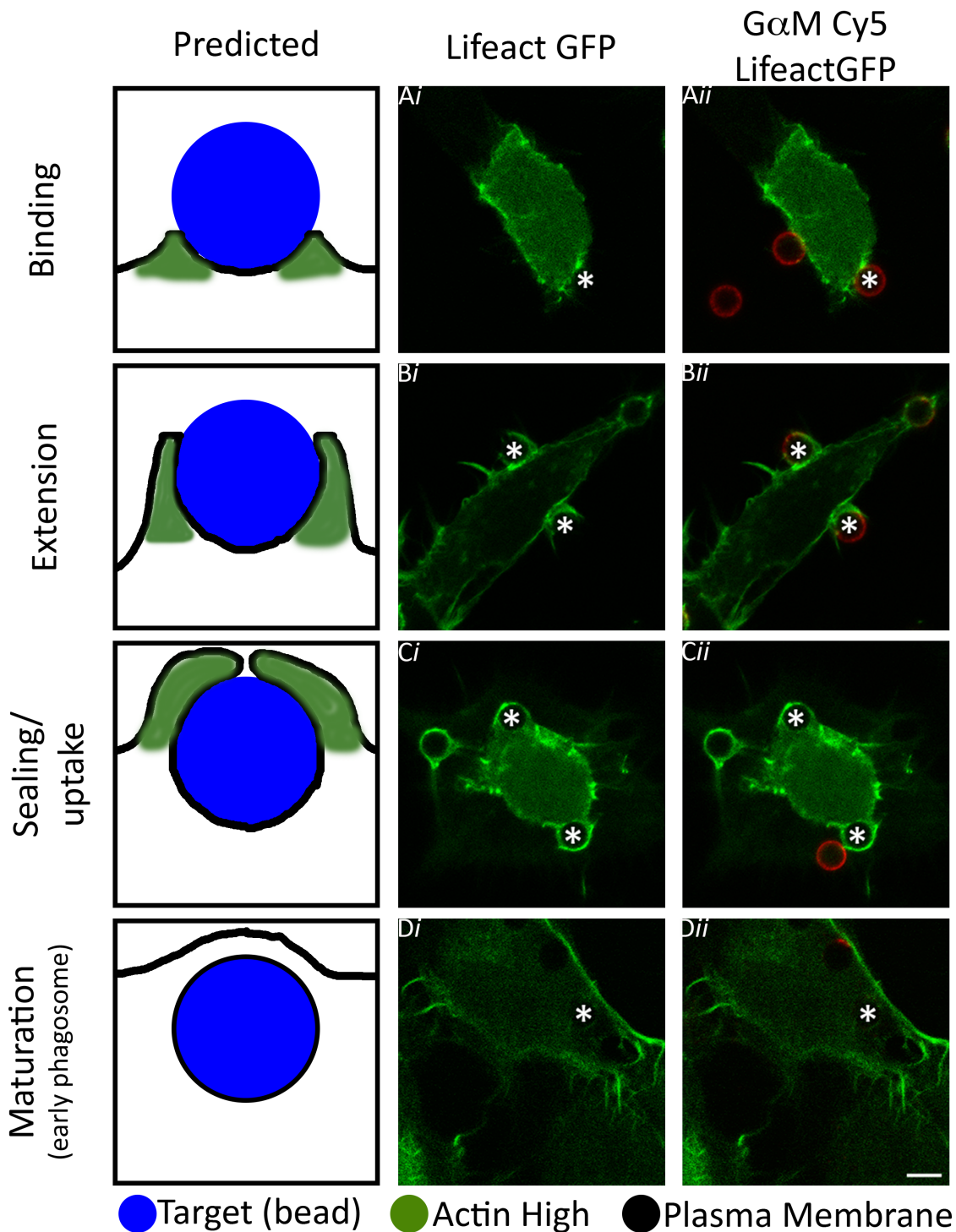
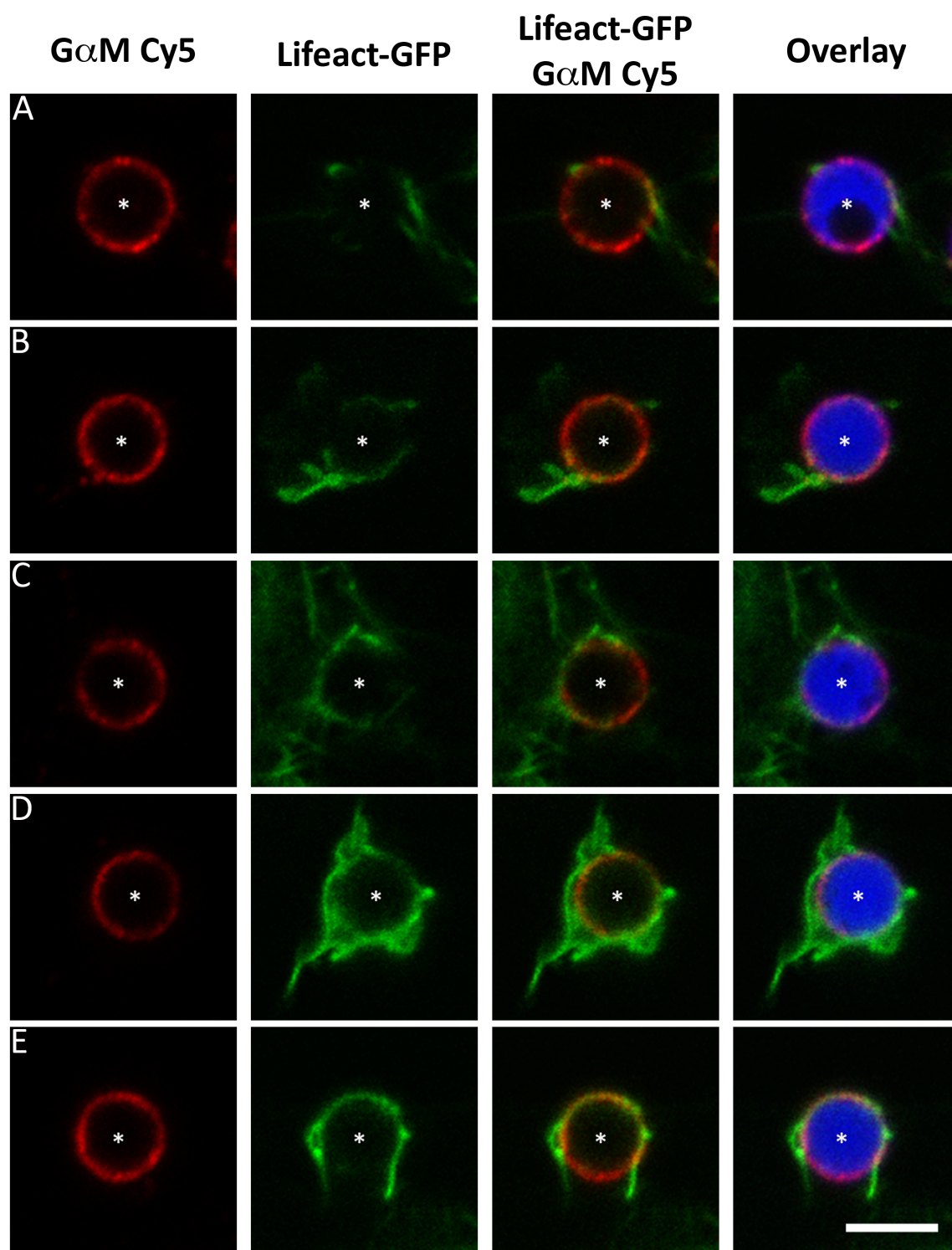
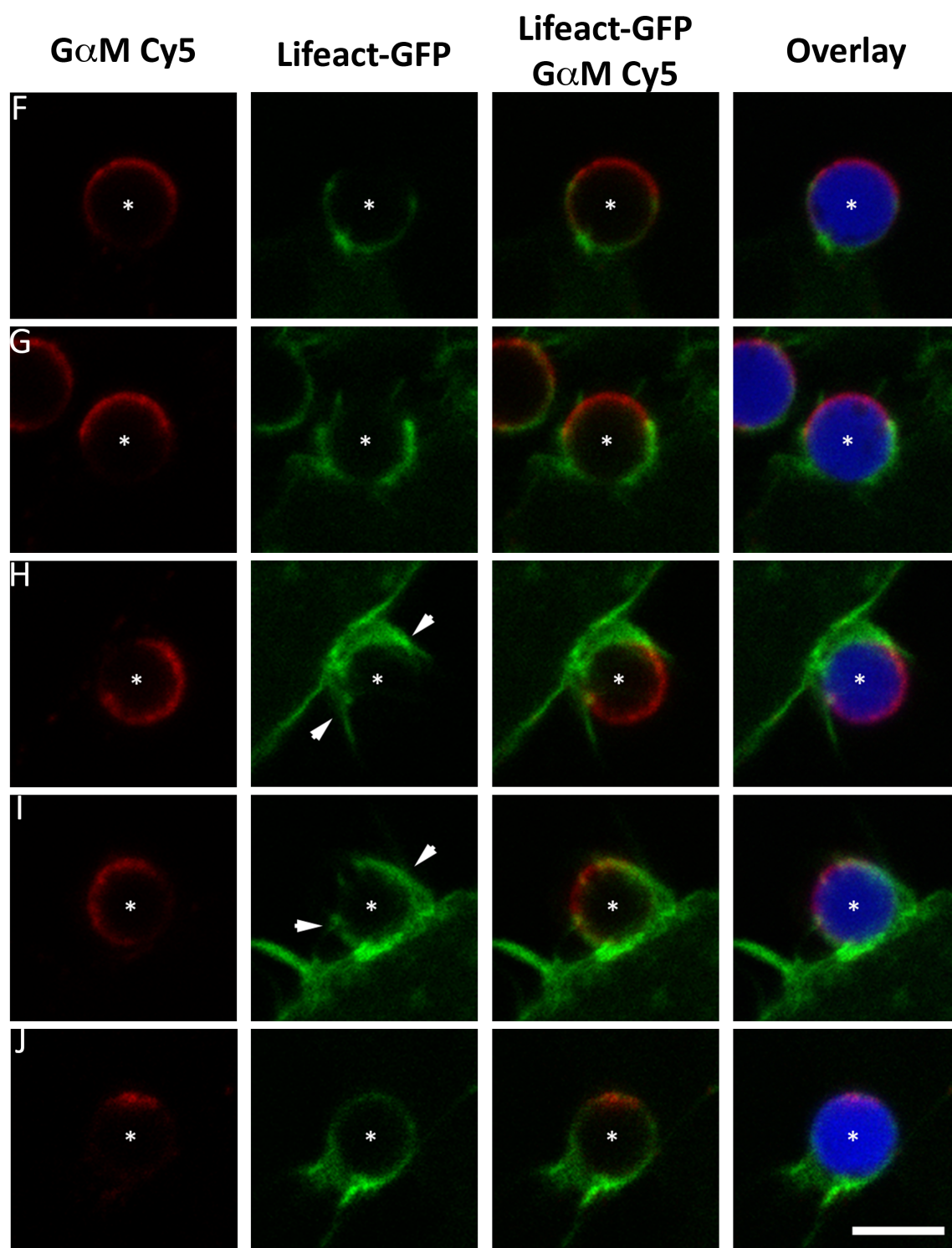


Figure 4. 7 Expected and observed actin distribution during IpLITR 1.1b/ITAM-mediated phagocytosis. Representative images of IpLITR 1.1b/ITAM –expressing cells binding beads (Ai-Aii), extending pseudopods (Bi-Bii), sealing phagosomes (Ci-Cii) or maturing phagosomes (Di-Dii). Asterisks show location of BB beads (not shown). Lifeact-GFP (actin) is shown in green and Cy5-conjugated goat anti-mouse (exposed αHA mAb on BB beads) shown in red. Scale bar 5 μm.





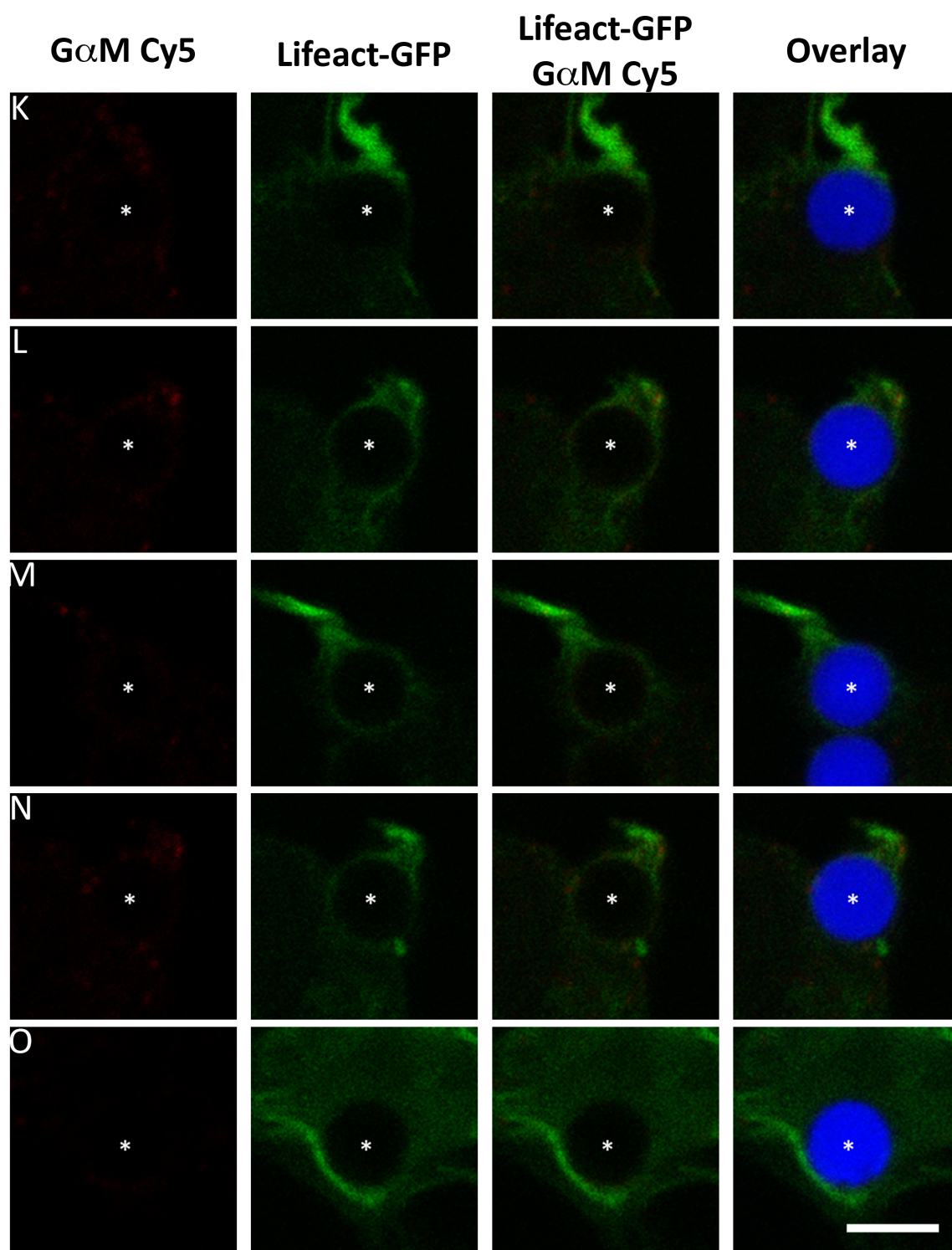


Figure 4. 8 Confocal microscopic visualization of Lifeact-GFP distribution at the phagocytic cup during IpLITR 1.1b/ITAM –mediated phagocytosis. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 1.5 \times 10^5$ cells) co-transfected with Lifeact-GFP were incubated with mAb anti-HA coated, 4.5 μm targets (3×10^5 beads) then fixed prior to imaging. External regions of beads were counterstained with a Cy5-conjugated goat anti-mouse mAb. Surface bound targets (A-E), partially engulfed (F-), and internalized (K-O) are representative images from three independent experiments with five replicates within each experiment. Scale bar, 5 μm .

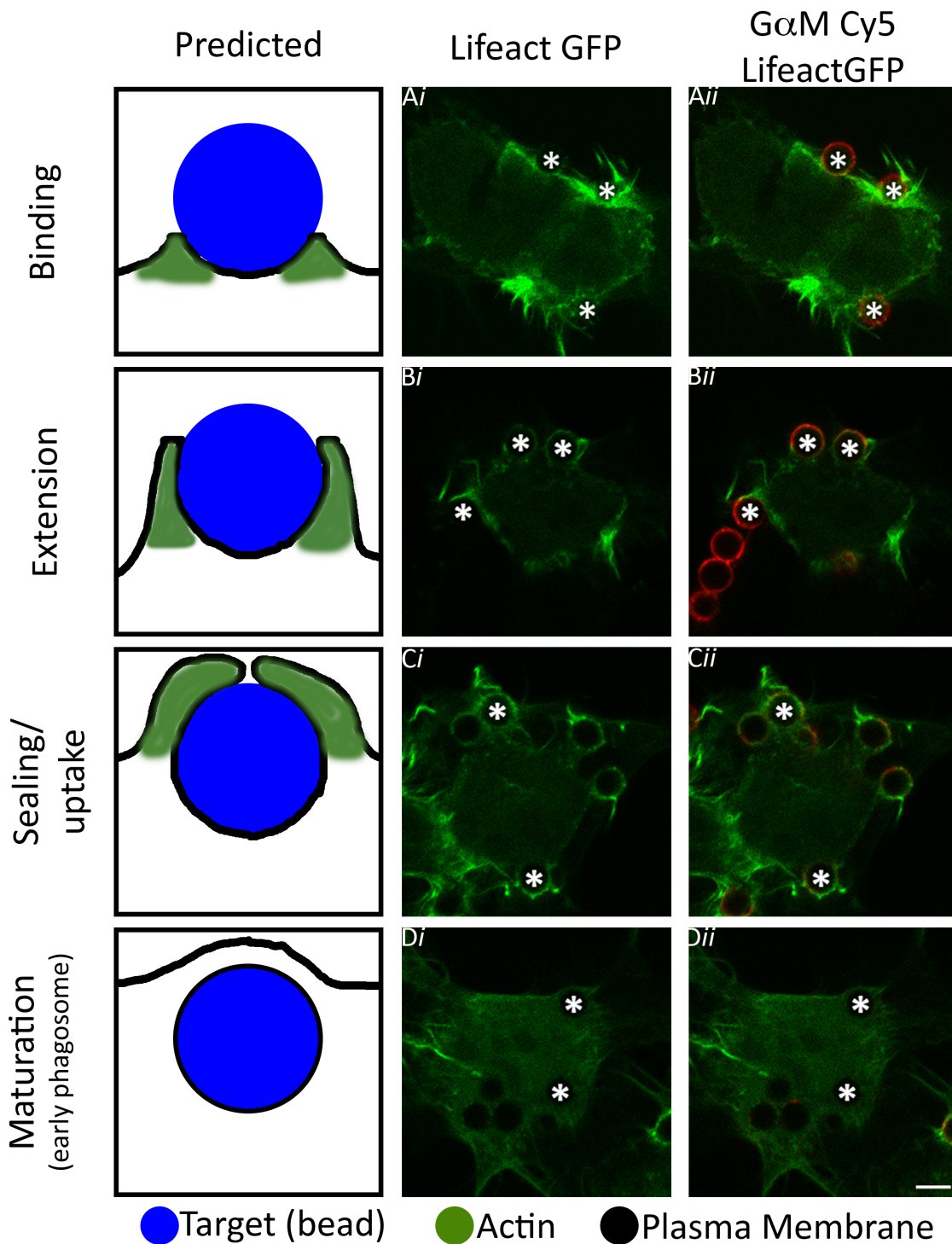
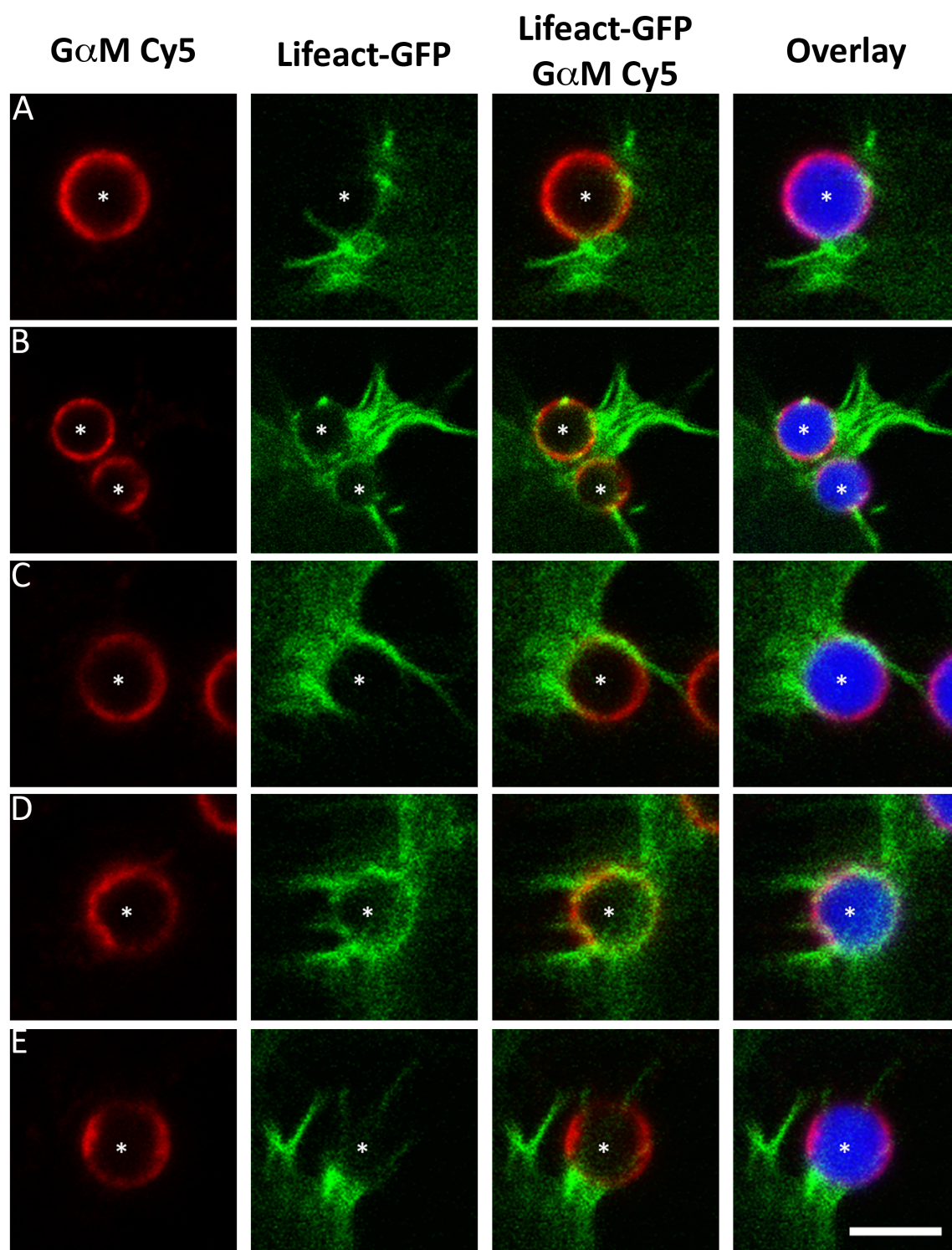
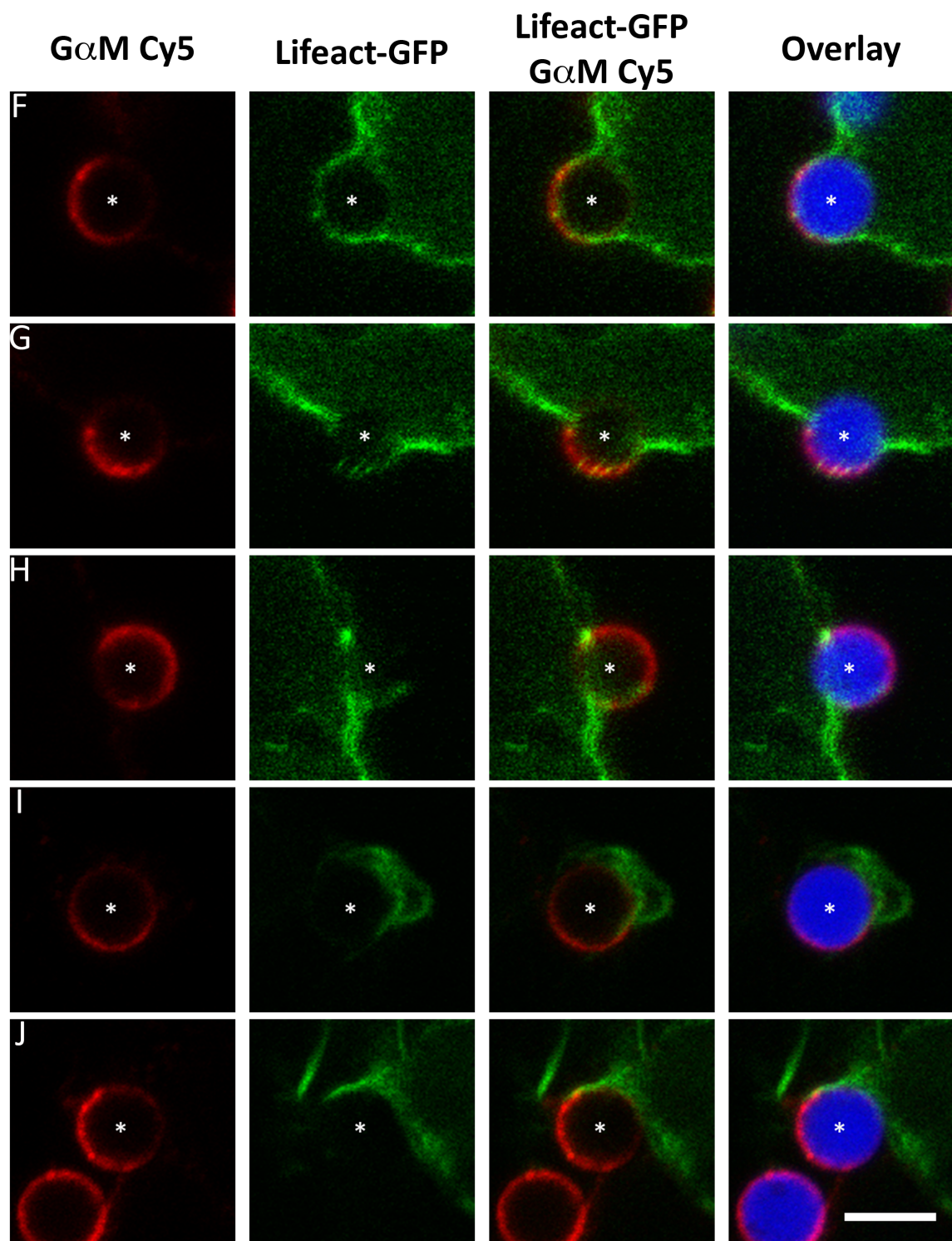


Figure 4. 9 Expected and observed actin distribution during IpLITR 1.1b/WT –mediated phagocytosis. Representative images of IpLITR 1.1b/WT –expressing cells binding beads (Ai-Aii), extending pseudopods (Bi-Bii), sealing phagosomes (Ci-Cii) or maturing phagosomes (Di-Dii). Asterisks show location of BB beads (not shown). Lifeact-GFP (actin) is shown in green and Cy5-conjugated goat anti-mouse (exposed anti-HA mAb on BB beads) shown in red. Scale bar 5 μ m.





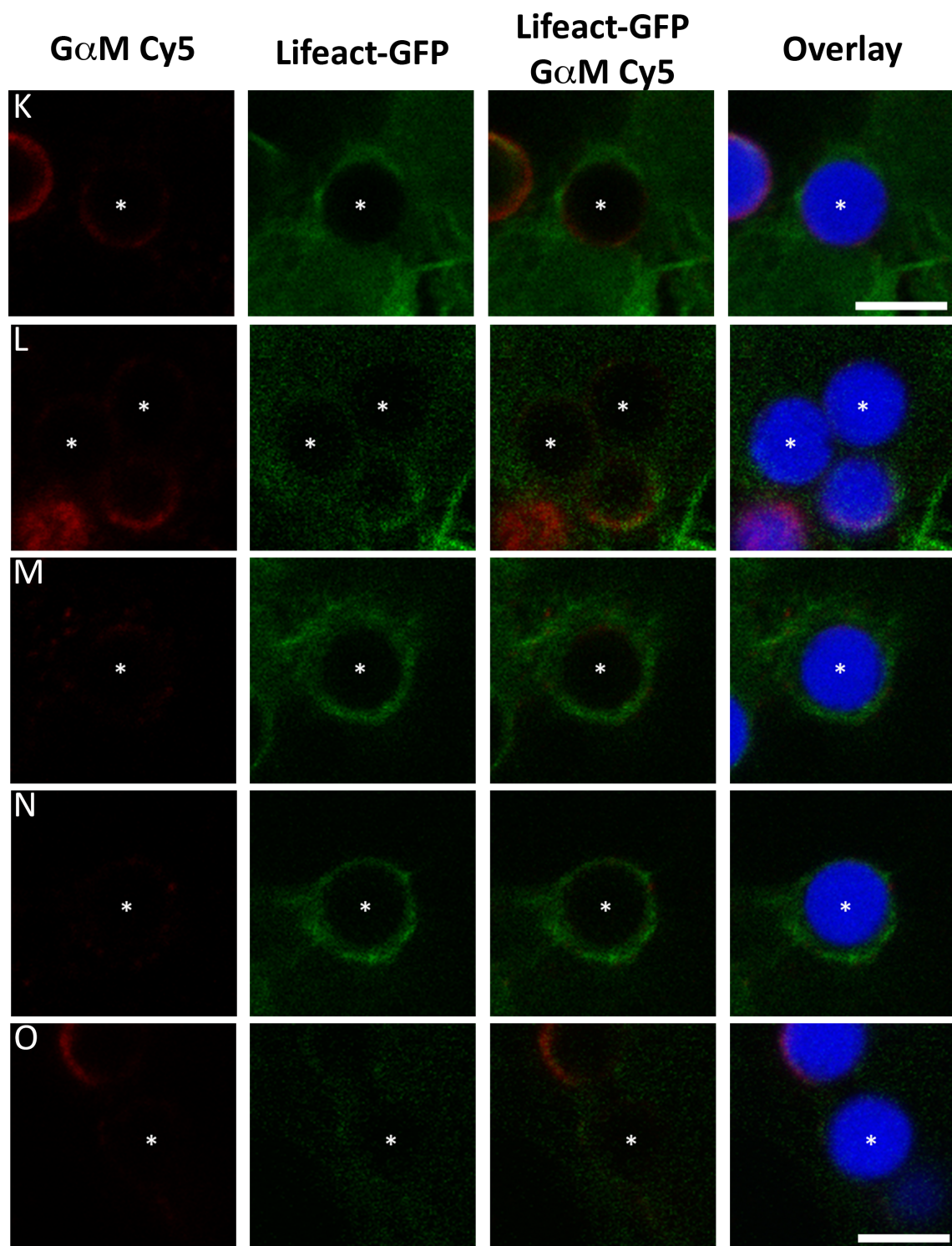


Figure 4. 10 Confocal microscopic visualization of Lifeact-GFP distribution at the phagocytic cup during IpLITR 1.1b/WT –mediated phagocytosis. AD293 cells stably expressing IpLITR 1.1b WT ($\sim 1.5 \times 10^5$ cells) co-transfected with Lifeact-GFP were incubated with mAb anti-HA coated, 4.5 μm targets (3×10^5 beads) then fixed prior to imaging. External regions of beads were counterstained with a Cy5-conjugated G α M mAb. Surface bound targets (A-E), partially engulfed (F-), and internalized (K-O) are representative images from two independent experiments with five replicates within each experiment. Scale bar, 5 μm .

CHAPTER 5

Development of an imaging-based platform to study IpLITR-mediated recruitment of phosphoinositides and other signaling molecules during the phagocytic process

5.1 Introduction

Phosphoinositides (PIs) are plasma membrane phospholipids that are predominantly found differentially and spatially distributed amongst the cytoplasmic leaflet of cellular membranes, generally in low abundance, and critical in recruiting effector proteins to membranes (Kutateladze, 2010; Levin et al, 2015; Swanson, 2014). PIs are composed of diacylglycerol linked to D-myo-inositol-1-phosphate via a phosphodiester bond. The hydrophobic diacylglycerol inserts into membranes and D-myo-inositol-1-phosphate is exposed to the intracellular environment. Hydroxyl groups on positions 3, 4, and 5 of the inositol ring can be reversibly phosphorylated (or dephosphorylated) to generate seven distinct PI species: monophosphorylated PI(3)P, PI(4)P, and PI(5)P; bisphosphorylated PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂; and trisphosphorylated PI(3,4,5)P₃ (depicted in Fig 5.1). PIs can also be irreversibly hydrolyzed by phospholipases to generate diacylglycerol and an inositol ring, which can also serve as potent intracellular signals (reviewed by Kutateladze, 2010; Schink et al, 2016; Levin et al, 2015). Differential inositol phosphorylation is responsible for mediating the recruitment of distinct effector molecules that have been implicated in various intracellular signaling networks responsible for membrane trafficking, cytoskeletal rearrangements, as well as lipid

metabolism. PI effector proteins recognize individual PI through binding domains such as pleckstrin homology (PH) and F-Y-V-E domains (Kutateladze, 2010). The FYVE domain is a ~70 amino acid zinc-binding domain with three positively charged, conserved sequences (WxxD, RR/KHHCR and RVC motifs), that specifically bind PI(3)P (Kutateladze, 2010). The amino acid sequence of PH domain is highly variable between PH domain-containing proteins but they form a conserved three-dimensional structure resulting in PI recognition by a diverse array of molecules (Kutateladze, 2010). At least eleven PI binding domains have been described (Kutateladze, 2010) but the focus of this thesis will be on the PH and FYVE domains.

Despite the importance of PIs in regulating many cellular processes, PI-effector protein interactions are difficult to study using traditional methods of exploring protein-protein interactions (Grinstein, 2010). Some PI species are only transiently found in cells and many PI interactions are ephemeral. Biochemical methods tend to destroy phospholipid species during processing while immunofluorescence requires permeabilization of the plasma membrane, which largely removes PIs (Grinstein, 2010). Due to the difficulty of studying phosphoinositide-mediated interactions, there has been a surge of imaging-based tools that mainly rely on the fluorescent protein (FP) tagging of phosphoinositide binding domains such as pleckstrin homology domain and FYVE. Confocal-microscopy-based examination of the distribution of phosphoinositides using these tools has provided much information regarding the localization of phosphoinositides during various cellular processes. For example, using a fusion construct containing the PH domain of phospholipase C $\delta 1$ (PH-PLC $\delta 1$ -GFP), a protein

known to selectively bind PI(4,5)P₂, Várnai and Balla (1998), demonstrated that PI(4,5)P₂ is present in the plasma membrane of resting cells. Additionally, by fusing two FYVE zinc finger domains from the PI(3)P-binding protein Hrs (a protein known to bind PI(3)P) to GFP (i.e. 2×FYVE-GFP), Gillooly et al (2000) demonstrated that PI(3)P localizes to early endosomes. This strategy of fusing fluorescent proteins to phosphoinositide-binding domains to visualize phosphoinositide distribution during cellular processes has revolutionized our understanding of the overall importance of lipid signaling-mediated regulation of cellular events such as phagocytosis.

During phagocytosis, PI species are critical for promoting cytoskeletal rearrangements, plasma membrane remodelling, and phagosome maturation (Swanson, 2008; Swanson, 2014; Levin et al, 2015). Specifically, during FcR-mediated phagocytosis, receptor-mediated signal transduction results in a transient but localized increase in PI(4,5)P₂ at sites where pseudopods form (Botelho et al, 2000). This increase in PI(4,5)P₂ occurs concomitantly with the activation of small RhoGTPases (Cdc42 and Rac1) that are responsible for activating actin nucleation-promoting factors WASP and WAVE, which bind to Arp2/3 and promote actin polymerization (Hoppe and Swanson, 2004; Takenawa & Suetsugu, 2007). Additionally, receptor activation results in the recruitment and activation of spleen tyrosine kinase (Syk) which is responsible for the activation of downstream adaptor molecules that provide a platform for the localization of PI(4,5)P₂ effector molecules to the phagocytic cup (Crowley et al, 1997; Tridandapani et al, 2000; Berton et al, 2005). As the phagocytic cup begins to close, PI(4,5)P₂ surrounding the phagosomal membrane disappears via two major pathways: irreversible cleavage to

diacylglycerol and inositol triphosphate (IP₃) (Botelho et al., 2000) and phosphorylation by PI-3-kinase to form PI(3,4,5)P₃ (Araki et al, 1996). This disappearance of PI(4,5)P₂ corresponds with actin depolymerisation at the base of the phagocytic cup and a concomitant increase in PI(3,4,5)P₃ (Levin et al, 2015). During FcR-mediated phagocytosis, PI(3)P is absent from pseudopodia and the plasma membrane but is predominantly detected surrounding maturing phagosomes (Levin et al, 2015).

We previously demonstrated that the teleost ITAM-dependent functional responses such as phagocytosis are dependent on many of the mediators that are involved in the prototypical, mammalian ITAM-mediated responses (Cortes et al, 2012; Lillico et al, 2015). Additionally, this teleost receptor induced a phagocytic response that was visually reminiscent of the mammalian FcR-mediated phagocytosis. On the other hand, the ITAM-independent, IpLITR 1.1b/WT receptor did not appear to require many of the signaling components of the prototypical ITAM-mediated signaling cascade including the activity of PI3K; however, IpLITR 1.1b-mediated phagocytosis did require actin polymerization as well as the activities of Src and Syk kinases (Lillico et al, 2015). This led to the hypothesis that IpLITR 1.1b-mediated phagocytosis could be mediated in a Syk-dependent manner whereby Syk kinase is recruited to the ITIMs (i.e. Y₄₇₇ and Y₄₉₉) within the distal cytoplasmic region of IpLITR 1.1b which are spaced twenty-two amino acids apart: a spacing previously demonstrated to facilitate Syk recruitment by the ITIM-containing receptor PECAM-1 (Wang et al, 2011). In addition, in RBL-2H3 cells, IpLITR 1.1b-induced target acquisition often (but not always) resulted in the formation of stalled phagocytic cups in which beads remained surface-bound: an observation that

might be explained by the independence of IpLITR 1.1b-mediated phagocytosis on the activity of PI3K (Lillico et al, 2015). This led to the hypothesis that IpLITR 1.1b exists in a primed state facilitating rapid engulfment of some targets. This primed state could be dependent on Nck1-mediated recruitment and activation of the WAVE2 complex to link IpLITR 1.1b binding to cytoskeletal reorganization (Lillico et al, 2015).

In Chapter 4, I visualized the distribution of actin when beads were surface-bound, partially engulfed, and fully internalized to gain insight into the cytoskeletal changes mediated by IpLITR 1.1b. I demonstrated that, during the IpLITR 1.1b/ITAM-mediated phagocytic process, actin dynamics followed the mammalian FcR-induced, linear progression from target binding, pseudopod extension, phagosome sealing, and phagosome maturation; conversely, the ITAM-independent receptor, IpLITR 1.1b/WT, promoted a unique distribution of actin during the receptor-mediated phagocytic process likely due to distinct signaling pathways between the two IpLITR-types. Specifically, I observed that IpLITR 1.1b did not promote the extension of long pseudopodia (actin-rich, membranous filopodia that extend and reach around targets to engulf them) that are characteristic of ITAM-mediated phagocytosis (i.e. FcR, Dectin-1, and IpLITR 1.1b/ITAM)(Flannagan et al, 2012, Mao & Finnemann, 2015). The difference in IpLITR 1.1b/ITAM and IpLITR 1.1b/WT –mediated reorganization of the cytoskeleton could be explained by distinct signaling pathways that translate receptor binding to F-actin nucleation.

In this chapter, I tested the hypothesis that IpLITR 1.1b/ITAM and IpLITR 1.1b/WT promote distinct distributions of signal transduction molecules during the

IpLITR-mediated phagocytic process. To test this hypothesis, I used GFP-tagged PH-PLC δ 1 and 2xFYVE, probes for PI(4,5)P₂ and PI(3)P respectively, as well as a GFP-tagged Syk kinase in order to visualize the distribution of these molecules during IpLITR-mediated phagocytosis. My first aim was to test whether IpLITR 1.1b/ITAM and IpLITR 1.1b/WT would induce a PI(4,5)P₂ distribution that would follow the pattern of actin dynamics that I previously observed in Chapter 4. Specifically, would IpLITR 1.1b/ITAM promote a transient increase in PI(4,5)P₂ at the location of receptor-binding that followed the length of pseudopodia while disappearing from the base of the nascent phagosome and disappear from maturing phagosome? Additionally, does PI(4,5)P₂ localize to the short pseudopodia previously observed during IpLITR 1.1b/WT-mediated phagocytosis? Secondly, to provide further evidence that IpLITR-mediated phagocytosis results in internalization of targets into a PI(3)P-enriched phagosome, I visualized the distribution of 2xFYVE-GFP during IpLITR-mediated phagocytosis (Fig 5.2). Lastly, I attempted to monitor the distribution of SykGFP during IpLITR-mediated phagocytosis to study the recruitment of this cytoplasmic signaling protein to the phagocytic cup. However, I was only able to provide preliminary observations regarding SykGFP localization to the IpLITR 1.1b/ITAM-mediated phagocytic cup. Overall, my studies provide the first insights into the distribution of PI(4,5)P₂ and PI(3)P during the IpLITR-mediated phagocytic process, which sets the stage for understanding the role of these lipid mediators during IpLITR-mediated signal transduction.

5.2 Results

5.2.1 Examination of the distribution of phosphatidylinositol 4,5-bisphosphate during IpLITR 1.1b/ITAM and 1.1b/WT –mediated phagocytosis using PH-PLC δ_1 -GFP

5.2.1.1 Imaging flow cytometry-based validation of IpLITR phagocytic phenotypes after AD293 co-transfections with PH-PLC δ_1 -GFP

To determine if IpLITR stable expression in AD293 cells was affected by co-transfection with PH-PLC δ_1 -GFP, receptor expression levels were compared to that of PH-PLC δ_1 -GFP co-transfected cells using imaging flow cytometry. Approximately 98% and 98.6% of AD293 cells stably expressing IpLITR 1.1b/ITAM and IpLITR 1.1b/WT, respectively, were positive for IpLITR; these cells did not express GFP (Figs 5.3A and 5.4A). Approximately 46% of PH-PLC δ_1 -GFP co-transfected, IpLITR 1.1b/ITAM-expressing, cell population fluoresced in both channels (Fig 5.3 B) while only 25% of co-transfected IpLITR 1.1b/WT cells were double positive (Fig 5.4 B). Importantly, the majority of cells not expressing GFP retained IpLITR co-expression. Although there was some background with the isotype control antibody (IgG3), the staining pattern did not reflect the surface expression of IpLITRs (i.e. it was diffuse and usually debris that fell within the gates used). Results of isotype control stain are in Figures A1, A3, A6, and A8 of the appendix. Simultaneously, the phagocytic activity of these cells was also verified by imaging flow cytometry. AD293 cells stably expressing IpLITRs retained their overall phagocytic phenotypes (Fig 5.5); that is, IpLITR 1.1b/ITAM demonstrated an overall engulfment phenotype while IpLITR 1.1b/WT had an overall tethering phenotype. Approximately 57% of IpLITR 1.1b/ITAM cells had fully internalized at least one bead and 4.3% of cells

only had surface bound beads. PH-PLC δ_1 -GFP co-transfected IpLITR 1.1b/ITAM cells retained a similar phenotype as 47.2% phagocytosed at least one bead and 4.3% had surface bound beads only (Fig 5.5A). IpLITR 1.1b/WT-expressing AD293 stables predominantly had surface bound targets (30.4%) and only 19.8% phagocytosed at least one bead. Co-transfected IpLITR 1.1b/WT cells also retained a similar phenotype with 18.3% fully internalizing at least one bead and 28.9% with only surface bound beads (Fig 5.5B). Neither IpLITR 1.1b/ITAM nor IpLITR 1.1b/WT-expressing cells engulfed beads coated in isotype control demonstrated by a lack of bead-cell association (~98% for all cell populations tested). These observations demonstrate that when AD293 cells stably expressing IpLITRs are co-transfected with PH-PLC δ_1 -GFP they retain IpLITR surface expression and previously documented phagocytic activities.

5.2.1.2 Confocal microscopy-based examination of the PH-PLC δ_1 -GFP distribution during IpLITR 1.1b/ITAM –mediated phagocytosis

To determine whether PI(4,5)P₂ is reorganized during IpLITR-mediated phagocytosis, I examined its distribution using PH-PLC δ_1 -GFP which detects PI(4,5)P₂. The progression of the distribution of PH-PLC δ_1 -GFP during IpLITR 1.1b/ITAM –mediated phagocytosis is shown in Fig 5.6. As was predicted based on classical FcR-mediated phagocytosis, there was a transient increase in PI(4,5)P₂ at the base of the phagocytic cup upon binding. As PI(4,5)P₂ –rich pseudopodia extended around targets, PI(4,5)P₂ disappeared from the base of the phagocytic cup and was not present in the early phagosomal membrane. Figure 5.7 shows representative images of the PH-PLC δ_1 -GFP distribution when beads

are surface bound (Fig 5.7 A-E), partially engulfed (Fig 5.7 F-), and internalized (Fig 5.7 K-O). In Figure 5.7, B-D show beads bound to the edge of a cell (along X-Z axis) while panel A shows a bead bound on the top of the cell (along the Y-Z axis). Panels K-O (Fig 5.7) demonstrate the loss of PI[4,5]P₂ surrounding beads once internalized. These results suggest that IpLITR 1.1b/ITAM-mediated changes in the redistribution of PI(4,5)P₂ follow a pattern reminiscent of the well-documented mammalian FcR-mediated phagocytosis (Botelho et al, 2000; Flannagan et al, 2012).

5.2.1.3 Confocal microscopy-based examination of the PH-PLCδ₁-GFP distribution during IpLITR 1.1b/WT –mediated phagocytosis

The distribution of PI(4,5)P₂ at the forming IpLITR 1.1b/WT –mediated phagocytic cup followed a pattern reminiscent to that of the F-actin patterning for these cells (Fig 5.8; compare with Fig. 4.9). For example, upon target binding, there was a transient increase in PI(4,5)P₂ at the base of the phagocytic cup that disappeared as the beads were becoming internalized – a pattern observed in the predicted ITAM-mediated pathway. However, as observed for the actin distribution during the pseudopod extension phase of IpLITR 1.1b/WT –mediated phagocytosis, long PI(4,5)P₂ –rich pseudopodia were not observed. Instead, IpLITR 1.1b/WT –induced phagosomes appeared to ‘sink’, or were retracted into the cells. Figure 5.9 shows representative IpLITR 1.1b/WT –induced phagocytic cups and the corresponding PH-PLCδ₁-GFP distribution for surface bound (A-E), partially engulfed (F-), and internalized targets (K-O). PH-PLCδ₁-GFP did not accumulate around the phagosomal membrane of internalized beads. These

observations demonstrate IpLITR 1.1b/WT –induced changes in the distribution of PI(4,5)P₂ that were unique from the ITAM-dependent receptor but akin to the IpLITR 1.1b-induced reorganization of F-actin previously reported in Chapter 4.

5.2.2 Examination of the phosphatidylinositol 3-phosphate distribution during IpLITR

1.1b/ITAM and 1.1b/WT –mediated phagocytosis using 2xFYVE-GFP

5.2.2.1 Imaging flow cytometry-based validation of IpLITR phagocytic phenotypes after AD293 co-transfections with 2xFYVE-GFP

To validate the stability of IpLITR expression during co-transfection with 2xFYVE-GFP, IpLITR expression was again monitored using imaging flow cytometry. When AD293 cells stably expressing IpLITRs were stained (positive control for IpLITR stable expression), 97.6% and 96.8% of cell populations were expressing IpLITR 1.1b/ITAM or 96.8% of IpLITR 1.1b/WT respectively (Figs. 5.10A and 5.11A). AD293 cells stably expressing IpLITR 1.1b/ITAM or 1.1b/WT were co-transfected with 2xFYVE-GFP then HA-epitope tagged IpLITR stained with anti-HA mAb to measure IpLITR and GFP co-expression. Over half of the co-transfected population of IpLITR 1.1b/ITAM cells (52.6%) were double positive for GFP and IpLITR expression and the majority of the population retained its IpLITR expression regardless of GFP expression (Fig 5.10B). The proportion of double positive cells was lower for IpLITR 1.1b/WT cells: seventeen percent of this population co-expressed GFP and IpLITR (Fig 5.11B). Regardless, IpLITR 1.1b/WT expression remained relatively stable after co-transfection. Lastly, when cells were treated with isotype control antibody, they did not stain (Appendix Figure A4 & A9).

When assessed for phagocytic activity, both cell types and their co-transfected counterparts retained their phagocytic phenotypes. For example, ~45.9% of cells stably expressing IpLITR 1.1b/ITAM internalized one or more beads and 2.7% had surface bound beads only (Fig 5.12A). Co-transfected cells internalized at least one bead 38.7% of the time and 2.4% had surface bound beads only (Fig 5.12A). Eighteen percent of the population of cells stably expressing IpLITR 1.1b/WT phagocytosed at least one bead while 26% of these cells had surface bound beads only (Fig 5.12B). In co-transfected cells, the proportion of cells with phagocytosed beads was 13.6% and 28.5% had surface bound beads only (Fig 5.12B). Isotype control-coated beads did not induce IpLITR-mediated internalization (Fig 5.12). These results confirm that 2xFYVE-GFP co-transfection does not affect IpLITR expression levels and their associated phagocytic phenotypes, suggesting that the co-transfection process does not alter the activity of the receptors.

5.2.2.2 Confocal microscopy-based examination of the 2xFYVE-GFP distribution during IpLITR 1.1b/ITAM –mediated phagocytosis

To determine whether targets of IpLITR-mediated phagocytosis are internalized into a PI(3)P-enriched phagosome, the distribution of this phosphoinositide was detected using 2xFYVE-GFP. The predicted distribution of PI(3)P during classical mammalian ITAM-mediated phagocytosis is shown in Fig 5.13: PI(3)P is only localized to early endosomal and phagosomal membranes and not the plasma membrane. This pattern was observed during IpLITR 1.1b/ITAM –mediated phagocytosis (Fig 5.13 and 5.14). Only fully internalized targets had recruited 2xFYVE-GFP to the maturing phagosome. The

beads within these phagosomes did not stain with GαM Cy5 since the antibody was excluded from the interior of the cell by cellular membranes (i.e. plasma and phagosomal membranes). Additionally, 2xFYVE-GFP localized to and intensely precipitated on PI(3)P-rich vesicles. Further supporting that PI(3)P is not present on all phagosomes (i.e. late phagosomes) was the finding that not all internalized targets in the same cell were bound by intensely GFP-fluorescent phagosomes. During IpLITR 1.1b/ITAM-mediated phagocytosis, beads that are surface bound or partially engulfed (Fig 5.14 A-E) do not recruit 2xFYVE-GFP. Conversely, internalized beads with 2xFYVE-GFP-demarcated phagosomes are shown in panels F- (Fig 5.14). The phagosomes in panel I (Fig 5.14) appear to be losing GFP intensity relative to phagosomes in the other panels possibly due to recently being internalized or a being in a later stage of phagosome maturation. Interestingly, punctate GFP fluorescence localizing to the phagosomal membranes can be visualized in panels F-H (Fig 5.14); these are PI(3)P-rich vesicles possibly being recruited to the maturing phagosome. This result demonstrated that IpLITR 1.1b/ITAM-mediated phagocytosis results in the engulfment of targets into a phagosome that gains PI(3)P identity after completion of the phagocytic process.

5.2.2.3 Confocal microscopy-based examination of the 2xFYVE-GFP distribution during IpLITR 1.1b/WT –mediated phagocytosis

Figure 5.15 demonstrates that IpLITR 1.1b/WT –mediated internalization results in the formation of a PI(3)P–enriched phagosomal membrane similar to that of IpLITR 1.1b/ITAM that loses its GFP fluorescence as the phagosome matures. Surface bound

and partially engulfed targets did not recruit 2xFYVE-GFP thus the nascent phagosome did not contain PI(3)P (Fig 5.16 A-E). Internalized targets were contained in 2xFYVE-GFP –rich phagosomes that were not accessible to GαM Cy5 (Fig 5.16 F-). Additionally, 2xFYVE-GFP-rich vesicles can be seen in proximity of phagosomes in panels F, H, I and (Fig 5.16). These observations confirm that IpLITR 1.1b/WT –mediated internalization leads to target engulfment into a PI3P-rich vesicle, which verifies that this non-traditional phagocytic pathway results in the formation of a PI(3)P-enriched phagosomal compartment that contains the phagocytosed targets.

5.2.3 Examination of spleen tyrosine kinase (Syk) distribution during IpLITR

1.1b/ITAM–mediated phagocytosis using GFP-conjugated Syk

5.2.3.1 Imaging flow cytometry-based validation of IpLITR phagocytic phenotypes after AD293 co-transfections with Syk-GFP

To demonstrate that co-transfection with Syk-GFP did not alter the stable expression of IpLITR 1.1b/ITAM or its overall engulfment phenotype in AD293 cells, these parameters were assessed at a populational level by imaging flow cytometry. The population of cells co-transfected with Syk-GFP overall maintained IpLITR expression (95.6%, Fig 5.17 B) compared to cells that were not treated with Syk-GFP (95.5%, Fig 5.17 A). Forty-eight percent of co-transfected cells co-expressed IpLITR 1.1b/ITAM and SykGFP (Fig 5.17 B). This population of co-transfected cells was simultaneously assessed for its phagocytic activity. When assessed for the ability to internalize αHA-coated beads, the majority of IpLITR 1.1b/ITAM cells associated with beads had phagocytosed at least one bead (Fig 5.18). Approximately 50.4% of AD293 cells stably expressing IpLITR 1.1b/ITAM had

phagocytosed at least one bead and 3.1% of cells only had surface bound beads. Like the parental population, cells co-transfected with Syk-GFP preferentially internalized at least one bead: 33.5% phagocytosed at least one bead and 2.2% only bound beads on their surface (Fig 5.18). Neither cell population internalized Isotype (IgG3)-coated beads. These results demonstrated that transfection with Syk-GFP did not alter the expression of IpLITR 1.1b/ITAM or the engulfment phenotype of this receptor.

5.2.3.2 Confocal microscopy-based examination of the Syk-GFP localization to the phagocytic cup during IpLITR 1.1b/ITAM –mediated phagocytosis

To visually assess the localization of Syk-GFP to the phagocytic cup, I co-transfected IpLITR 1.1b/ITAM –expressing cells with Syk-GFP and assessed GFP distribution in cells fixed during internalization (Fig 5.19). Shown in Figure 5.19 (A-C) are cells that have bound beads to their surface. The top cell in panel D (Fig 5.19) has two beads that are partially engulfed; arrow indicates which bead is shown in inset. In panel E and F are two cells that are no longer exposed to GαM Cy5 and are therefore internalized. Insets show exposure of beads to GαM Cy5 (i), distribution of Syk-GFP (ii), and overlay. This preliminary examination of Syk recruitment during IpLITR 1.1b/ITAM-mediated phagocytosis was performed twice and further experiments will be required to make conclusions about Syk-GFP recruitment to the IpLITR 1.1b/ITAM-mediated phagocytic cup.

5.3 Discussion and Conclusions

My research focuses on understanding IpLITR-mediated regulation of immune responses such as phagocytosis. In order to achieve this, I developed imaging-based assays to monitor the distribution of actin and phosphoinositides (PIs) during the IpLITR-mediated phagocytic process. In this chapter, I further confirmed that co-transfection of AD293 cells stably expressing IpLITRs is a useful way to monitor the distribution of fluorophore-tagged signaling molecules during IpLITR-mediated signaling. Additionally, my confocal microscopy-based examinations of PI(4,5)P₂ and PI(3)P distribution during IpLITR-mediated phagocytic process represent the first evidence of IpLITR-mediated changes in the distribution of PIs upon engagement of IpLITRs. I demonstrated that a teleost ITAM-containing receptor induced PI distributions reminiscent of the mammalian FcR-mediated phagocytic process while the ITAM-independent receptor displayed a unique distribution of PI(4,5)P₂.

During mammalian FcR-mediated phagocytosis, PI(4,5)P₂ is transiently enriched at the phagocytic cup and this enrichment follows the length of pseudopodia as they extend over targets while disappearing from the base of the phagocytic cup (Botelho et al, 2000). PI(4,5)P₂ is depleted from the phagosomal membrane before phagosome closure (Botelho et al, 2000). These dynamic changes in PI(4,5)P₂ distribution are important for F-actin polymerization because PI(4,5)P₂ directly interacts with many actin-binding proteins responsible for promoting actin polymerization while inhibiting proteins that mediate actin depolymerisation (Saarikangas et al, 2010). For example, during FcR-mediated phagocytosis, the increased PI(4,5)P₂ concentration leads to the

activation of RhoGTPases, Cdc42 and Rac1 (Hoppe & Swanson, 2004). RhoGTPases activate WASP and WAVE which in turn promote Arp2/3-mediated actin polymerization (Higgs et al, 2000). The disappearance of PI(4,5)P₂ coincides with the disappearance of F-actin at the base of forming phagosomes (Scott et al, 2005). Depletion of PI(4,5)P₂ is mainly due its conversion to PI(3,4,5)P₃ (Araki et al, 1996) and its cleavage to diacylglycerol and inositol (1,4,5)-trisphosphate (Botelho et al, 2000). Critical to the recruitment of RhoGTPases is the activation and activity of spleen tyrosine kinase (Syk), which is responsible for phosphorylation of adaptor proteins that localize RhoGTPases to the phagocytic cup (Flannagan et al, 2012; Berton et al, 2005). Loss of Syk activity in macrophages has been associated with failure to close phagocytic cups (Crowley et al 1997). Once internalized, the phagosomal membrane gains PI(3)P identity, an important regulator of phagosome maturation (Levin et al, 2015; Schink et al, 2016). PI(3)P is absent in the pseudopodia and plasma membrane of phagocytes but it is mainly found on endosomes.

Akin to the distribution of PI(4,5)P₂ and PI(3)P during mammalian FcR-mediated phagocytosis, the teleost ITAM-dependent receptor (i.e. IpLITR 1.1b/ITAM) induced a similar distribution of these phosphoinositides during internalization (Summarized in Figure 5.20). This observation further supported the notion that a teleost ITAM-containing receptor mediates phagocytosis through a canonical ITAM-mediated pathway. The ITAM-independent, IpLITR 1.1b/WT appeared to induce a very transient increase in PI(4,5)P₂ upon target binding that disappeared rapidly. In addition, IpLITR 1.1b/WT did not induce the formation of PI(4,5)P₂-rich pseudopods that extended or

reached around the phagocytic targets. Instead, targets appeared to be pulled into cells (Fig 5.7). This observation was reminiscent of the IpLITR 1.1b/WT –induced cytoskeletal reorganization observed in Chapter 4 of this thesis. Once internalized, phagosomes containing targets were enriched with PI(3)P during the IpLITR 1.1b/ITAM and 1.1b/WT-mediated phagocytic process. More detailed examination of the IpLITR 1.1b/ITAM and 1.1b/WT-mediated signaling events is required to understand how these changes in PI distribution are coordinated and how they coordinate with IpLITR-mediated signal transduction to result in F-actin redistribution and phagosome maturation. A common theme observed during mammalian phagocytic receptor signaling is that receptor binding results in the formation of signaling complexes initiated by kinases (e.g. spleen tyrosine kinase, Syk) during mammalian FcR-signaling that localize small RhoGTPases, PI(4,5)P₂ effectors, to the plasma membrane (Flannagan et al, 2012). These spatiotemporally regulated events are critical for the appropriate coordination of events that lead to receptor-mediated dynamic changes in the cytoskeletal organization required for phagocytosis.

The finding that IpLITR 1.1b/WT could mediate inhibitory signaling through two independent mechanisms (i.e. Csk [Y₄₅₃] and SHP-2 [Y₄₇₇ and Y₄₉₉] –dependent) yet promote phagocytosis in RBL-2H3 cells were pivotal in providing testable models to understand how this unique receptor may regulate immune responses (Montgomery et al, 2009; Montgomery et al, 2012; Cortes et al, 2014; Lillico et al, 2015). These models were based on the observation that IpLITR 1.1b –mediated target acquisition was rapid, was reliant on actin polymerization, Src family kinase and Syk kinase activities that link

phosphorylation of IpLITR 1.1b to effectors of actin polymerization (Lillico et al, 2015; Fei et al, 2016). It was predicted that ligation of IpLITR 1.1b induced variable signaling protein recruitment to the proximal and distal cytoplasmic regions of this receptor; thus the two regions would differentially regulate phagocytosis. Furthermore, the presence of an Nck –consensus-binding sequence (HIYDEV, Frese et al, 2006) within the IpLITR 1.1b proximal cytoplasmic region (Y₄₃₃) suggested that this receptor could recruit Nck when engaged by its ligand. The recruitment of Nck would then facilitate the recruitment and activation of the actin polymerization-promoting WAVE2 complex to promote IpLITR 1.1b-mediated actin reorganization: a short-circuited signaling pathway employed by mammalian CEACAM3 to promote the rapid phagocytosis of bacteria (Pils et al, 2012). Additionally, it was predicted that the twenty-two amino acid spacing between the tandem ITIMs (Y₄₇₇ and Y₄₉₉) within the distal cytoplasmic region of IpLITR 1.1b could accommodate and recruit the two SH2 domains of Syk kinase: a unique ability of the PECAM-1 receptor (Wang et al, 2011). Syk kinase would then induce the recruitment and activation of guanine exchange factors, and promote the activity of small RhoGTPases and lipid kinases, such as PI3K. This collection of events would regulate the activation of WAVE2 or WASP and, by extension, activation of the F-actin-nucleation promoting Arp2/3 complex. Using fusion proteins encoding the IpLITR 1.1b proximal, distal, or full-length cytoplasmic region fused to GST, Zwozdesky et al (2016) biochemically examined the association of SHP-2, Csk, Syk, Grb2, Nck1, PI3K p85 α , Vav1 and Vav3 with each cytoplasmic region. This demonstrated that the IpLITR1.1b proximal cytoplasmic region associated with Csk, Grb2, Nck1, Vav1 and PI3K p85 α but not SHP2,

Syk, or Vav3. In contrast, the distal region recruited SHP2, Syk and PI3K p85 α , but not Csk, Grb2, Nck1, or Vav. The recruitment of Csk to the proximal region and SHP-2 to the distal region concurred with previous observations (Montgomery et al, 2012) providing validity to this biochemical approach. These results supported the hypotheses that upon receptor phosphorylation, Nck1 is recruited to the proximal region of the IpLITR 1.1b cytoplasmic tail while Syk is recruited to the distal cytoplasmic region. Furthermore, the hypothesis that the distal and proximal cytoplasmic regions of IpLITR 1.1b independently regulate IpLITR 1.1b-mediated functional responses (i.e. phagocytosis) was also examined in AD293 cells stably expressing mutant IpLITR 1.1b constructs encoding either the distal or proximal regions of the receptor. Using imaging flow cytometry, Zwozdesky et al (2016) demonstrated that the two cytoplasmic regions independently induced IpLITR 1.1b-mediated phagocytosis and reproduced the overall tethering (i.e. predominantly surface bound targets versus fully internalized) phenotype previously reported for the full-length IpLITR 1.1b receptor in RBL-2H3 cells (Lillico et al, 2015). Collectively these results supported two overarching hypotheses: (1) the distal and proximal region of the IpLITR 1.1b cytoplasmic tail function independently of one another to promote the same functional response (2) IpLITR 1.1b could recruit Nck1 and Syk to its proximal and distal regions respectively (Zwozdesky et al, 2016). However, these results do not confirm the precise role that these molecules play during IpLITR 1.1b-mediated phagocytosis. The remaining questions are: (1) are these molecules recruited to the IpLITR 1.1b-mediated phagocytic cup? (2) If so, when (temporal resolution)? (3) And where along the phagocytic cup (spatial resolution)? (4) Most

importantly, what molecular mechanisms drive the IpLITR 1.1b-mediated phagocytic response?

This biochemical assessment of potential IpLITR associations provided useful insights into molecules that are recruited to the artificially phosphorylated receptors. The role of these signaling molecules during IpLITR-mediated signal transduction can be validated using microscopy techniques and phagocytosis assays to visualize the spatiotemporal recruitment of signaling components to the phagocytic cup: the location at which IpLITR-mediated signaling is taking place. In order to visually demonstrate a role for Syk kinase distribution during IpLITR 1.1b/ITAM and 1.1b/WT-mediated signal transduction, I examined the distribution of GFP-tagged Syk kinase during the IpLITR-mediated phagocytic process. I was only able to provide preliminary demonstrations of Syk-GFP localization to the IpLITR 1.1b/ITAM-induced phagocytic cup during the early stage of target binding; however, a role for Syk in teleost ITAM-mediated phagocytosis must be further assessed in order to propose a distribution pattern during the IpLITR-induced phagocytic process. Future studies will concentrate on determining the dynamics of SHP2, Csk, Syk, Grb2, Nck1, PI3K p85 α , and Vav1 during the IpLITR 1.1b/WT-mediated phagocytic process using imaging-based techniques. Furthermore, using receptor constructs encoding the distal or proximal tail independently as well as tyrosine to phenylalanine mutants will aid in understanding the role each of the signaling cassettes play in regulating IpLITR-mediated signal transduction.

By capturing cells fixed in early stages of target acquisition and engulfment, I attempted to identify recruitment, generation, localization, or redistribution of signaling

molecules during the IpLITR-mediated phagocytic process. Despite the wealth of visual information regarding redistribution of PI(4,5)P₂ and the generation of PI(3)P during IpLITR-mediated phagocytic process, these experiments only suggest a role for these molecules during the IpLITR-mediated signal transduction. However, the mechanism by which these molecules may be involved in the IpLITR –mediated signal transduction will require more detail experimentation. For example, confocal microscopy-based comparison of IpLITR-mediated phagocytosis in the presence and absence of inhibitors (either pharmacological or inhibitory RNA-based) may shed light on the requirement for certain molecules. My experiments are limited by being snapshots in time, yet signal transduction and phagocytosis are dynamic and temporal processes. Early events of target capture are difficult to appreciate in fixed cells for multiple reasons: (1) it is difficult to know when targets are captured by thin membrane extensions (e.g. filopodia) versus not associated with cells (2) cellular events that would have occurred after the stage being visualized are lost. Live cell imaging is a valuable tool to understand the temporal regulation of IpLITR-mediated signaling that was recently used to monitor the F-actin organization during the IpLITR –mediated phagocytic process in RBL-2H3 cells (Lillico et al, unpublished observations). These observations demonstrated that, in RBL-2H3 cells, IpLITR 1.1b induced multiple phenotypes of actin reorganization that uniquely differed from the linear ITAM-mediated progression from receptor binding, pseudopod extension, and uptake. This demonstrated the dynamic nature of IpLITR 1.1b/WT-mediated phagocytosis and the lack of temporal resolution when assessing static images. Nevertheless, static images of the phagocytic process are useful

for the initial of testing potential models of IpLITR-mediated signal transduction. It is more feasible to assess a larger population of cells when visualizing static images as opposed to single cells when imaging live cells. Furthermore, static images have the advantage of being able to detect bead position using a stain that exclusively labels extracellular, or non-membrane bound areas of beads. This technique provides measurable sensitivity that can be standardized using image analysis software such as Imaris or ImageJ to precisely calculate the area of a bead that remains extracellular (Yeo et al, 2013; Condon et al, 2017). This strategy can potentially be used as a high throughput screen to decipher whether signaling molecules of interest are being recruited to the IpLITR-induced phagocytic cup. Overall, static imaging provides value as a qualitative technique to assess whether signaling molecules are involved in IpLITR-mediated signal transduction; these observations can then be examined by live cell imaging to understand the temporal component of specific IpLITR-mediated signaling events during the phagocytic process.

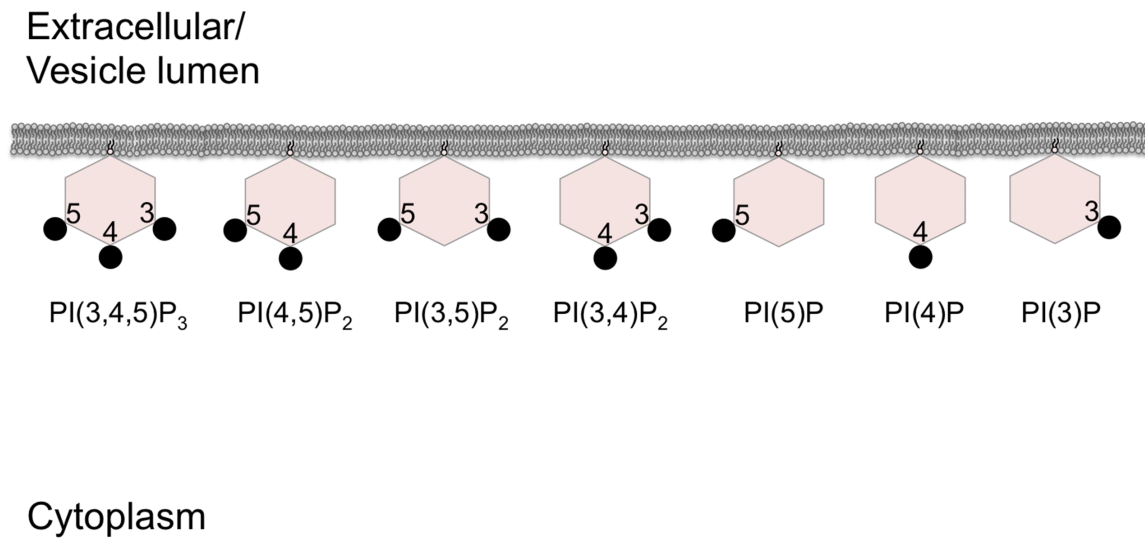


Figure 5. 1 Phosphoinositide species found in the cytoplasmic leaflet of eukaryotic cellular membranes. Hydroxyl groups within the inositol head-groups (red hexagons) are phosphorylated (black circles) or dephosphorylated (kinase versus phosphatase activity) at position 3, 4, and/or 5 to generate seven distinct phosphoinositide (PI) species: phosphatidylinositol 3,4,5-trisphosphate PI(3,4,5)P₃; phosphatidylinositol 4,5-bisphosphate PI(4,5)P₂; phosphatidylinositol 3,5-bisphosphate PI(3,5)P₂; phosphatidylinositol 3,4-bisphosphate PI(3,4)P₂; phosphatidylinositol 5-phosphate PI(5)P; phosphatidylinositol 4-phosphate PI(4)P; and phosphatidylinositol 3-phosphate PI(3)P.

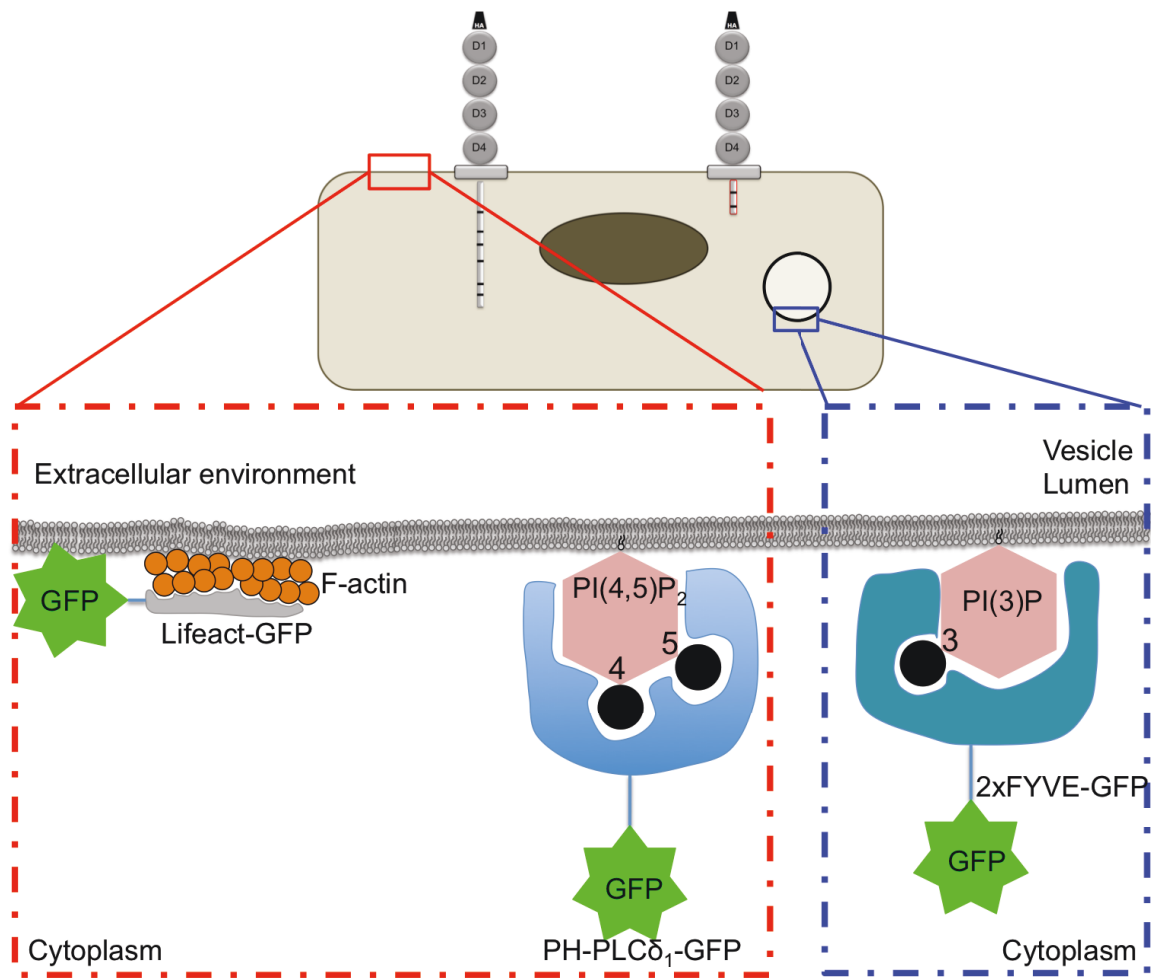


Figure 5. 2 Probes used in this thesis. In order to visualize the distribution of filamentous actin, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], and phosphatidylinositol 3-phosphate [PI(3)P] during the IpLITR-mediated phagocytic process, green fluorescent protein (GFP)-tagged probes were used. Lifeact-GFP detects filamentous actin. PH-PLCδ₁-GFP detects PI(4,5)P₂ present in the cytoplasmic leaflet of the plasma membrane (Várnai & Balla, 1998). 2xFYVE-GFP detects PI(3)P in the cytoplasmic leaflet of maturing phagosomes (Gillooly et al, 2000).

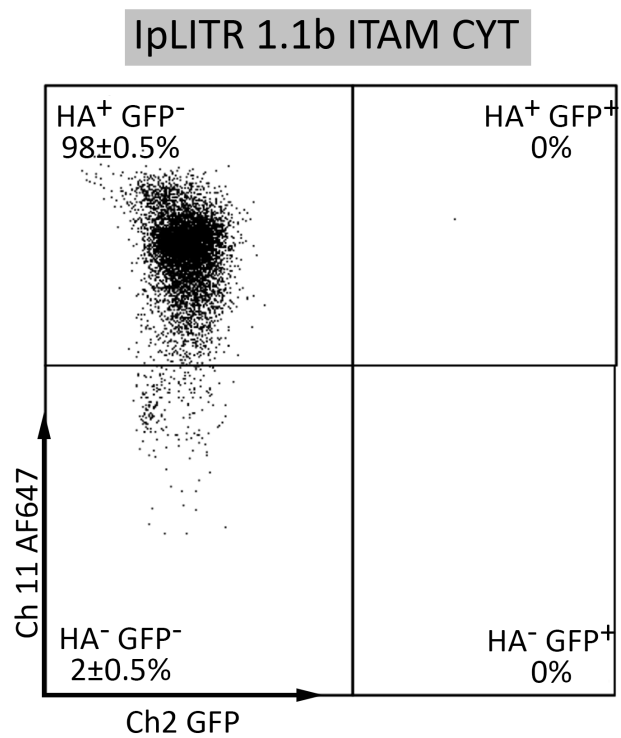
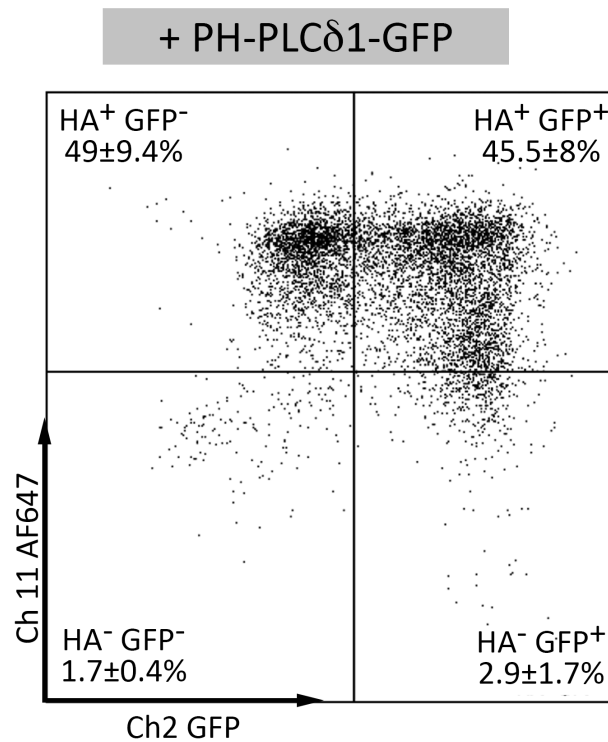
A**B**

Figure 5. 3 Imaging flow cytometry-based co-expression analysis of PH-PLC δ 1-GFP and IpLITR 1.1b/ITAM in AD293 cells. In order to demonstrate that transient co-transfection with PH-PLC δ 1-GFP did not alter the stable expression of IpLITR 1.1b/ITAM in AD293 cells, co-expression of PH-PLC δ 1-GFP and IpLITR 1.1b/ITAM was assessed at a populational level using imaging flow cytometry. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) were transiently co-transfected with PH-PLC δ 1-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g anti-hemagglutinin (HA) mAb followed by staining with 0.5 μ g AF647-conjugated rabbit anti-mouse IgG. PH-PLC δ 1-GFP and IpLITR co-expression was compared to AD293 cells stably expressing IpLITR 1.1b/ITAM that were not transfected with PH-PLC δ 1-GFP. IpLITR 1.1b/ITAM expression in non-transfected cells (A) and co-transfected cells (B) shown. Axes represent intensity of fluorescence in Channel 11 (AF647 IpLITR 1.1b/ITAM) and Channel 2 (PH-PLC δ 1-GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown (8×10^3 collected events per experiment).

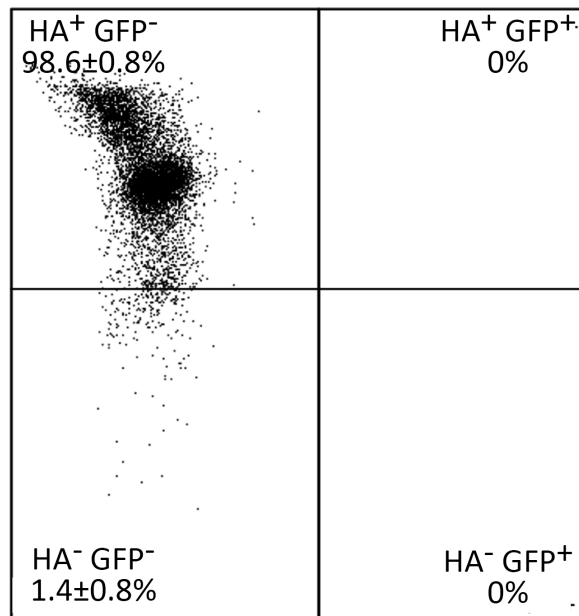
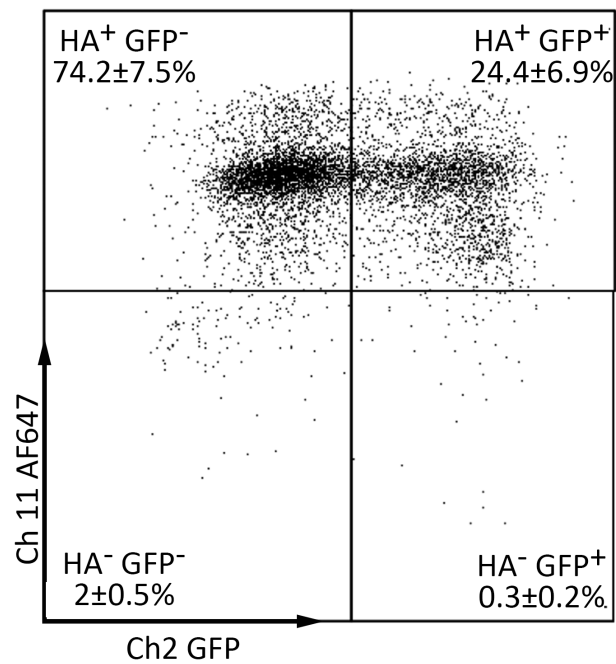
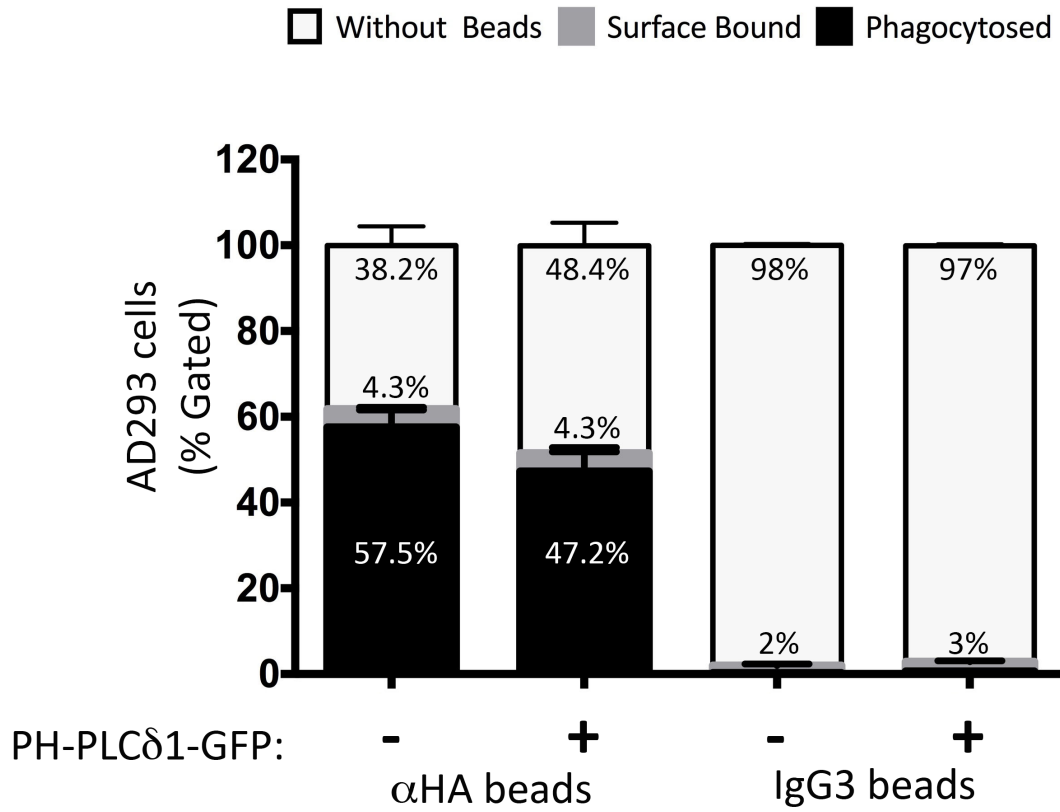
A**IpLITR 1.1b WT CYT****B****+ PH-PLCδ1-GFP**

Figure 5. 4 Imaging flow cytometry-based co-expression analysis of PH-PLC δ 1-GFP and IpLITR 1.1b/WT in AD293 cells. In order to demonstrate that transient co-transfection with PH-PLC δ 1-GFP did not alter the stable expression of IpLITR 1.1b/WT in AD293 cells, co-expression was assessed at a populational level using imaging flow cytometry. AD293 cells stably expressing IpLITR 1.1b/WT ($\sim 3 \times 10^5$ cells) were transiently co-transfected with PH-PLC δ 1-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g anti-hemagglutinin (HA) mAb followed by staining with 0.5 μ g AF647-conjugated rabbit anti-mouse IgG. PH-PLC δ 1-GFP and IpLITR co-expression was compared to AD293 cells stably expressing IpLITR 1.1b/WT that were not transfected with PH-PLC δ 1-GFP. IpLITR 1.1b/WT expression in non-transfected cells (A) and co-transfected cells (B) shown. Axes represent intensity of fluorescence in Channel 11 (AF647 IpLITR 1.1b/WT) and Channel 2 (PH-PLC δ 1-GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown (8×10^3 collected events per experiment).

A

IpLITR 1.1b ITAM CYT



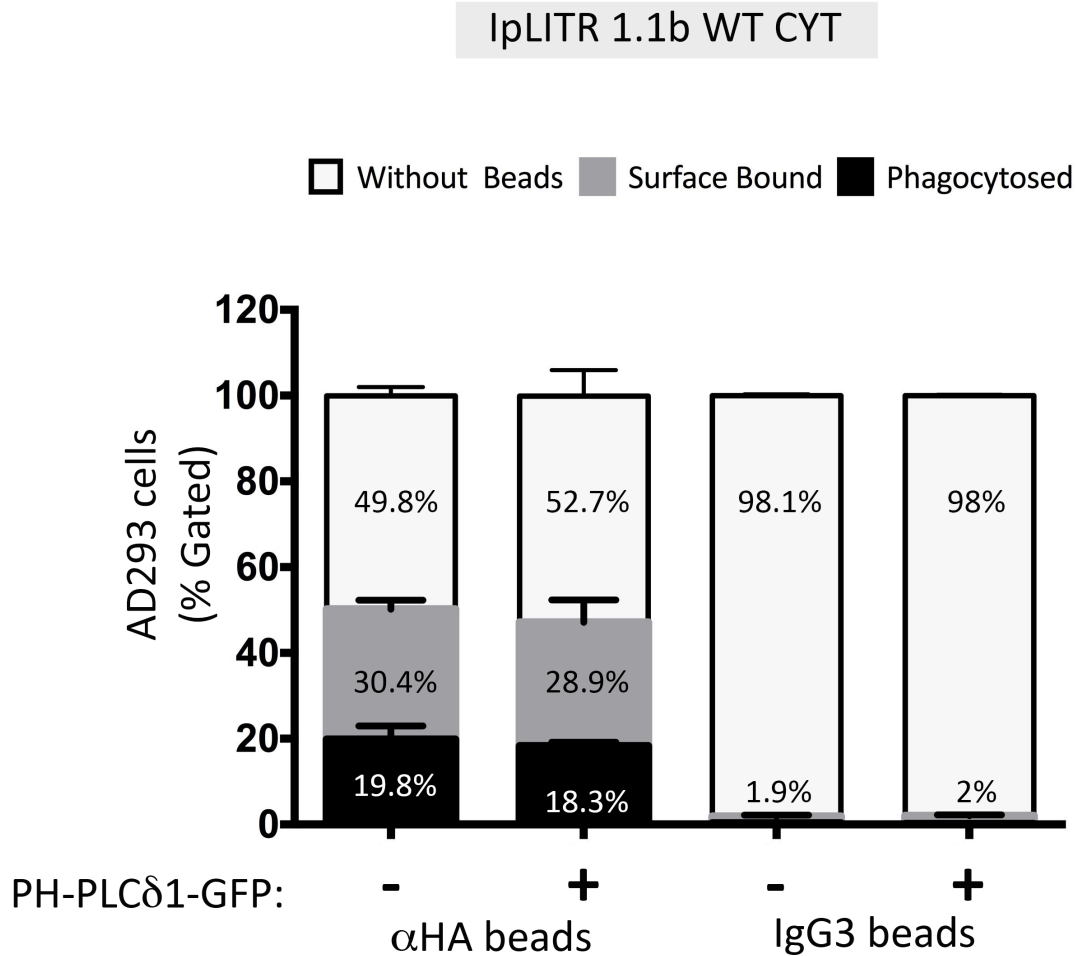
B

Figure 5. 5 IpLITR 1.1b/ITAM and 1.1b/WT retain their phagocytic phenotype after co-transfection with PH-PLCδ1-GFP. To demonstrate that transient co-transfection of AD293 cells stably expressing IpLITR with PH-PLCδ1-GFP did not alter the receptors' overall phenotype of target acquisition and engulfment, the phagocytic activities of co-transfected cells was measured at a populational level using imaging flow cytometry. AD293 cells stably expressing IpLITR 1.1b/ITAM (A) and 1.1b/WT (B) were co-transfected with PH-PLCδ1-GFP (+) for twenty-four hours. The ability of co-transfected cells (3×10^5 cell/sample) to internalize αHA mAb or IgG3 isotype control –coated beads (9×10^5 beads/sample) at 37°C for one hour was assessed by imaging flow cytometry in comparison to cells without PH-PLCδ1-GFP (-). Numbers represent the mean percent of population that had internalized at least one bead (black), only had surface bound beads (gray), or were not associated with beads (white). Error bars represent mean $\pm$ SEM of three independent experiments.

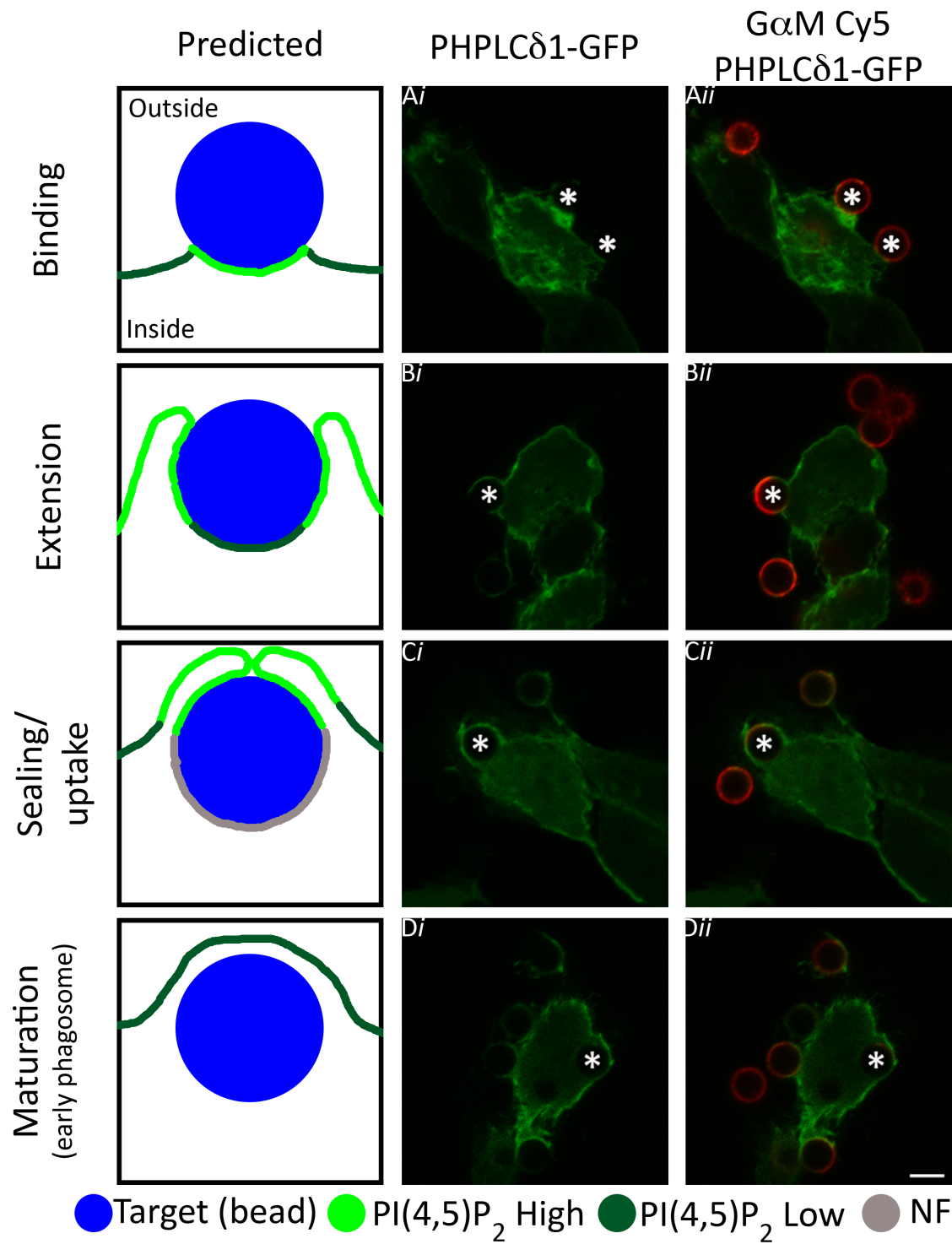
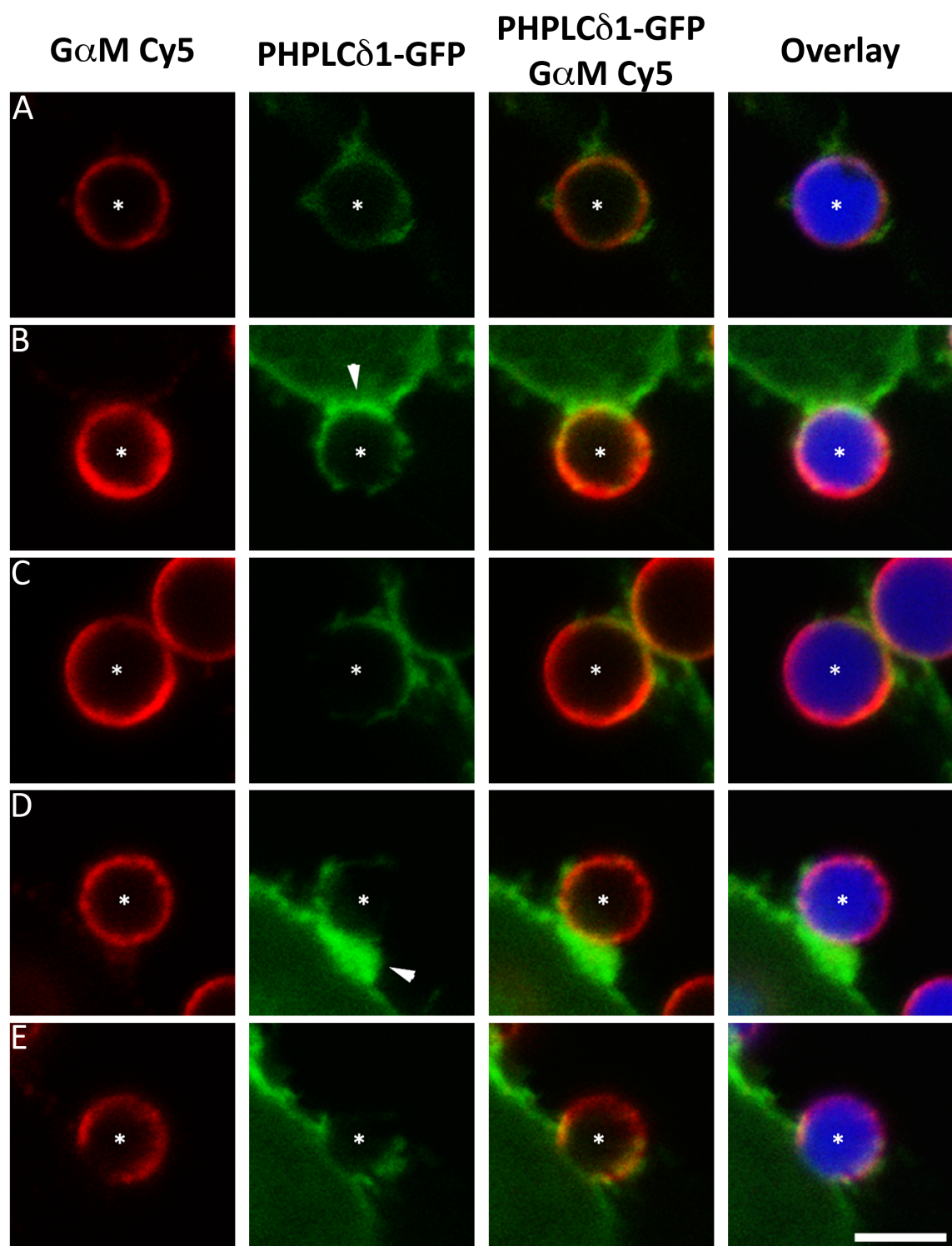
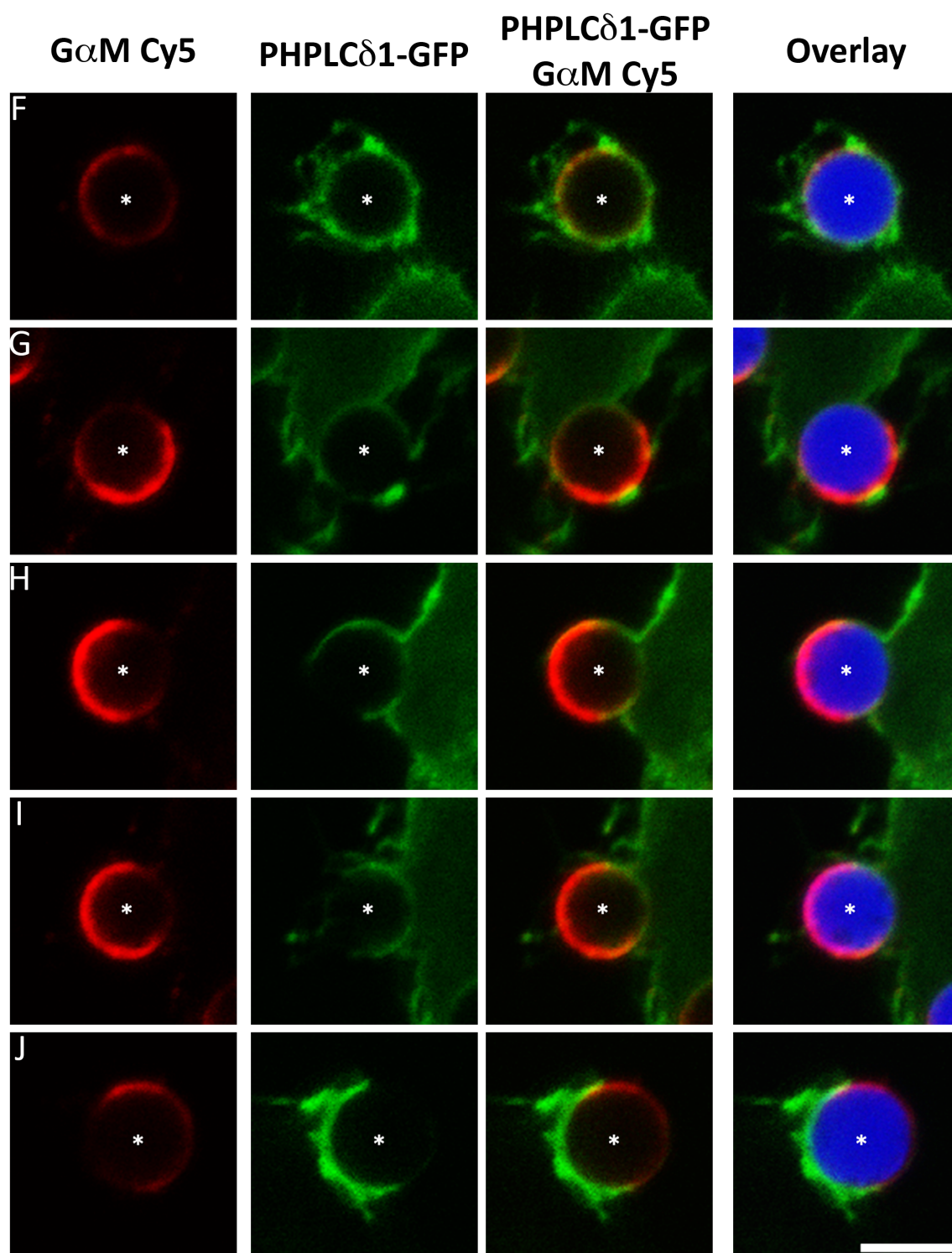


Figure 5. 6 Expected and observed phosphatidylinositol (4,5)-bisphosphate (PI[4,5]P₂) distribution during IpLITR 1.1b /ITAM –mediated phagocytosis. Representative images of IpLITR 1.1b/ITAM –expressing cells binding beads (Ai-Aii), extending pseudopods (Bi-Bii), sealing phagosomes (Ci-Cii) or maturing phagosomes (Di-Dii). Asterisks show location of BB beads. PH-PLCδ1-GFP (a marker of PI[4,5]P₂) is shown in green and Cy5-conjugated goat anti-mouse (detects exposed anti-HA mAb on BB beads) shown in red. Predicted PI[4,5]P₂ based on mammalian FcR-mediated phagocytosis. Basal levels of PI[4,5]P₂ in plasma membrane shown in dark green; transient increase in PI[4,5]P₂ is shown in bright green; absence of PI[4,5]P₂ from nascent phagosomal membrane shown in gray. Scale bar 5 μm.





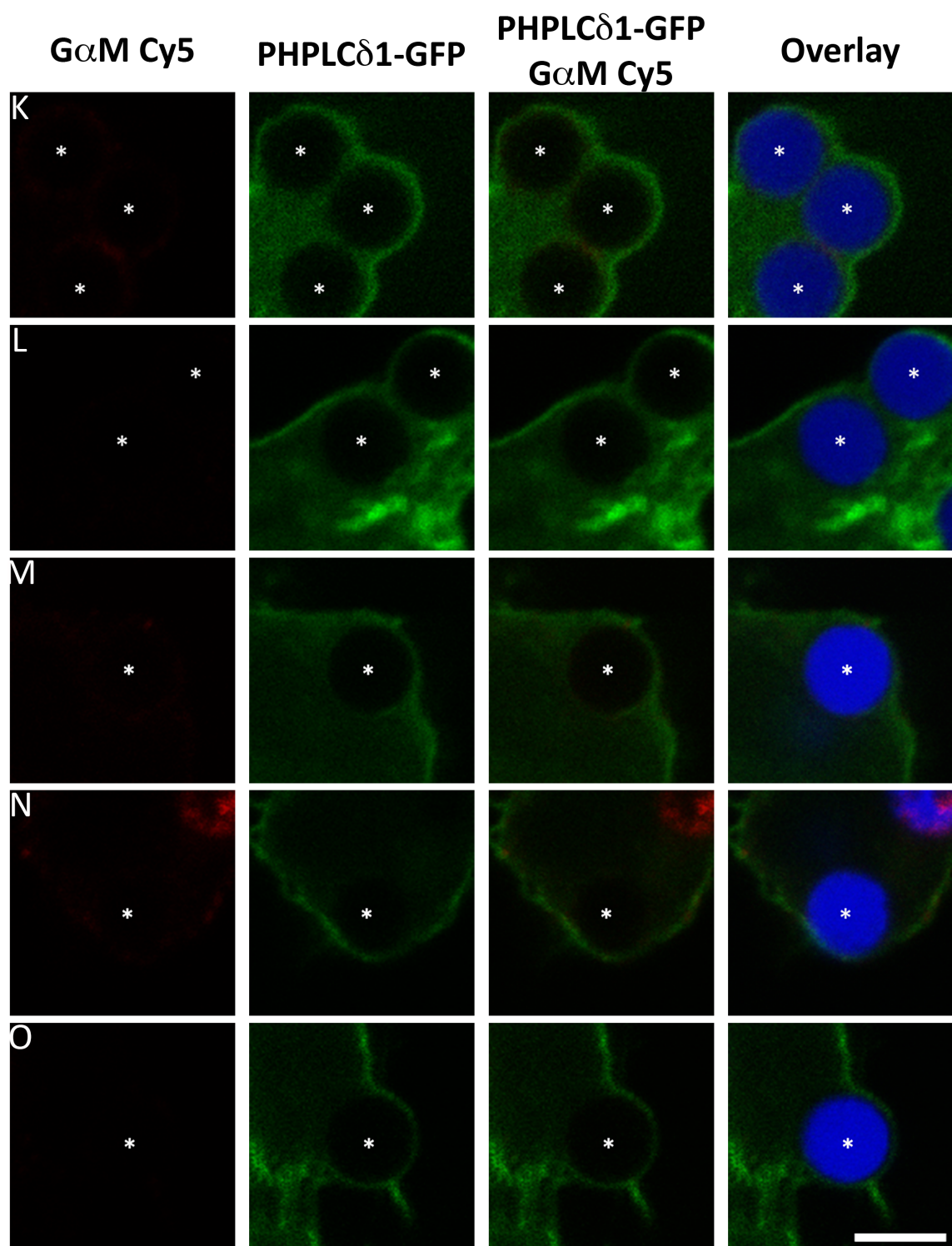


Figure 5. 7 Confocal microscopic visualization of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] distribution at the phagocytic cup during IpLITR 1.1b/ITAM –mediated phagocytosis. To monitor the distribution of PI(4,5)P₂ during the IpLITR 1.1b/ITAM-mediated phagocytic process, AD293 cells stably expressing IpLITR 1.1b/ITAM (~1.5x10⁵ cells) co-transfected with PH-PLCδ1-GFP were incubated with mAb anti-HA coated, 4.5 μm targets (3x10⁵ Bright Blue beads) then fixed prior to imaging. PH-PLCδ1-GFP (green) is a marker of PI(4,5)P₂. External regions of beads were counterstained with a Cy5-conjugated goat anti-mouse polyclonal antibody (GαM Cy5; red). Surface bound targets (A-E), partially engulfed (F-), and internalized (K-O) are representative images from three independent experiments with five replicates within each experiment. Scale bar, 5 μm.

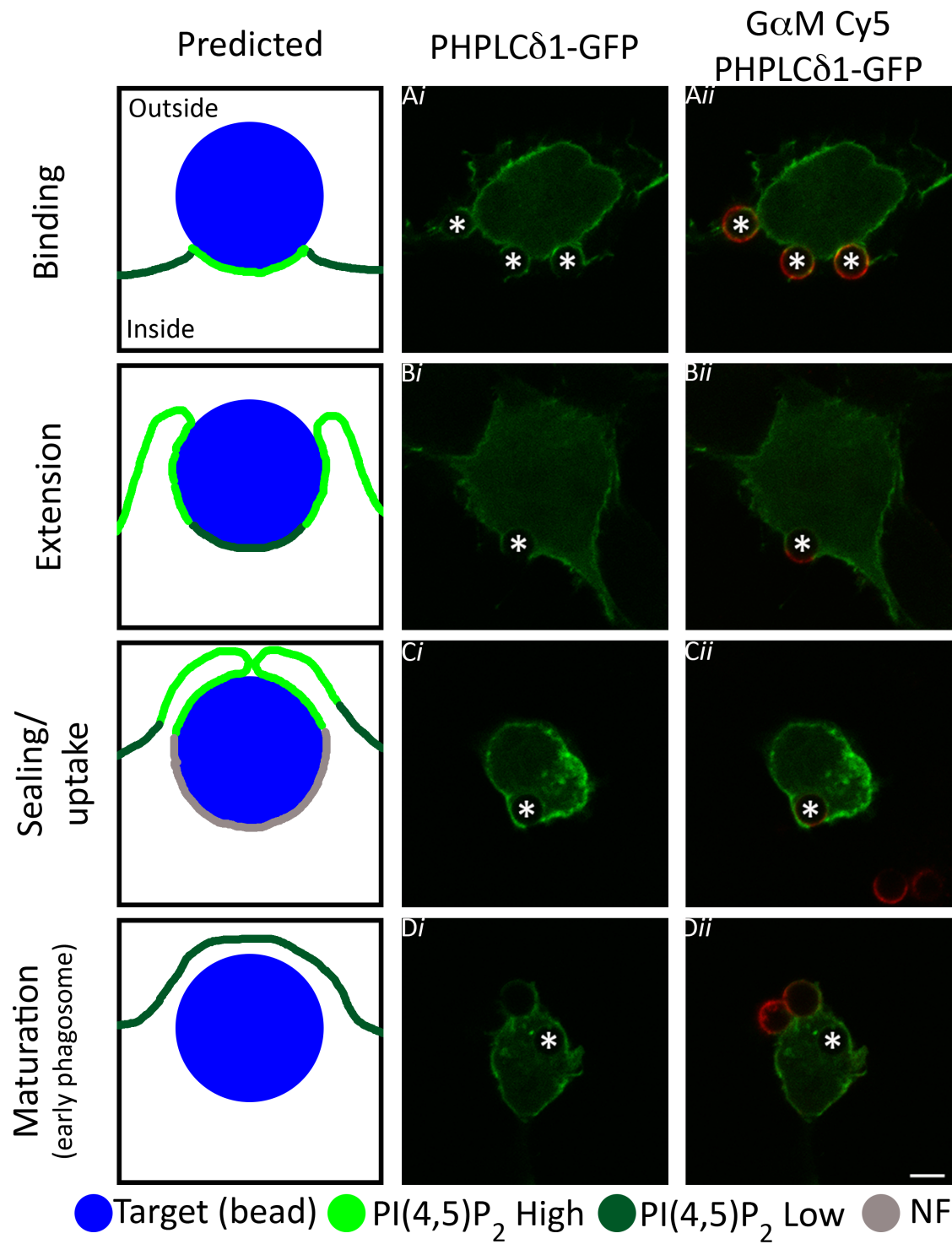
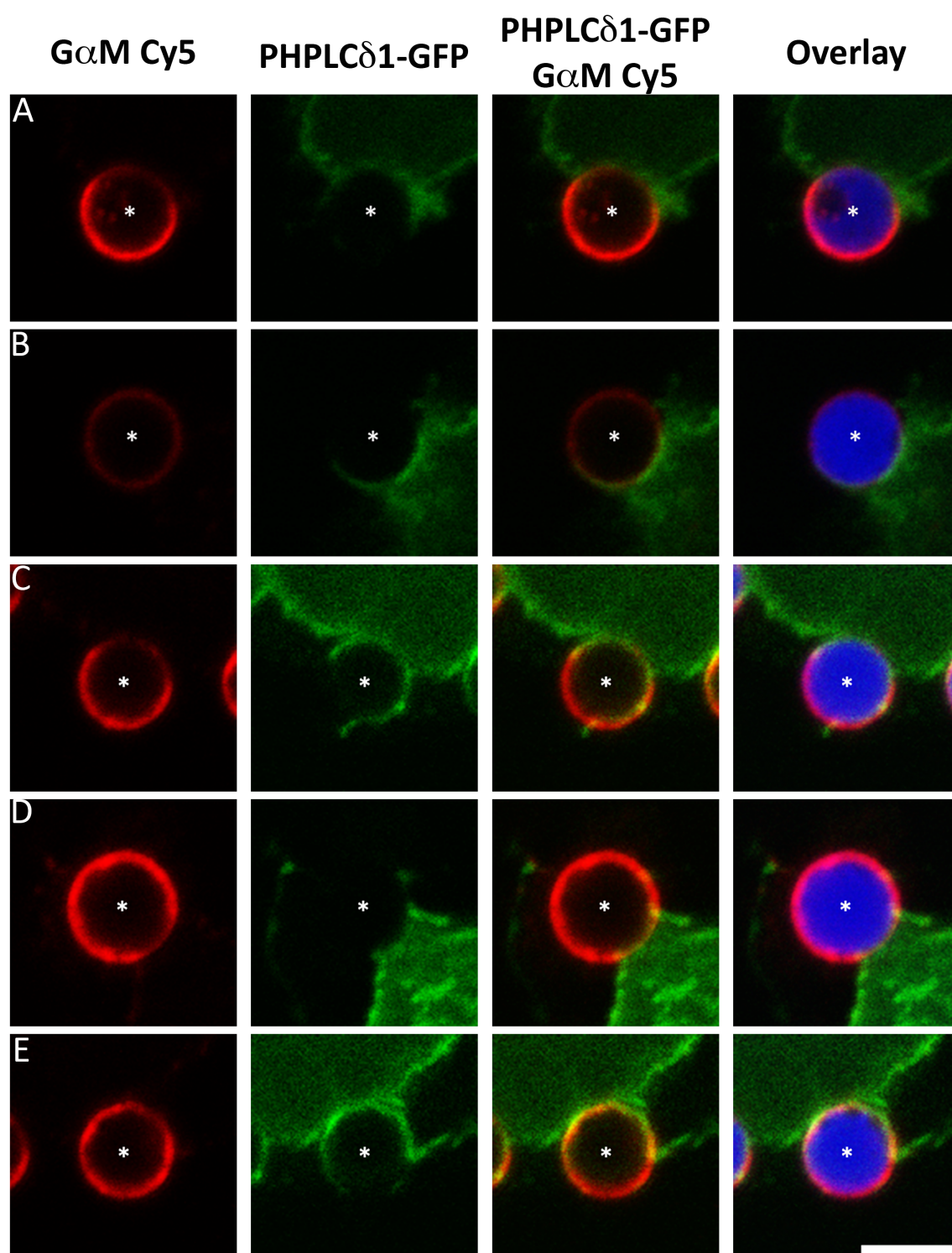
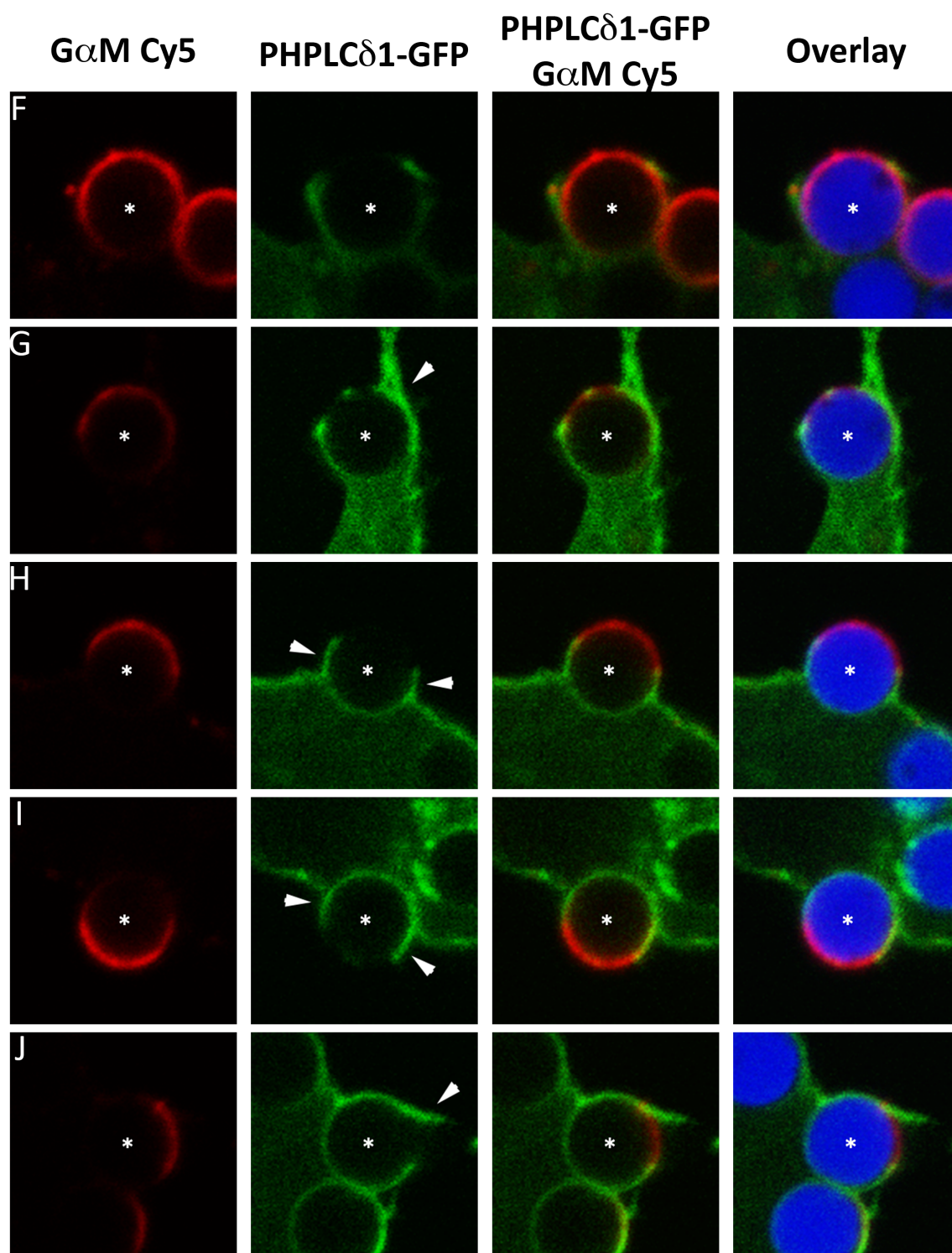


Figure 5. 8 Expected and observed phosphatidylinositol (4,5)-bisphosphate (PI[4,5]P₂) distribution during IpLITR 1.1b /WT –mediated phagocytosis. Representative images of IpLITR 1.1b/WT –expressing cells binding beads (Ai-Aii), extending pseudopods (Bi-Bii), sealing phagosomes (Ci-Cii) or maturing phagosomes (Di-Dii). Asterisks show location of BB beads. PH-PLCδ1-GFP (a marker of PI[4,5]P₂) is shown in green and Cy5-conjugated goat anti-mouse (detects exposed anti-HA mAb on BB beads) shown in red. Predicted PI[4,5]P₂ based on mammalian FcR-mediated phagocytosis. Basal levels of PI[4,5]P₂ in plasma membrane shown in dark green; transient increase in PI[4,5]P₂ is shown in bright green; absence of PI[4,5]P₂ from nascent phagosomal membrane shown in gray. Scale bar 5 μm.





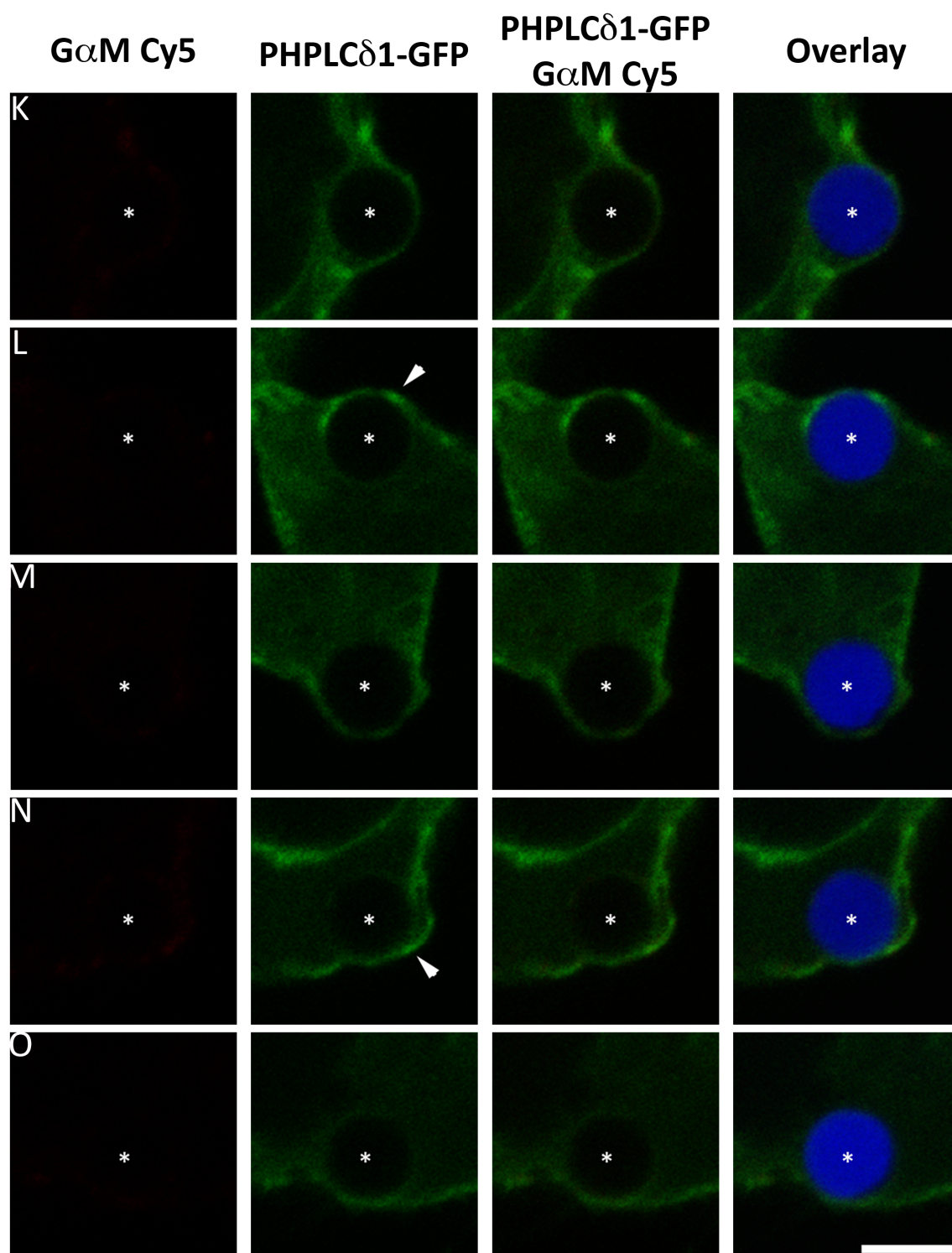
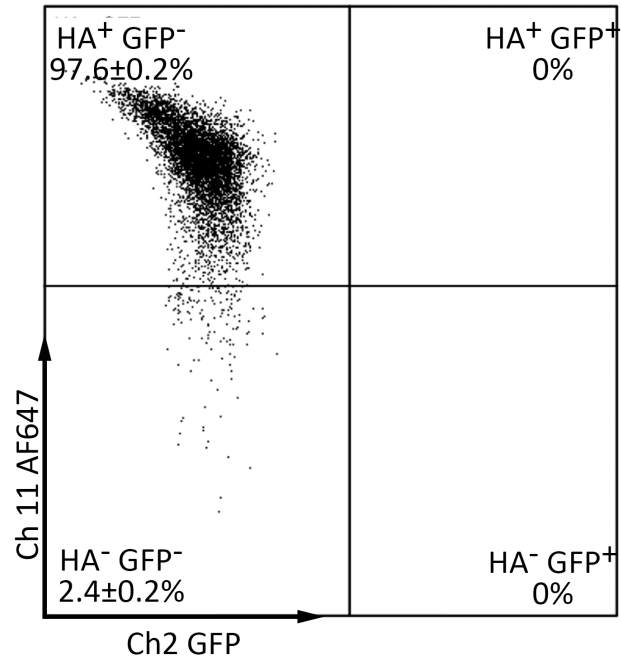


Figure 5. 9 Confocal microscopic visualization of phosphatidylinositol (4,5)-bisphosphate (PI[4,5]P₂) distribution at the phagocytic cup during IpLITR 1.1b/WT – mediated phagocytosis. To visualize the distribution of PI(4,5)P₂ during the IpLITR 1.1b/WT-mediated phagocytic process, AD293 cells stably expressing IpLITR 1.1b WT (~1.5x10⁵ cells) co-expressing PH-PLCδ1-GFP were incubated with mAb anti-HA coated, 4.5 μm targets (3x10⁵ Bright Blue beads) then fixed prior to imaging. PH-PLCδ1-GFP (green) is a marker of PI(4,5)P₂. External regions of beads were counterstained with a Cy5-conjugated goat anti-mouse polyclonal antibody (GαM Cy5; red). Surface bound targets (A-E), partially engulfed (F-), and internalized (K-O) are representative images from two independent experiments with five replicates within each experiment. Arrowheads identify extending pseudopodia. Scale bar, 5 μm.

A

IpLITR 1.1b ITAM CYT

**B**

+ 2xFYVE-GFP

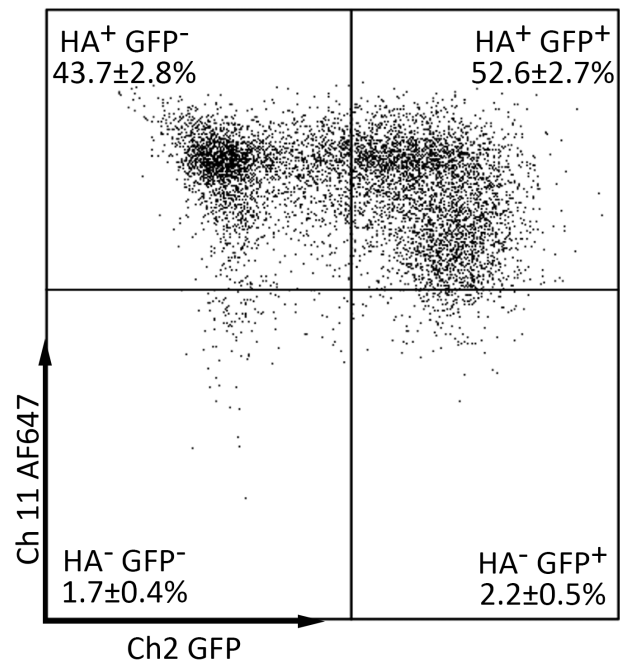


Figure 5. 10 Imaging flow cytometry-based co-expression analysis of 2xFYVE-GFP and IpLITR 1.1b/ITAM in AD293 cells. In order to demonstrate that transient co-transfection with 2xFYVE-GFP did not alter the stable expression of IpLITR 1.1b/ITAM in AD293 cells, co-expression was assessed at a populational level using imaging flow cytometry. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) were transiently co-transfected with 2xFYVE-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g anti-hemagglutinin (HA) mAb followed by staining with 0.5 μ g AF647-conjugated rabbit anti-mouse IgG. 2xFYVE-GFP and IpLITR co-expression was compared to AD293 cells stably expressing IpLITR 1.1b/ITAM that were not transfected with 2xFYVE-GFP. IpLITR 1.1b/ITAM expression in non-transfected cells (A) and co-transfected cells (B) shown. Axes represent intensity of fluorescence in Channel 11 (AF647 IpLITR 1.1b/ITAM) and Channel 2 (2xFYVE-GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown (8×10^3 collected events per experiment).

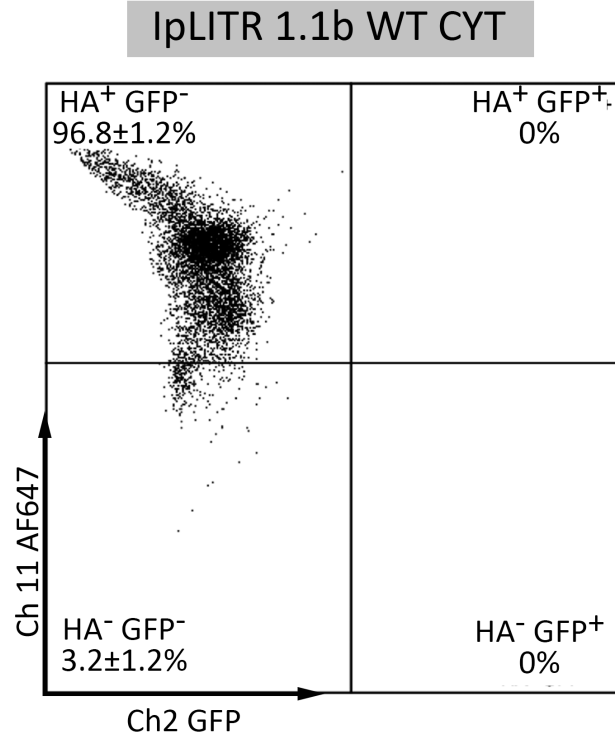
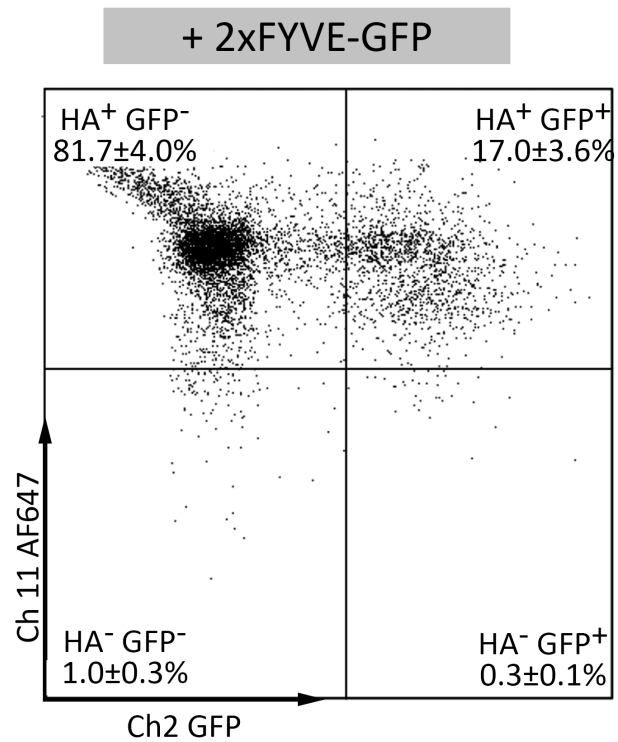
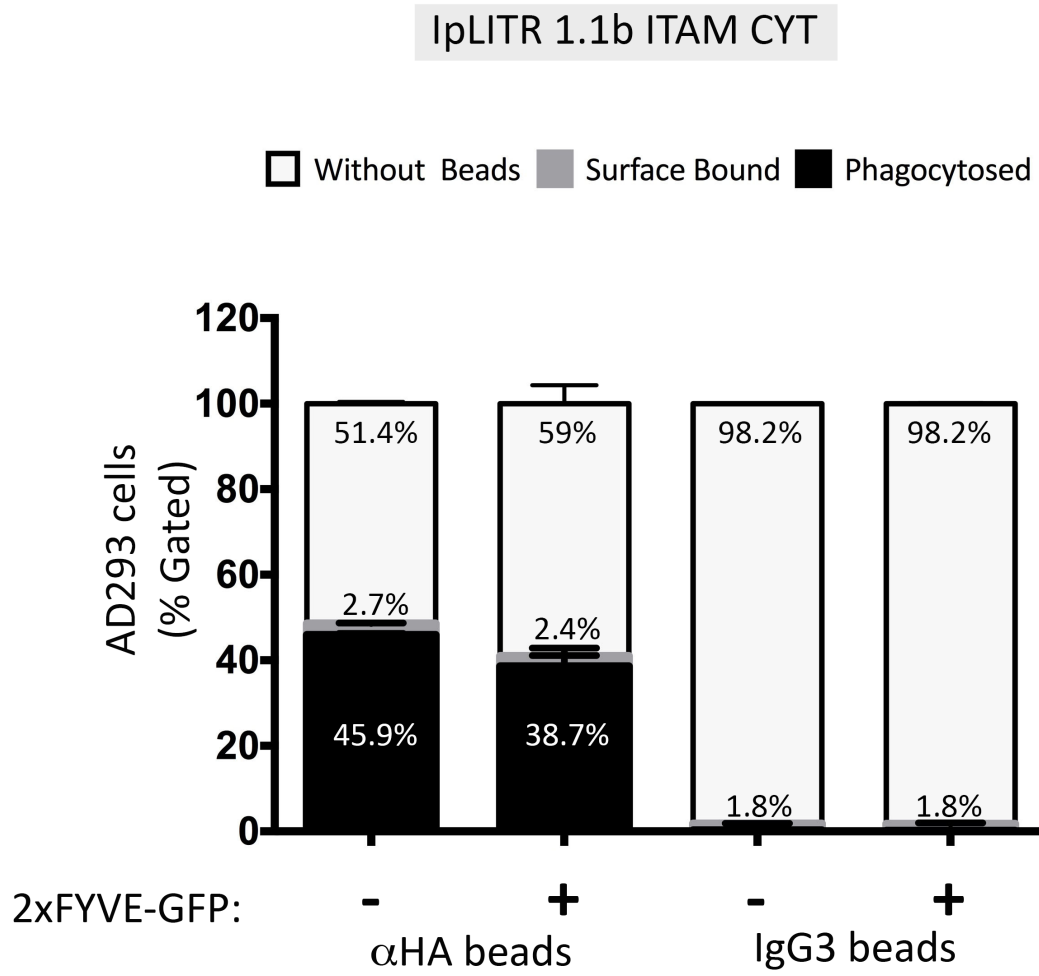
A**B**

Figure 5. 11 Imaging flow cytometry-based co-expression analysis of 2xFYVE-GFP and IpLITR 1.1b/WT in AD293 cells. In order to demonstrate that transient co-transfection with 2xFYVE-GFP did not alter the stable expression of IpLITR 1.1b/WT in AD293 cells, co-expression was assessed at a populational level using imaging flow cytometry. AD293 cells stably expressing IpLITR 1.1b/WT ($\sim 3 \times 10^5$ cells) were transiently co-transfected with 2xFYVE-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μg anti-hemagglutinin (HA) mAb followed by staining with 0.5 μg AF647-conjugated rabbit anti-mouse IgG. 2xFYVE-GFP and IpLITR co-expression was compared to AD293 cells stably expressing IpLITR 1.1b/WT that were not transfected with 2xFYVE-GFP. IpLITR 1.1b/WT expression in non-transfected cells (A) and co-transfected cells (B) shown. Axes represent intensity of fluorescence in Channel 11 (AF647 IpLITR 1.1b/WT) and Channel 2 (2xFYVE-GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown (8×10^3 collected events per experiment).

A



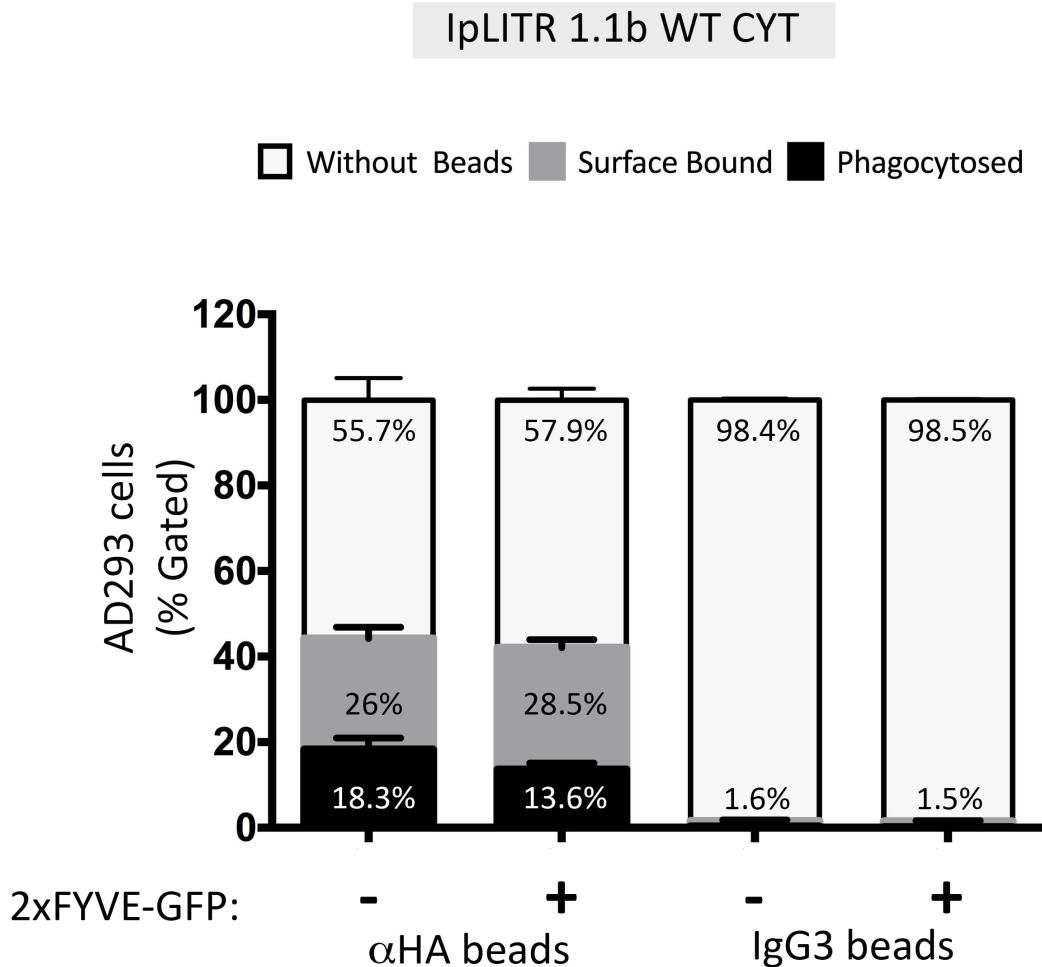
B

Figure 5. 12 IpLITR 1.1b/ITAM and 1.1b/WT retain their phagocytic phenotype after co-transfection with 2xFYVE-GFP. To demonstrate that transient co-transfection of AD293 cells stably expressing IpLITR with PH-PLCδ1-GFP did not alter the receptors' overall phenotype of target acquisition and engulfment, the phagocytic activities of co-transfected cells was measured at a populational level using imaging flow cytometry. AD293 cells stably expressing IpLITR 1.1b/ITAM (A) and 1.1b/WT (B) were co-transfected with 2xFYVE-GFP (+) for twenty-four hours. The ability of co-transfected cells (3×10^5 cell/sample) to internalize αHA mAb or IgG3 isotype control –coated beads (9×10^5 beads/sample) at 37°C for one hour was assessed by imaging flow cytometry in comparison to cells without 2xFYVE-GFP (-). Numbers represent the mean percent of population that had internalized at least one bead (black), only had surface bound beads (gray), or were not associated with beads (white). Error bars represent mean $\pm$ SEM of three independent experiments.

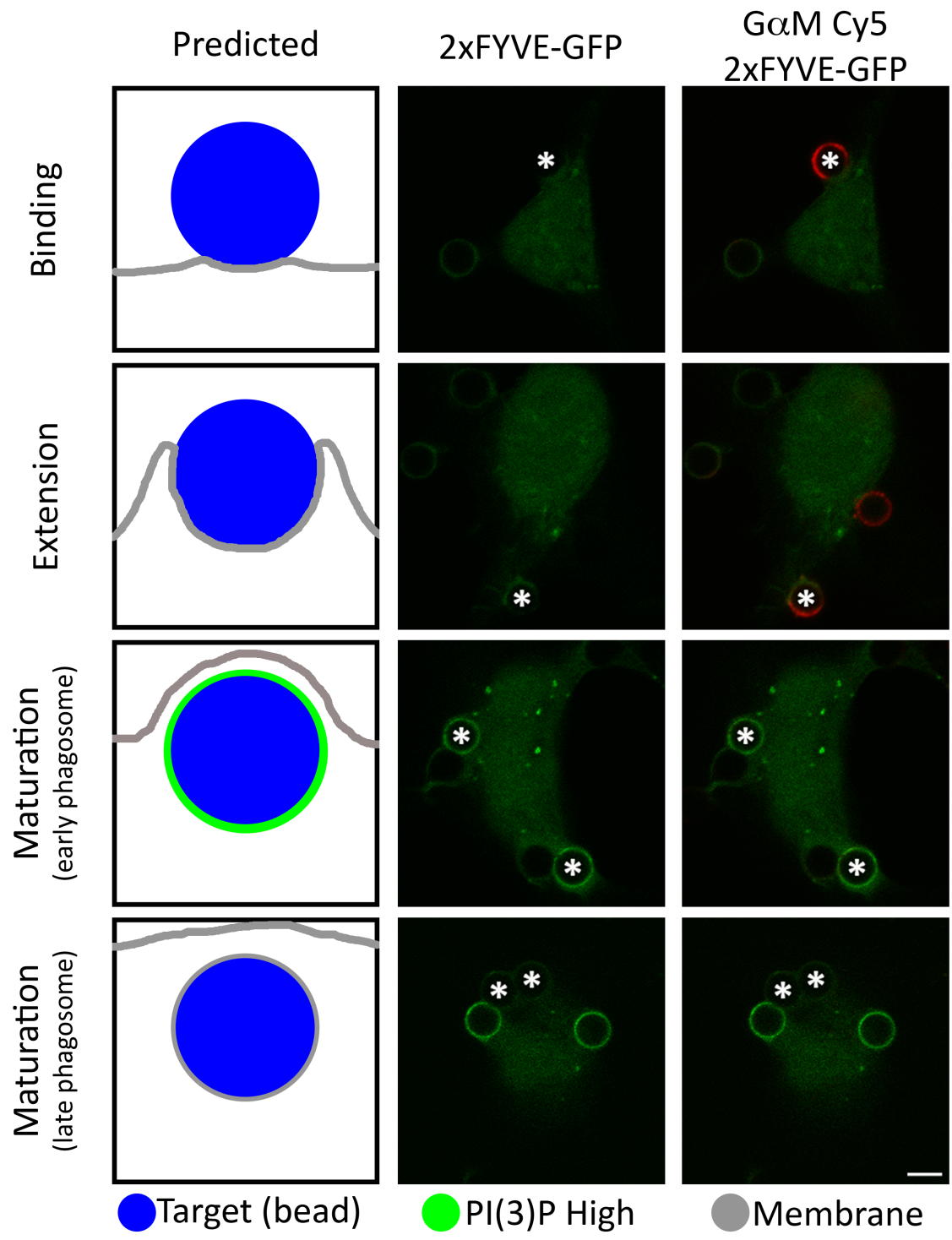
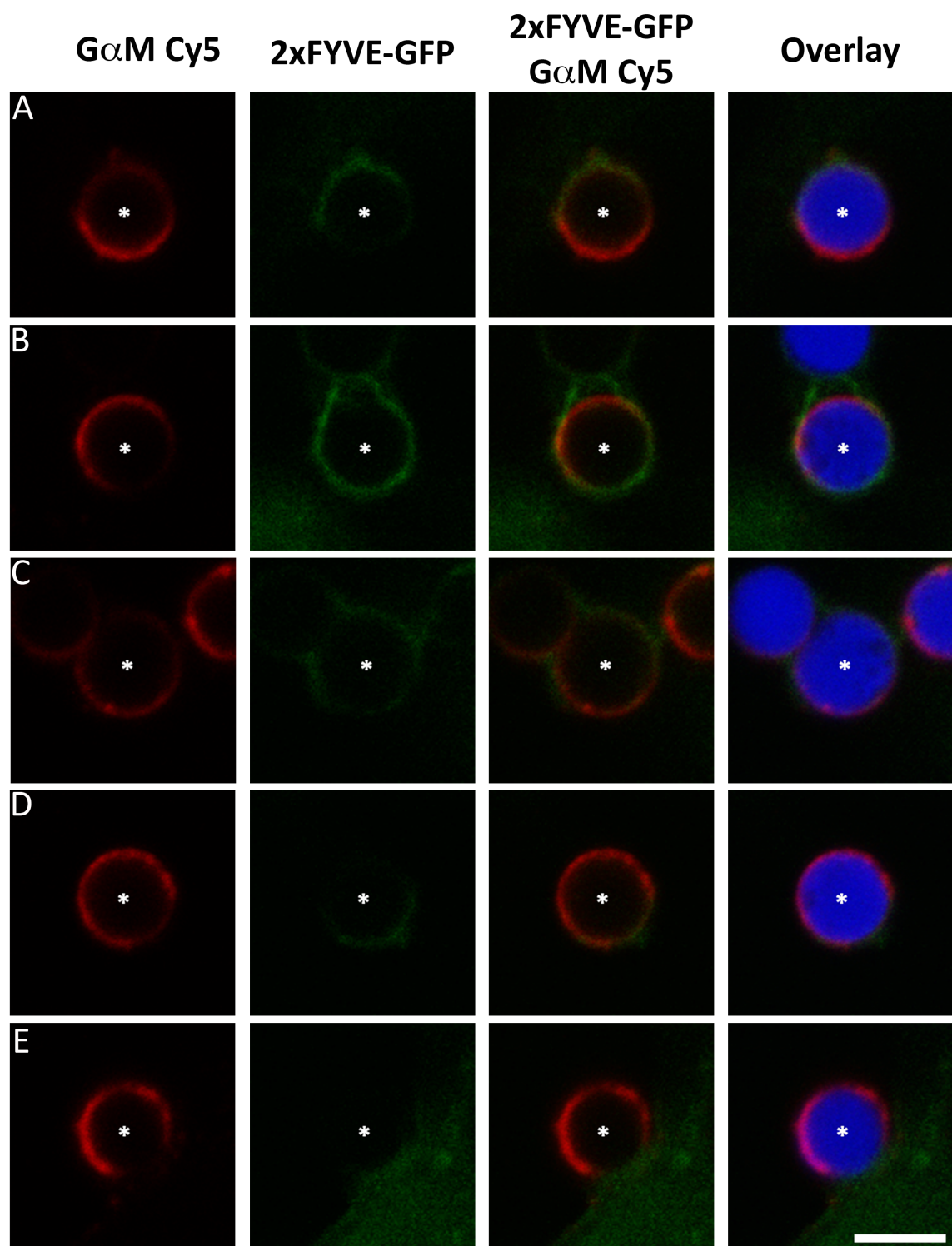


Figure 5. 13 Expected and observed phosphatidylinositol (3)-phosphate (PI[3]P) distribution during IpLITR 1.1b /ITAM –mediated phagocytosis. Representative images of IpLITR 1.1b/ITAM –expressing cells binding beads (Ai-Aii), extending pseudopods (Bi-Bii), in early phagosome maturation (Ci-Cii) or later maturation (Di-Dii). Asterisks show location of BB beads. 2xFYVE-GFP (PI[3]P) is shown in green and Cy5-conjugated goat anti-mouse (exposed anti-HA mAb on BB beads) shown in red. Predicted PI[3]P based on mammalian FcR-mediated phagocytosis. PI[3]P is not detectable in plasma membrane (gray); transient increase in PI[3]P during early phagosomal maturation shown in bright green; absence of PI[3]P from phagosomal membrane in later stages of maturation shown in gray. Scale bar 5 μ m.



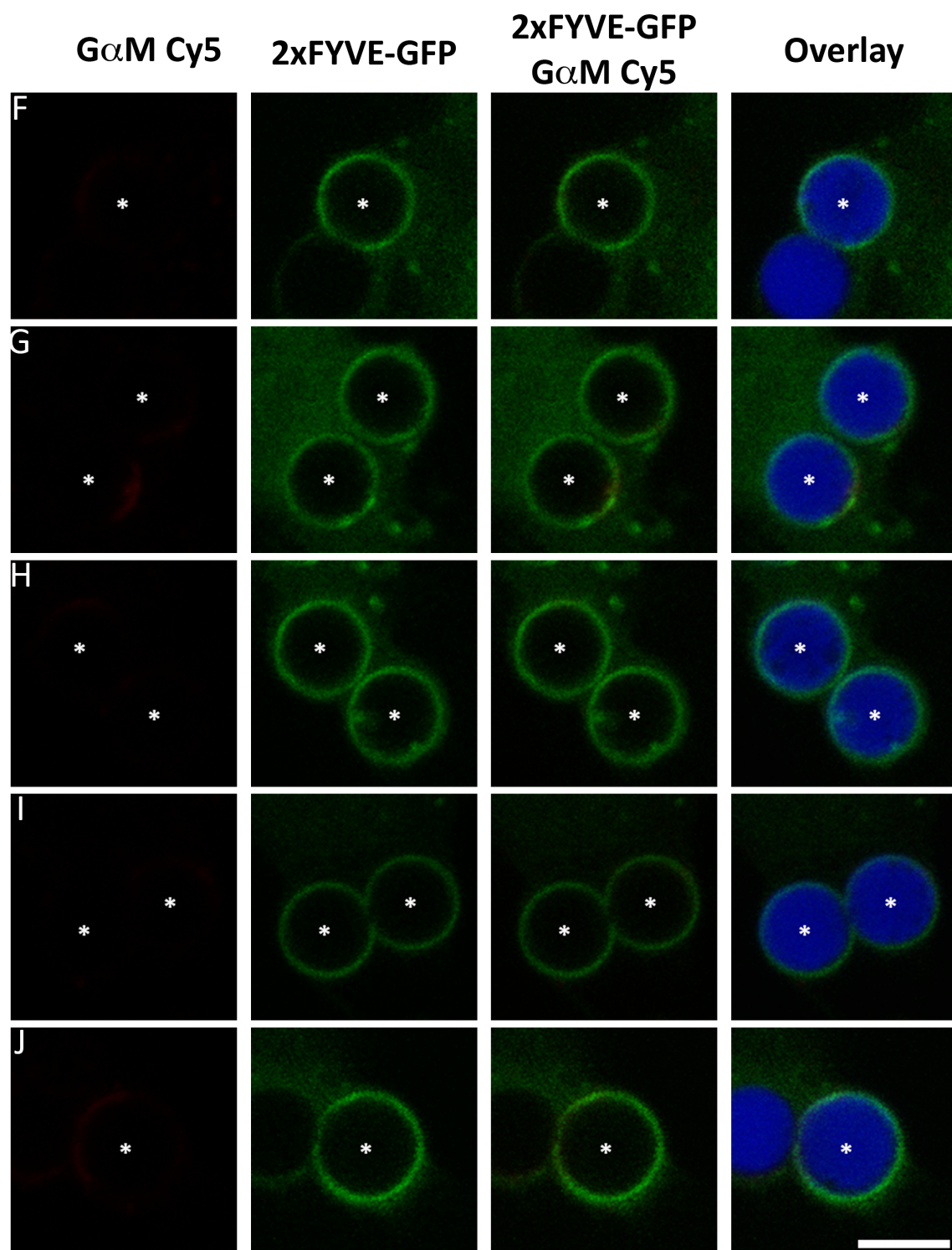


Figure 5. 14 Confocal microscopic visualization of phosphatidylinositol 3-phosphate [PI(3)P] maturing phagosomes during IpLITR 1.1b/ITAM-mediated phagocytosis. To demonstrate that targets of IpLITR 1.1b/ITAM-mediated phagocytosis are internalized into a PI(3)P-enriched phagosomes, AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 1.5 \times 10^5$ cells) co-transfected with 2xFYVE-GFP (a marker of PI(3)P; green) were incubated with mAb anti-HA coated, 4.5 μm targets (3×10^5 beads) then fixed. External regions of beads were counterstained with a Cy5-conjugated goat anti-mouse polyclonal antibody (G α M Cy5; red). Surface bound and partially engulfed targets (A-E), and internalized (F-) are representative images from three independent experiments with five replicates within each experiment. Scale bar, 5 μm .

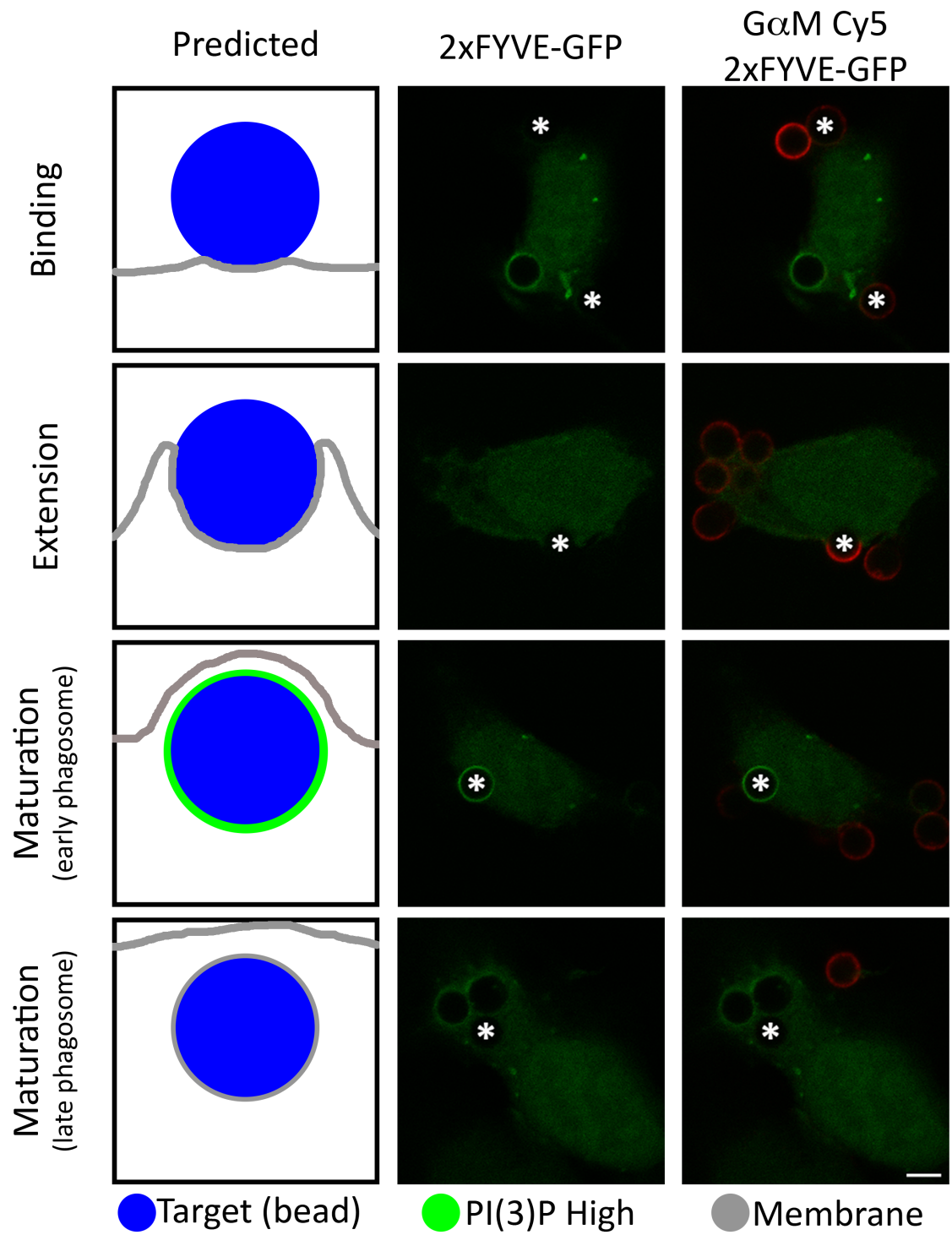
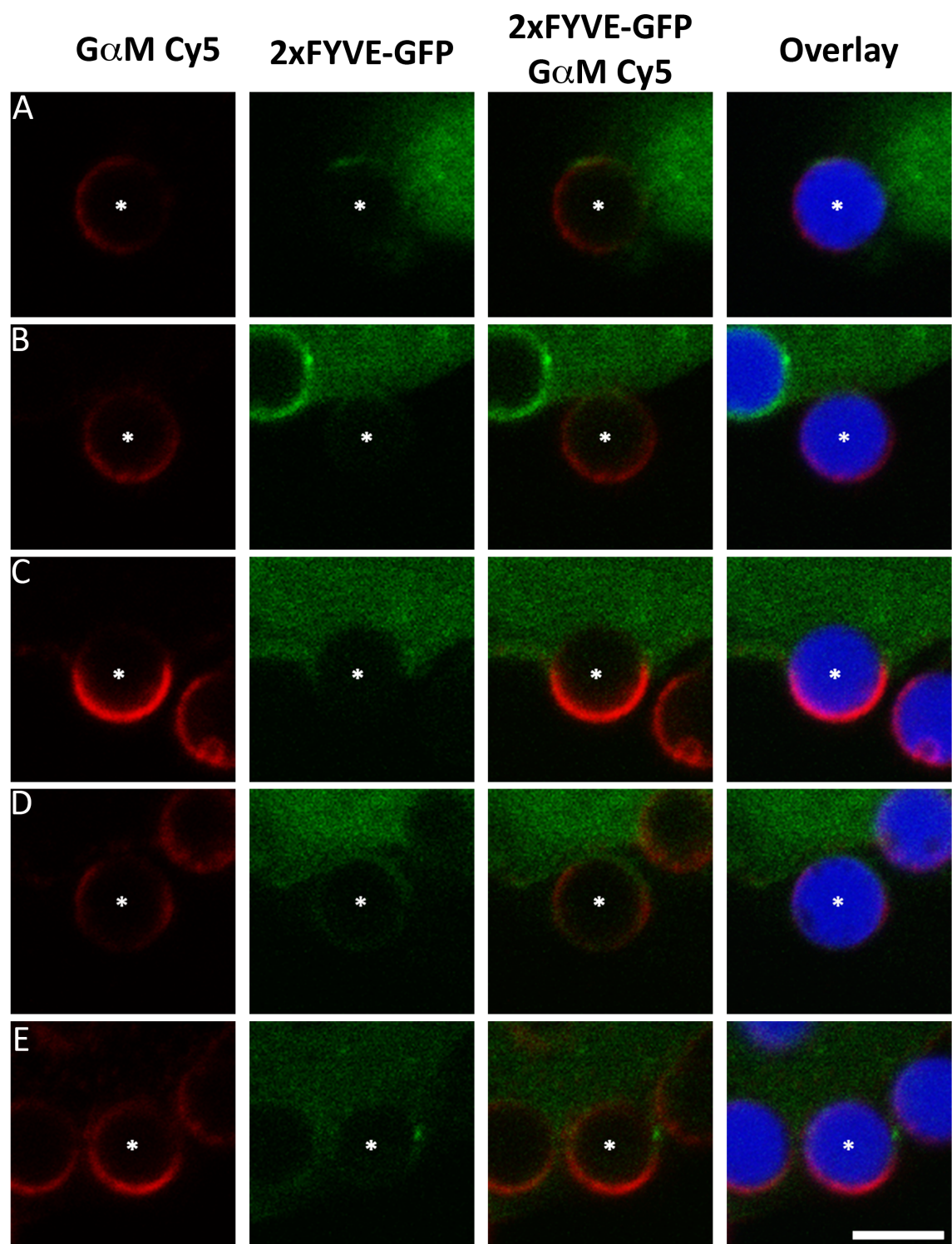


Figure 5. 15 Expected and observed phosphatidylinositol (3)-phosphate (PI[3]P) distribution during IpLITR 1.1b/WT –mediated phagocytosis. Representative images of IpLITR 1.1b/WT–expressing cells binding beads (Ai-Aii), extending pseudopods (Bi-Bii), in early phagosome maturation (Ci-Cii) or later maturation (Di-Dii). Asterisks show location of BB beads. 2xFYVE-GFP (green) detects PI[3]P surrounding early maturing phagosomes. Cy5-conjugated goat anti-mouse (GαM Cy5) detects exposed anti-hemagglutinin antibody on Bright Blue beads and is shown in red. Predicted PI[3]P based on mammalian FcR-mediated phagocytosis. PI[3]P is not detectable in plasma membrane (gray); transient increase in PI[3]P during early phagosomal maturation shown in bright green; absence of PI[3]P from phagosomal membrane in later stages of maturation shown in gray. Scale bar 5 μ m.



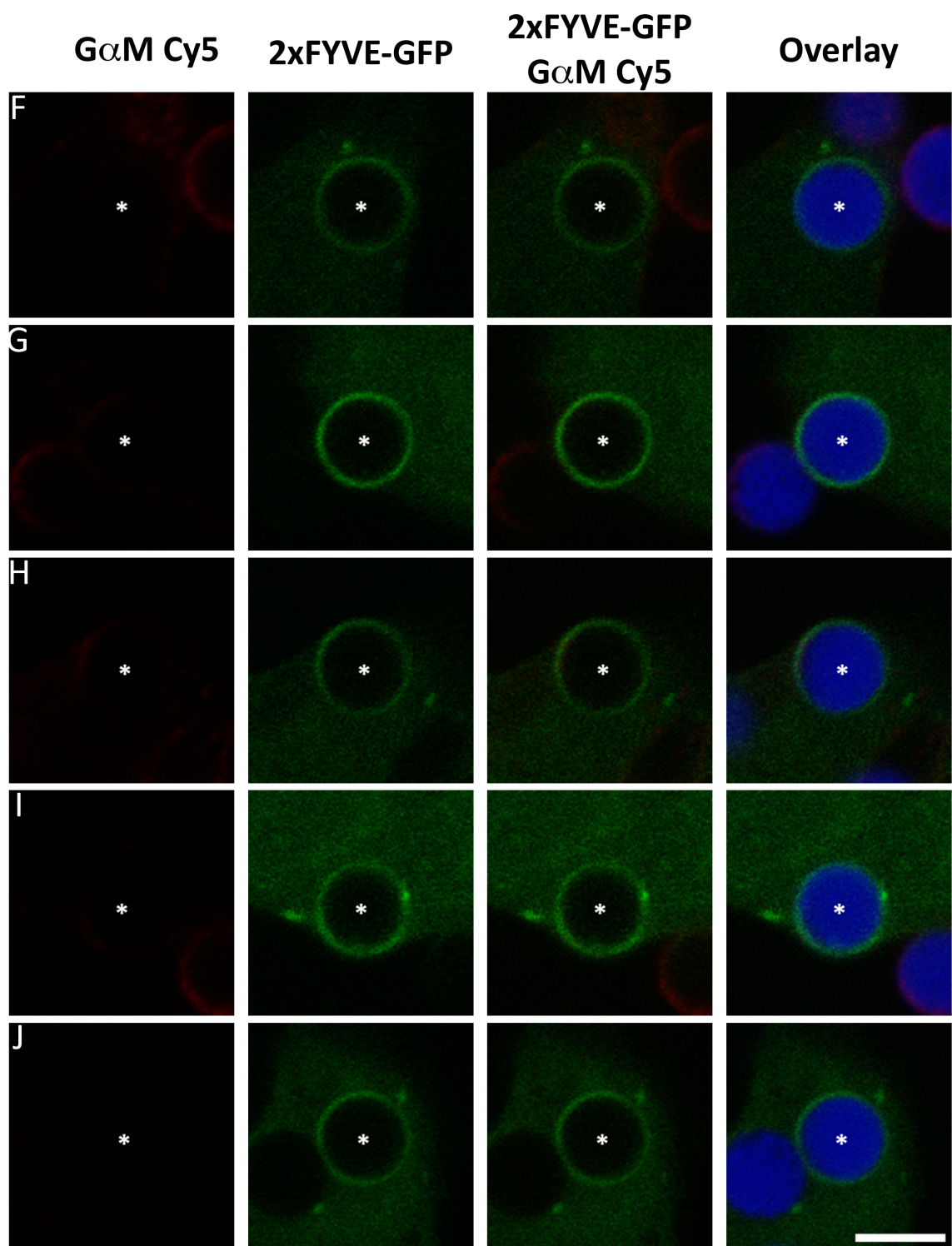


Figure 5. 16 Confocal microscopic visualization of phosphatidylinositol 3-phosphate distribution on maturing phagosomes during IpLITR 1.1b/WT-mediated phagocytosis.

To determine whether targets of IpLITR 1.1b/WT-mediated phagocytosis are internalized into PI(3)P-enriched phagosomes, the distribution of this phospholipid was monitored with 2xFYVE-GFP (a marker of PI(3)P; green) during the IpLITR 1.1b/WT-induced phagocytic process. AD293 cells stably expressing IpLITR 1.1b/WT ($\sim 1.5 \times 10^5$ cells) co-transfected with 2xFYVE-GFP were incubated with mAb anti-HA coated, 4.5 μm targets (3×10^5 Bright Blue beads) then fixed prior to imaging. External regions of beads were counterstained with a Cy5-conjugated goat anti-mouse polyclonal antibody (G α M Cy5; red). Surface bound and partially engulfed targets (A-E), and internalized (K-O) are representative images from three independent experiments with five replicates within each experiment. Scale bar, 5 μm .

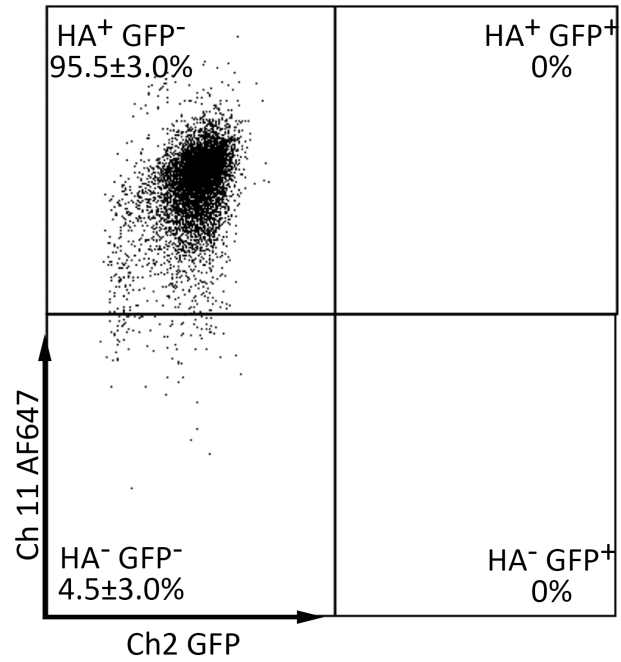
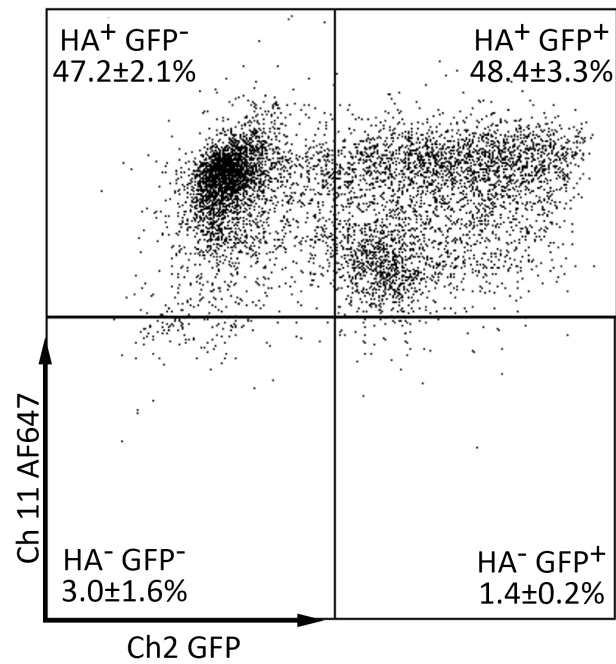
A**IpLITR 1.1b ITAM CYT****B****+ Syk-GFP**

Figure 5. 17 Imaging flow cytometry-based co-expression analysis of Syk-GFP and IpLITR 1.1b/ITAM in AD293 cells. To demonstrate that transient co-transfection with Syk-GFP did not alter the stable expression of IpLITR 1.1b/ITAM in AD293 cells, IpLITR 1.1b/ITAM and SykGFP co-expression was analyzed using imaging flow cytometry. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) were transiently co-transfected with Syk-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μg anti-HA mAb followed by staining with 0.5 μg AF647-conjugated rabbit anti-mouse IgG. Syk-GFP and IpLITR co-expression was compared to AD293 cells stably expressing IpLITR 1.1b/ITAM that were not transfected with Syk-GFP. IpLITR 1.1b/ITAM expression in non-transfected cells (A) and co-transfected cells (B) shown. Axes represent intensity of fluorescence in Channel 11 (AF647 IpLITR 1.1b/ITAM) and Channel 2 (Syk-GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown (8×10^3 collected events per experiment).

IpLITR 1.1b ITAM CYT

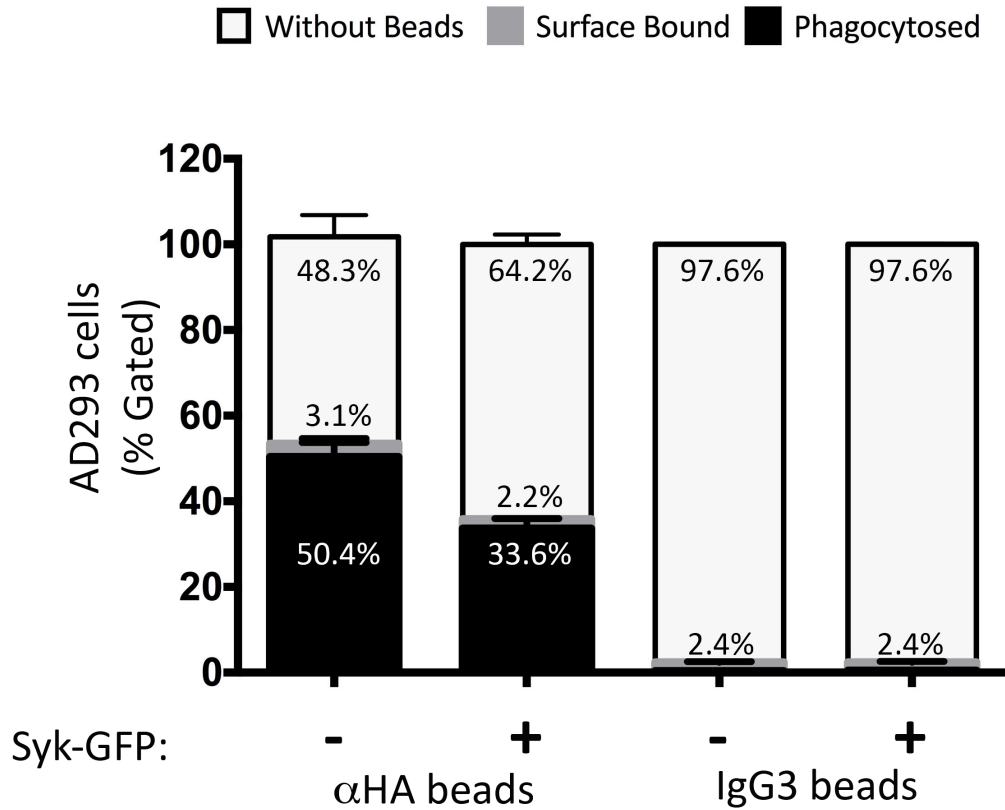


Figure 5. 18 IpLITR 1.1b/ITAM retains its phagocytic phenotype after co-transfection with Syk-GFP. To show that transient co-transfection of AD293 cells stably expressing IpLITR 1.1b/ITAM did not alter the receptor's overall phagocytic phenotype, the phagocytic activity of co-transfected cells was measured at a populational level. AD293 cells stably expressing IpLITR 1.1b/ITAM were co-transfected with Syk-GFP (+) for twenty-four hours. The ability of co-transfected cells (3×10^5 cell/sample) to internalize α HA mAb or IgG3 isotype control –coated beads (9×10^5 beads/sample) at 37°C for one hour was assessed by imaging flow cytometry in comparison to cells without Syk-GFP (-). Numbers represent the mean percent of population that had internalized at least one bead (black), only had surface bound beads (gray), or were not associated with beads (white). Error bars represent mean $\pm$ SEM of three independent experiments.

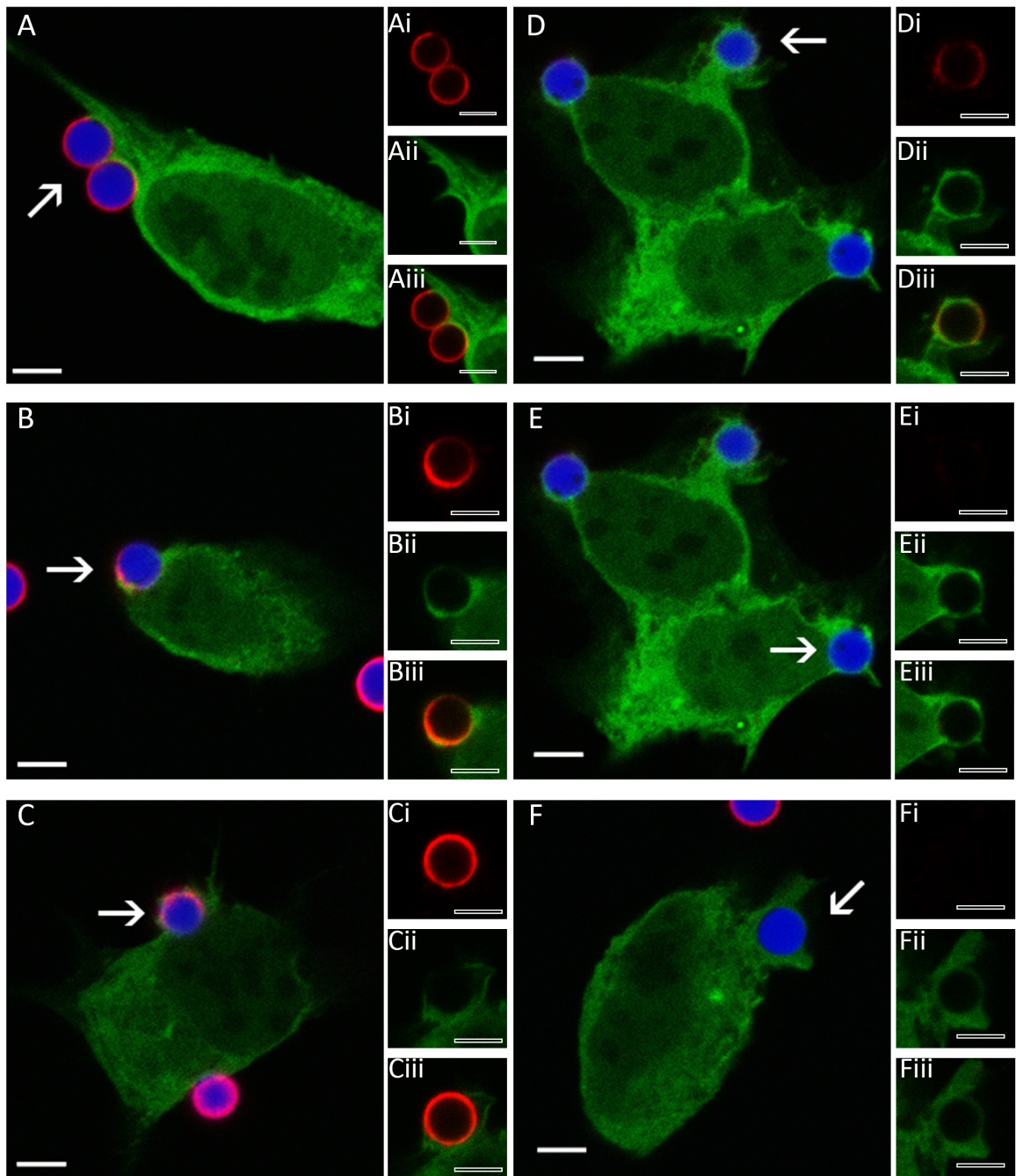


Figure 5. 19 Confocal microscopy visualization of IpLITR 1.1b/ITAM-mediated changes in SykGFP distribution during internalization. To visualize the distribution of spleen tyrosine kinase (Syk) during IpLITR 1.1b/ITAM-mediated phagocytosis, AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 1.5 \times 10^5$ cells) co-expressing Syk-GFP were incubated with anti-hemagglutinin coated, 4.5 μm targets (3×10^5 beads) then fixed prior to imaging. External regions of beads were counterstained with Cy5-conjugated goat anti-mouse (G α M Cy5) polyclonal antibody. A-F show cells engulfing targets. Arrows point to beads shown in insets. (i) G α M Cy5 (ii) SykGFP (iii) Cy5/GFP overlay. Scale bars, 5 μm .

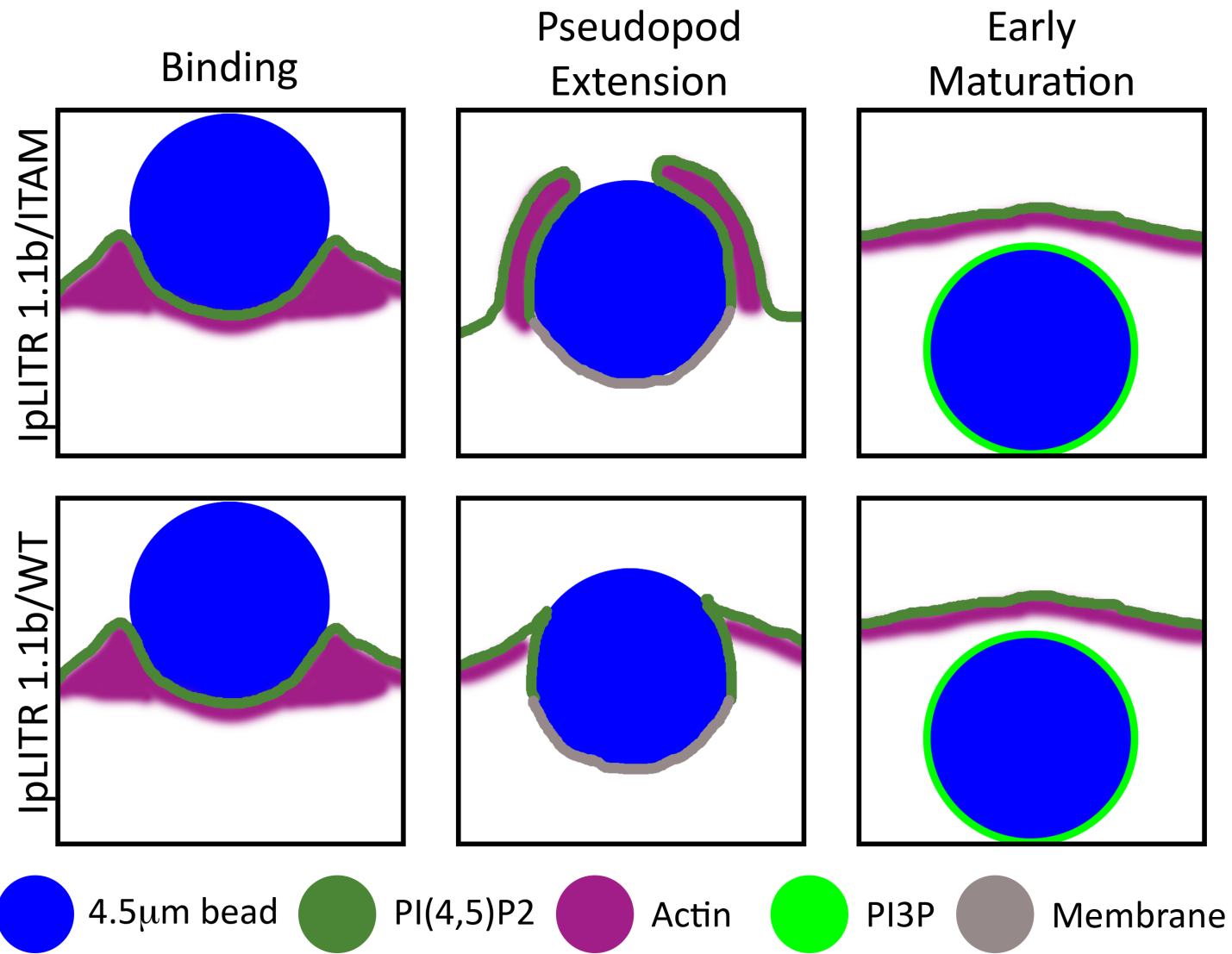


Figure 5. 20 Proposed model for the distribution of actin, PI(4,5)P₂, and PI(3)P during IpLITR –mediated phagocytosis. The blue circle represents a α HA coated, 4.5 μ m bead. The dark green color represents PI(4,5)P₂ in the plasma membrane of AD293 cells. The magenta color represents filamentous actin. Bright green represents PI(3)P present in the membrane of the early phagosome. Gray color represents membrane (e.g. plasma membrane or nascent phagosome) that is not enriched in either PI(4,5)P₂ or PI(3)P.

CHAPTER 6

General Discussion and Conclusions

6.1 Summary of findings

Phagocytosis is an important cell biological process used by all animals for innate immune protection from pathogens (Greenberg & Grinstein, 2002; Underhill & Ozinsky, 2002; Henneke & Golenbock, 2004; Arandjelovic & Ravichandran, 2015; Barreda et al., 2016). The process of phagocytosis is regulated by dedicated phagocytic receptors that bind distinct targets and induce their internalization via cytoskeletal changes that drive plasma membrane movements. These movements are coordinated through diverse intracellular signaling pathways facilitated by the cytoplasmic signaling domain of phagocytic receptors that link receptor activation with F-actin polymerization (reviewed by Flannagan et al, 2012; Swanson, 2014). The early stages of phagocytosis are classically described as (1) target acquisition (or binding), (2) formation of a phagocytic cup and membrane extension around targets, (3) scission of target containing vesicle (phagosome) from the plasma membrane, (4) and phagosome maturation (reviewed by Flannagan et al, 2012).

Much effort has been focused on understanding the phagocytic process in mammalian model systems but little is known of this process in other vertebrates such as fish. It was recently reported that channel catfish LITRs can induce phagocytosis in the context of mammalian myeloid cells (Cortes et al, 2012; 2014), and that two IpLITR

types induce distinct modes of target acquisition and engulfment (Lillico et al, 2015). An ITAM-encoding IpLITR type was able to efficiently induce phagocytosis of large targets (i.e. 4.5 μm) in an ITAM-dependent manner; this demonstrated that a canonical ITAM pathway is conserved in fish (Cortes et al, 2012; Lillico et al, 2015). Surprisingly, an ITIM-containing IpLITR type also induced phagocytosis in mammalian myeloid cells (Cortes et al, 2014). However, ligation of this same IpLITR type, known as IpLITR 1.1b, demonstrated an inhibitory function in the context of mammalian lymphoid NK cells (Montgomery et al, 2009; 2012); a concept described as functional plasticity (Cortes et al, 2014). In the context of the myeloid cells, IpLITR 1.1b induced a mode of target acquisition and engulfment that was distinct to the ITAM-containing IpLITR-type (Lillico et al, 2015). Specifically, IpLITR 1.1b induced the formation of membranous protrusions that anchored targets and retracted them to the cell surface (Lillico et al, 2015). In some cases these targets were fully engulfed but, in many cases, targets remained tethered at the cell surface (Lillico et al, 2015). The overall goal of my thesis work was to develop techniques to further examine the IpLITR 1.1b-induced mechanisms that coordinate target acquisition and engulfment. In order to achieve this, I developed imaging flow cytometry and confocal microscopy –based techniques to observe the IpLITR-mediated phagocytic process temporally using F-actin and PI distribution as markers for the discrete stages of IpLITR-mediated phagocytosis. First, I successfully developed a counterstaining strategy to identify beads that were surface bound, partially engulfed, or fully internalized. Using imaging flow cytometry, I demonstrated that, at the population level, AD293 cells stably expressing IpLITRs retained previously described

IpLITR-mediated modes of target acquisition and engulfment phenotypes (Lillico et al, 2015). Additionally, cells co-expressing IpLITRs and fluorophore-tagged markers for F-actin, PI(4,2)P₂, and PI(3)P also retained their phagocytic activities. Subsequently, I showed that IpLITR 1.1b/WT induced a unique redistribution of F-actin during the phagocytic process that was mirrored by the distribution of PI(4,5)P₂. The ITAM-dependent receptor induced F-actin and PI(4,5)P₂ distributions that were reminiscent of the well-characterized mammalian FcR-mediated phagocytosis. Lastly, I showed that IpLITR targets are internalized into a PI(3)P-enriched phagosome. These findings provide new information regarding the reorganization of the cytoskeleton and phosphoinositide species during the IpLITR 1.1b/ITAM and IpLITR 1.1b/WT –mediated phagocytic processes.

6.1.1 Development of imaging based-assays to monitor IpLITR-mediated phagocytic process using F-actin as a surrogate for location of signal transduction

IpLITRs were previously demonstrated to induce phagocytosis (targets 4.5 µm in size) in the context of a mammalian immune cell line (i.e. RBL-2H3 cells; Cortes et al, 2012 and 2014). Two representative receptor-types, IpLITR 1.1b and IpLITR 2.6b, demonstrated vastly different modes of target acquisition and engulfment likely due to their distinct signalling potential; however, it was unknown how the two receptors accomplished these activities. Additionally, we did not know whether this IpLITR-mediated activity was an RBL-2H3 –specific phenomenon or if this activity was

conserved amongst different cell-types. This was important because our model was dependent on the heterologous expression of teleost receptors in mammalian cell lines.

The main objective of my thesis was to develop imaging based bioassays to examine the IpLITR-induced phagocytic process with the goal to apply these experiments to test whether candidate-signaling molecules are recruited to the IpLITR-induced phagocytic cup, as well as characterize spatial and temporal distributions of these molecules during the IpLITR-mediated phagocytic process. First, I developed a counterstaining method to discriminate surface-bound, partially engulfed, and internalized beads. This technique has been used in a variety of ways (e.g. biotinylation, quantum dots, immunofluorescence) to examine the phagocytic process induced by mammalian receptor-types (examples: Flannagan, et. al., 2014; Schlam et. al., 2015; Jaumouillé et al, 2014). In the preparation of IpLITR targets (i.e. 4.5 μm microspheres/beads), beads are coated with anti-hemagglutinin monoclonal IgG3 isotype antibody raised in mouse (i.e. αHA mAb). Therefore, I reasoned that I could specifically detect beads using a fluorophore-conjugated secondary antibody raised against mouse fragment crystallisable (Fc) of IgG3. I showed that when beads are surface-bound, they are mostly exposed to the secondary antibody and thus fluoresce in two colors: blue (bead emission wavelength) and red (antibody emission wavelength). When beads are partially engulfed, areas of beads that are tightly associated with the cellular plasma membrane do not fluoresce in red because the antibody is excluded from this area. However, the areas of beads that are exposed to the secondary antibody

fluoresce red. Lastly, Internalized beads are not accessible to the secondary antibody and thus only fluoresce in the blue wavelength.

To address the question regarding the ability of IpLITRs to mediate phagocytic activity in other cell-types, I transfected IpLITRs into the human epithelioid, non-immune, non-phagocytic (i.e. do not express phagocytic receptors) AD293 cell line. AD293 cells are easily transfectable and have been shown to support receptor-mediated phagocytosis when heterologously transfected with phagocytic receptors (Flannagan et al, 2014); therefore, by demonstrating that IpLITRs can mediate phagocytosis in non-phagocytic cells, we could establish that these receptors are ‘bona-fide’ phagocytic receptors. In mammals, ‘bona-fide’ phagocytic receptors are phagocytic receptors (e.g. FcR and Dectin-1) that, when heterologously expressed in non-phagocytic cells, induce phagocytosis (Reviewed by Flannagan et al, 2012; Underhill & Goodridge, 2012). Molecules that participate in the polymerization of actin are highly conserved through evolution due to the requirement for cytoskeletal changes that facilitate cell-cell or cell-matrix adhesion as well as cell migration (Barreda et al, 2016; Cougoule et al, 2004); thus making it possible to induce receptor-mediated phagocytosis in otherwise non-phagocytic cells.

Using imaging flow cytometry, I demonstrated that the co-transfection approach did not alter the overall expression of IpLITRs and their ability to mediate target acquisition and engulfment phenotypes that were previously described (Lillico et al, 2015). This strategy allowed me to specifically identify cells that had engulfed at least one bead from those that only had surface bound beads. This was made possible by

modifying the counterstaining strategy that I previously developed for microscopy to accommodate the laser and filter requirements of the imaging flow cytometer.

The signal transduction events that take place during phagocytosis are localized to the site where receptors are triggered (i.e. phagocytic cup) and culminate in actin redistribution, allowing for the plasma membrane remodelling required for accommodating target engulfment. Since these events culminate in cytoskeletal rearrangements, I chose to develop my microscopy-based assay using F-actin as a surrogate for the location at which signal transduction will take place. I monitored IpLITR 1.1b/ITAM and IpLITR 1.1b/WT –induced changes in F-actin polymerization to gain insight into the spatial and temporal distribution of these structures. I found that IpLITR 1.1b/WT –induced a very unique pattern of actin reorganization that I did not observe during the IpLITR 1.1b/ITAM –induced response. Specifically, IpLITR 1.1b/WT did not extend F-actin rich pseudopodia. Instead, F-actin depolymerized from the base of the phagocytic cup and essentially allowed targets to sink or be retracted into the cell. This was vastly different from the IpLITR 1.1b/ITAM response, which did induce the formation of long pseudopodia to facilitate entry.

These findings provided new information regarding the F-actin changes induced by two IpLITR-types to promote target entry, while also validating that IpLITR-mediated induction of F-actin polymerization is conserved across cell-types. Lastly, I provided a method for detecting bead position relative to the cell membrane as a method to discriminate the phagocytic activities of the two receptors.

6.1.2 Imaging-based examination of phosphoinositides during IpLITR-mediated phagocytosis

In Chapter 4 of this thesis, I demonstrated that two IpLITR-types induced distinct patterns of actin reorganization. Phosphoinositides serve as molecular addresses to localize very specific subsets of molecules to membranes and thus are directly involved in the regulation of many cellular responses including phagocytosis. PI(4,5)P₂ is found in the inner leaflet of the plasma membrane of cells and has been shown to play a role in mammalian FcR-mediated phagocytosis (Botelho et al, 2000). PI(4,5)P₂ cooperation with small RhoGTPases is important for the activation of the actin polymerization machinery (Reviewed by Levin et al, 2015). To gain further insight into IpLITR-mediated changes in F-actin distribution, I developed imaging-based assays to monitor the distribution of phosphoinositides during the IpLITR-induced phagocytic process. I hypothesized that IpLITRs-induced PI reorganization would mirror my previous observations regarding the distribution of F-actin during the IpLITR-mediated phagocytic process (Figure 5.19).

I showed that IpLITR 1.1b/ITAM induced PI(4,5)P₂ redistribution similar to what has been reported during the mammalian FcR-induced phagocytic process. That is, upon target binding, IpLITR 1.1b/ITAM induced a transient increase of phosphoinositides at the base of the phagocytic cup. PI(4,5)P₂ enrichment followed the leading edges of pseudopodia until the phagosome sealed. Concurrently, PI(4,5)P₂ disappeared from the base of the phagocytic cup (i.e. the nascent phagosome) and this disappearance followed behind the extending pseudopodia. PI(4,5)P₂ did not surround the phagosomal membranes of any internalized targets.

Once again, the IpLITR 1.1b/WT did not induce the formation of long pseudopodia. That is, PI(4,5)P₂ transiently increased at the base of the phagocytic cup but disappeared rapidly (during the 'surface bound' stage). As signaling precedes function, a possible reason for the lack of F-actin rich pseudopodia during IpLITR 1.1b-mediated phagocytosis is the rapid disappearance of PI(4,5)P₂, however, further studies are required to confirm this.

PI(3)P is predominantly found on the cytoplasmic leaflet of intracellular vesicles (Gillooly et al, 2000) and on phagosomal membranes where it plays a role in the maturation of these vesicles (Swanson, 2014; Levin et al, 2015). As was expected, targets of both IpLITR-types were internalized into a phagosome that became transiently enriched in PI(3)P. These results confirmed the complete internalization of particles.

Taken together, these findings provide new information regarding IpLITR-induced reorganization of the cytoskeleton and redistribution of phosphoinositide species during the phagocytic process. Here I demonstrate that a teleost ITAM-dependent receptor promotes canonical ITAM-mediated changes in F-actin polymerization and PI(4,5)P₂ redistribution. However, the ITAM-independent receptor induced distinctly different patterns of F-actin and PI(4,5)P₂ redistribution (when compared to the teleost ITAM receptor), which is likely due to the ability of this receptor to induce unique signaling pathways. Additionally, IpLITR-induced phagocytosis results in target internalization into PI(3)P-enriched phagosomes; during an immune response PI(3)P enrichment of phagosomes is important phagosome maturation (i.e. fusion with

other vesicles such as lysosomes and endosomes) into a potent antimicrobial compartment (Jeschke and Haas, 2016). I could have also monitored other phosphoinositides (i.e. $\text{PI}(3,4,5)\text{P}_3$) using a GFP chimeric construct fused to the PH domain of Akt (kindly provided by Dr. Touret); however, I did not have time to accomplish this. Examination of $\text{PI}(3,4,5)\text{P}_3$ levels during IpLITR-mediated phagocytosis would have demonstrated PI3K activity since it is believed to be the main contributor to the conversion of $\text{PI}(4,5)\text{P}_2$ to $\text{PI}(3,4,5)\text{P}_3$ during mammalian FcR-mediated phagocytosis (Levin et al, 2015).

6.1.3 Phagocytosis as a bioassay to map IpLITR-mediated signal transduction

Phagocytosis is a conserved physiological process that is highly dependent on filamentous (F)-actin remodelling (Reviewed by Levin et al, 2015; Flannagan et al, 2012). The cytoskeletal network actively participates in the capture and engulfment of phagocytic targets (e.g. pathogens). Therefore, in order for phagocytic receptors to mediate target internalization; they must be able to translate extracellular binding into dynamic F-actin reorganization. Interestingly, other cellular processes such as cell-cell adhesion, cell-matrix adhesion, cell migration, as well as synapse formation all require F-actin remodelling (Reviewed by Cougoule et al, 2004; Niedergang et al, 2016). As a result, there is a striking similarity in the types of molecules that phagocytic receptors and adhesion molecules (e.g. integrins) recruit and activate upon ligation (Cougoule et al, 2004). These signaling molecules include: (1) guanine exchange factors which promote activation of (2) small RhoGTPases (3) GTPases activate WASP and or WAVE (4) then activated WAVE and/or WASP play pivotal roles in activating the Arp2/3-

dependent polymerization of F-actin. Although the precise identity of molecules that different receptors use can vary, their coordinate signaling activities achieve the same objective in that they network cell surface binding into F-actin mediated cytoskeletal rearrangements. The importance of F-actin polymerization is underscored by the ability of many pathogens to exploit the phagocytic process to induce infection. For example, *Salmonella* induces F-actin polymerization in host cells to promote its internalization. This is accomplished by injecting a virulence factor (SopE) that functionally mimics host guanine exchange factors: molecules that promote the activation of small RhoGTPases (Humphreys et al, 2012). The ability of primitive organisms such as bacteria to manipulate eukaryotic cellular functions demonstrates the importance and conservation of signaling networks that promote cytoskeletal remodelling.

The ability of teleost receptors to promote F-actin remodelling in two distinctly different surrogate cells (i.e. rat immune cells and human epithelioid cells) is highly suggestive of the ability of these receptors to mediate similar activities in teleost cells due to the conserved nature of signaling that culminates in F-actin remodelling. Therefore it is tempting to predict that these receptors are capable of mediating cytoskeletal remodelling that is required for innate immune activities such as immunological synapse formation, phagocytosis, cell migration, and degranulation in teleost. In addition to being related to human LRC encoded proteins and FcR/FCRLs, IpLITRs are related to adhesion molecules such as the human SIGLECs and CEACAMs (Fei et al, 2016). These LRC-related proteins are involved in adhesion as well as pathogen sensing (Trowsdale, 2001). Therefore it is plausible that IpLITR roles in teleost immunity

may range from immune sensing and immune-modulation to adhesion, all activities that likely require the ability of receptors to translate extracellular binding to cytoskeletal reorganization. However, without knowing the natural ligands of IpLITRs and the lack of IpLITR-specific reagents poses a challenge to examine the precise roles that IpLITRs undertake. As a result, characterization of IpLITRs relies on heterologous overexpression. The ability of IpLITRs to mediate phagocytosis in surrogate cell systems can be exploited to elucidate the precise signaling mechanisms that IpLITRs employ to network to the actin polymerization machinery. Phagocytosis provides a useful bioassay to gain insight into IpLITR-mediated signal transduction because these events are localized to the specific area of the plasma membrane that is engaged (i.e. the 'phagocytic synapse') and the size of this structure can be manipulated by the size of targets (Grinstein, 2010). Furthermore, these signaling events occur within seconds to minutes and thus can be captured using microscopy techniques to achieve spatial and temporal resolution for the recruitment and activation of molecules. As a result, valuable information regarding the spatiotemporal regulation of IpLITR-mediated signal transduction can be gained using phagocytic assays coupled with confocal microscopy to understand how these receptors promote dynamic actin remodelling and provide insight into how these receptors may function in their native cell types.

6.2 Future Directions

The unique organization of the IpLITR 1.1b cytoplasmic region gives this receptor the ability to control functional responses that are independently mediated by the

proximal and distal cytoplasmic domains (Montgomery et al, 2009; Montgomery et al, 2012; Cortes et al, 2014; Lillico et al, 2015; Zwozdesky et al, 2016). Montgomery et al demonstrated that IpLITR 1.1b induced an inhibitory function in the context of mouse NK cells via two independent mechanisms: a Csk-dependent (2012) and SHP-2 – dependent manner (2009). The uniqueness of this receptor was further reinforced by the discovery that IpLITR 1.1b could stimulate phagocytosis in RBL cells (Cortes et al, 2014) in a manner biochemically and morphologically unique to classical ITAM-mediated phagocytosis (Lillico et al, 2015). Zwozdesky et al (2016) recently confirmed the independent functionality of the proximal and distal IpLITR 1.1b cytoplasmic regions to mediate IpLITR 1.1b-induced target acquisition and engulfment. Using biochemical techniques, Zwozdesky et al (2016) also explored the recruitment of candidate signaling molecules and showed that the proximal region associated with Csk, Grb2, Nck1, Vav and PI3K p85 α but not SHP-2, Syk or Vav3. The distal region associated with SHP-2, Syk and PI3K p85 α but not Csk, Grb2, Nck1, or Vav (Zwozdesky et al, 2016). These observations contributed to the development of multiple models to explain potential signalosomes that IpLITR 1.1b engages in order to translate extracellular binding to F-actin polymerization: a requirement for IpLITR 1.1b-mediated phagocytosis (Cortes et al, 2014; Lillico et al, 2014) and the formation of an immunological synapse between an NK cell and its target cell (Carpen et al, 1983; Wülfing et al, 2003; Orange et al, 2003). These models are based on the differential recruitment of Nck1, Grb2, Syk and SHP-2 to the individual IpLITR 1.1b cytoplasmic regions (Zwozdesky et al, 2016). Importantly, the

proposed models are supported by signaling pathways that have been deciphered for other receptors capable of networking to the F-actin polymerization machinery.

Cooperative signaling between the proximal and distal regions could coordinate IpLITR 1.1b-mediated target acquisition and engulfment. In this model, receptor engagement and phosphorylation of proximal (Y₄₃₃) and distal (Y₄₇₇ & Y₄₉₉) could result in the recruitment and activation of Nck1 and Syk to the proximal and distal regions respectively. Activation of Nck1 could localize the WAVE2 complex in close proximity to the site of receptor engagement. Activated Syk could bind to a guanine exchange factor such as Vav and/or PI3K p85 α that can activate the WAVE2 complex. In turn, the activated WAVE2 complex activates Arp2/3, a nucleator of F-actin polymerization. This proposed signaling mechanism mimics a short-circuit pathway recently described for human CEACAM3, a phagocytic receptor for *Neisseria gonorrhoeae* (Pils et al, 2012). In an alternative model, based on the reported human NKG2D signaling pathway (Upshaw et al., 2006), Nck1 and Grb2 are recruited to Y₄₃₃ and Y₄₆₃, respectively, within the distal cytoplasmic region. As in the previous model, Nck1 would localize the WAVE2 complex in the vicinity of the activated receptor. Syk recruitment to the tandem ITIMs in the distal cytoplasmic region of IpLITR 1.1b (Y₄₇₇ and Y₄₉₉) could phosphorylate Grb2 and induce Grb2-dependent recruitment of Vav1 and Syk-dependent activation of Vav1. Vav1 would then activate a small rho GTPase that would activate the WAVE2 complex leading to WAVE2-mediated activation of the actin polymerization machinery.

The observation that the distal and proximal cytoplasmic regions could independently mediate acquisition and engulfment suggest that each domain can

independently network to the F-actin polymerization machinery (Zwozdesky et al, 2016). The proximal cytoplasmic domain of IpLITR 1.1b could recruit Nck1 to Y₄₃₃ and Grb2 to Y₄₆₃. Grb2-dependent recruitment of the guanine exchange factor Sos1 can lead to the activation of the small GTPase Ras; activated Ras can lead to Arp2/3-dependent F-actin polymerization (Gomez-Cambronero, 2011). Formation of a Grb2-Gab2-PI3K complex is another potential mechanism of promoting activation of F-actin polymerization activities (Bennett et al., 1994; Li et al., 1994; Gu et al., 1998; Brummer et al., 2010). In this model, phosphorylated Y₄₆₃ would result in the Grb2-dependent formation of this heterotrimeric complex and promote the activation of Vav, Vav activates Rac, and Rac facilitates WAVE2-mediated activation of Arp2/3. In this model, WAVE2 recruitment would be mediated by PI3K-dependent production of PI(3,4,5)P₃ (Rougerie et al., 2013). SHP-binding is generally associated with inhibitory signaling, however, phosphorylation of a C-terminal tyrosine can give SHP-2 the ability to scaffold the recruitment and activation of the Grb2-Gab2-PI3K signaling complex (Gu et al., 2003). SHP-2 recruitment to the ITIMs or ITSM within the distal region (Y₄₇₇, Y₄₉₉ and Y₅₀₃ respectively) could promote PI3K-dependent IpLITR 1.1b-induced F-actin polymerization. An alternate SHP-2 –dependent mechanism could also be initiated when SHP-2 with a C-terminal phosphorylated tyrosine (Y₅₄₂). Phosphorylation at this position can reveal a cryptic ITAM (Deng et al., 2015). Recruitment of two Y₅₄₂ – phosphorylated SHP-2 molecules to two tandem IpLITR 1.1b molecules could facilitate the recruitment and activation of Syk. Syk activation would then trigger Syk-dependent recruitment of PI3K and PI(3,4,5)P₃-dependent localization of a guanine exchange factor,

such as Vav1. Vav1 could facilitate WAVE2-dependent activation of Arp2/3. It is likely that other signaling molecules play a role in IpLITR 1.1b-mediated signal transduction; however, the proposed models provide a basis for the design of new experiments to test the spatiotemporal recruitment and activation of these molecules using microscopy-based techniques.

Confocal microscopy-based studies allow for direct, visual examination of cellular processes; therefore a plethora of tools and techniques have been developed and extensively used in the quest to characterize mammalian phagocytic receptors (e.g. Várnai & Balla, 1998; Botelho et al, 2000; Lu et al, 2017; Riedl et al, 2008; Gillooly et al, 2000). PH, 2xFYVE, and Lifeact constructs have been fused to many fluorophores in order to monitor the activities of multiple molecules (or phagocytic targets) during the phagocytic process. For example, Jaumouillé et al (2014) used red fluorescent protein – tagged Lifeact to monitor the cytoskeleton and FcR-GFP when cells were treated with a pharmacological drug. In the same research article, the researchers used GFP-tagged Lifeact to monitor actin during the FcR-mediated internalization of red beads. The main objective of my thesis was to develop and apply many of these imaging-based tools to monitor the IpLITR-mediated phagocytic process. I used markers for F-actin and PI(4,5)P₂ distribution in conjunction with a counterstain for extracellular targets to visualize discrete stages of the IpLITR 1.1b-induced phagocytic process. My work provided preliminary observations regarding the patterns of F-actin polymerization and PI(4,5)P₂ distribution at various stages of the IpLITR 1.1b-mediated phagocytic process. In addition, I cloned human Csk, Grb2, Nck1, SH2D1A, SHP-2, and PI3K p85 α into a GFP-

encoding plasmid to facilitate future studies regarding the role of these molecules during the IpLITR-mediated phagocytic process. These studies using fixed cells can be complemented with live-cell imaging to temporally define the phagocytic activity of IpLITR 1.1b in AD293 cells.

The actin distribution during the IpLITR 1.1b-mediated phagocytic process was recently monitored in live RBL-2H3 cells and demonstrated that this receptor mediates a variety of actin reorganization patterns (i.e. modes of target acquisition and engulfment) (Lillico & Stafford, unpublished). These studies demonstrated that in RBL-2H3 cells, IpLITR induced a similar mode of engulfment (lack of long pseudopodia during the process of internalization) further confirming my observations in AD293 cells. One downside of live-cell imaging is the inability to monitor large populations of cells at one time. This pitfall gives value to using static images as well as imaging flow cytometry, which provide a broader snapshot in time. Nevertheless, these strategies make it more difficult to appreciate the dynamic nature of cellular processes such as phagocytosis. Using confocal microscopy, Lillico et al (2015) demonstrated in the RBL-2H3 system, IpLITR 1.1b induced the formation of membranous extensions that captured targets. I was unable to reproduce this observation in AD293 cells: possibly due to the different nature of the two cell types (i.e. innate immune cell versus epithelial cell). Being an innate immune cell line, RBL-2H3 may be more motile than epithelial cells. Nevertheless, IpLITR 1.1b I was able to reproduce the overall tethering phenotype using both imaging flow cytometry and confocal microscopy of fixed cells. As a result, a combination of imaging flow cytometry, fixed cell imaging and live-cell imaging –based

experiments will be a useful approach to examine the recruitment and spatiotemporal distribution of candidate signaling molecules during the IpLITR 1.1b phagocytic process.

CHAPTER 7

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doi://dx.doi.org/10.1016/j.dci.2016.10.011

Appendix

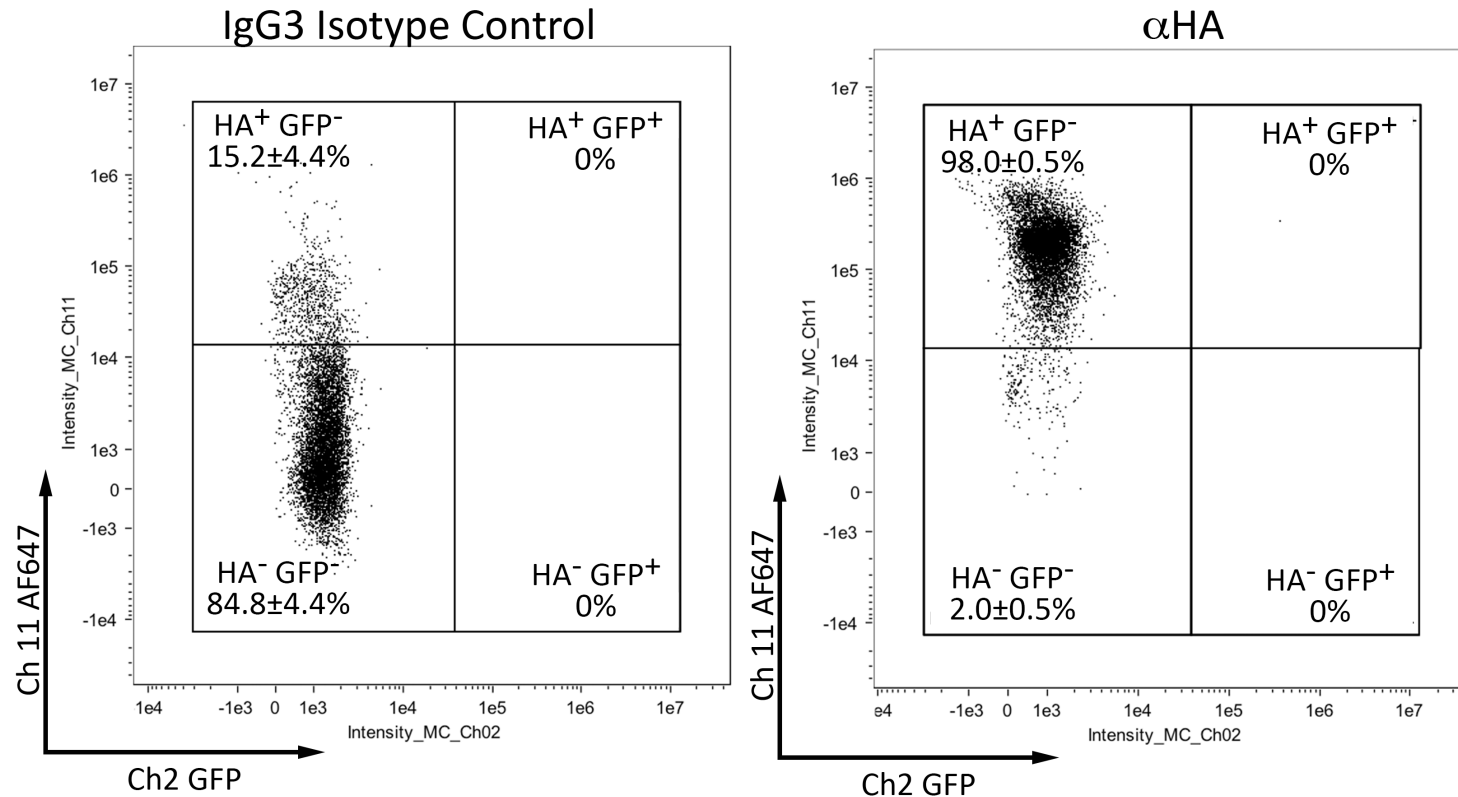


Figure A 1. Representative imaging flow-cytometry based dot plots of IpLITR 1.1b/ITAM stable expression in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) surface stained for IpLITR co-expression with 0.1 μ g of IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g R α M AF647. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

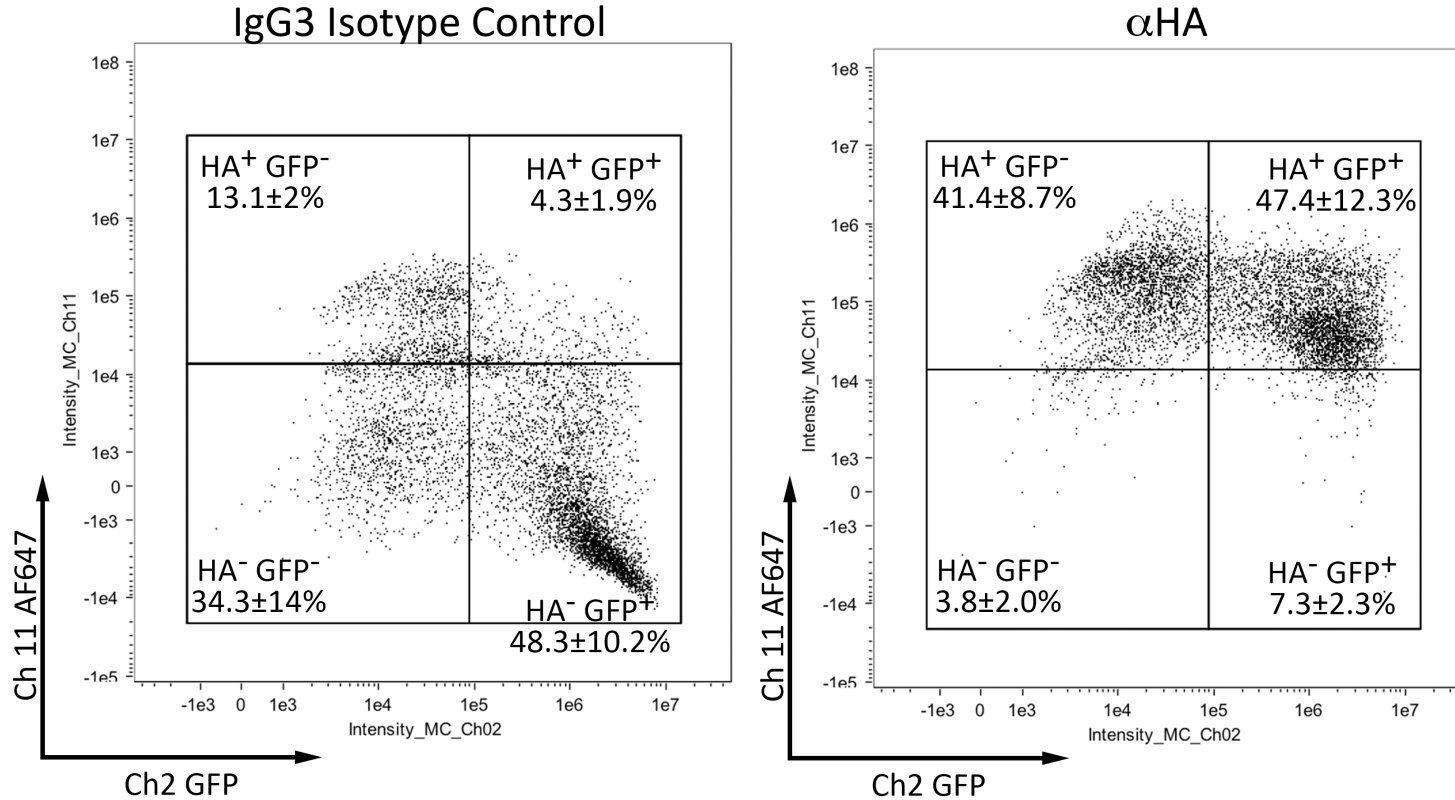


Figure A 2. Imaging flow cytometry-based co-expression analysis of Lifeact-GFP and IpLITR 1.1b/ITAM in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) were transiently co-transfected with Lifeact-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g of IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g AF647-conjugated rabbit α -mouse IgG. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

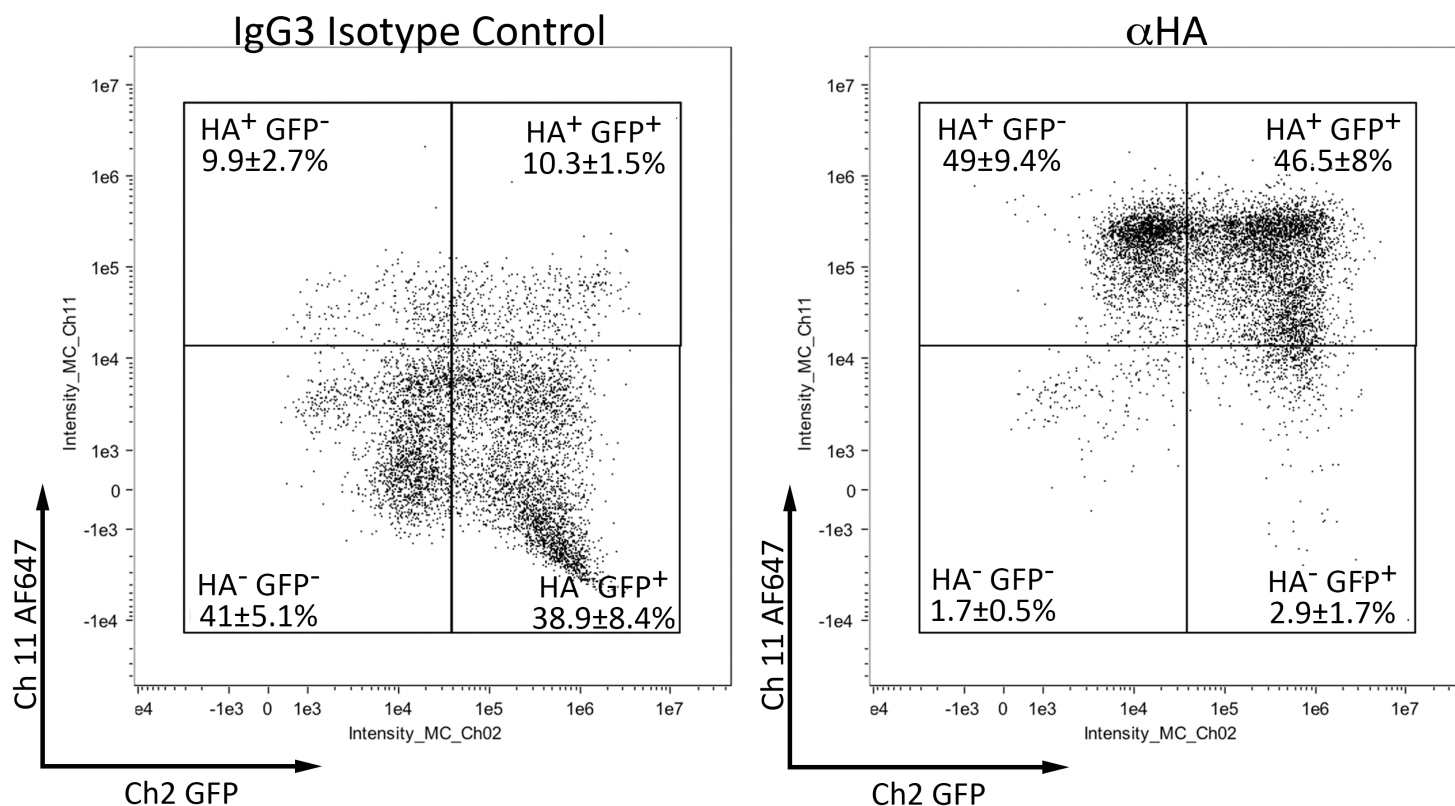


Figure A 3. Imaging flow cytometry-based co-expression analysis of PH-PLC δ_1 -GFP and IpLITR 1.1b/ITAM in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) were transiently co-transfected with PH-PLC δ_1 -GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g of IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g R α M AF647. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

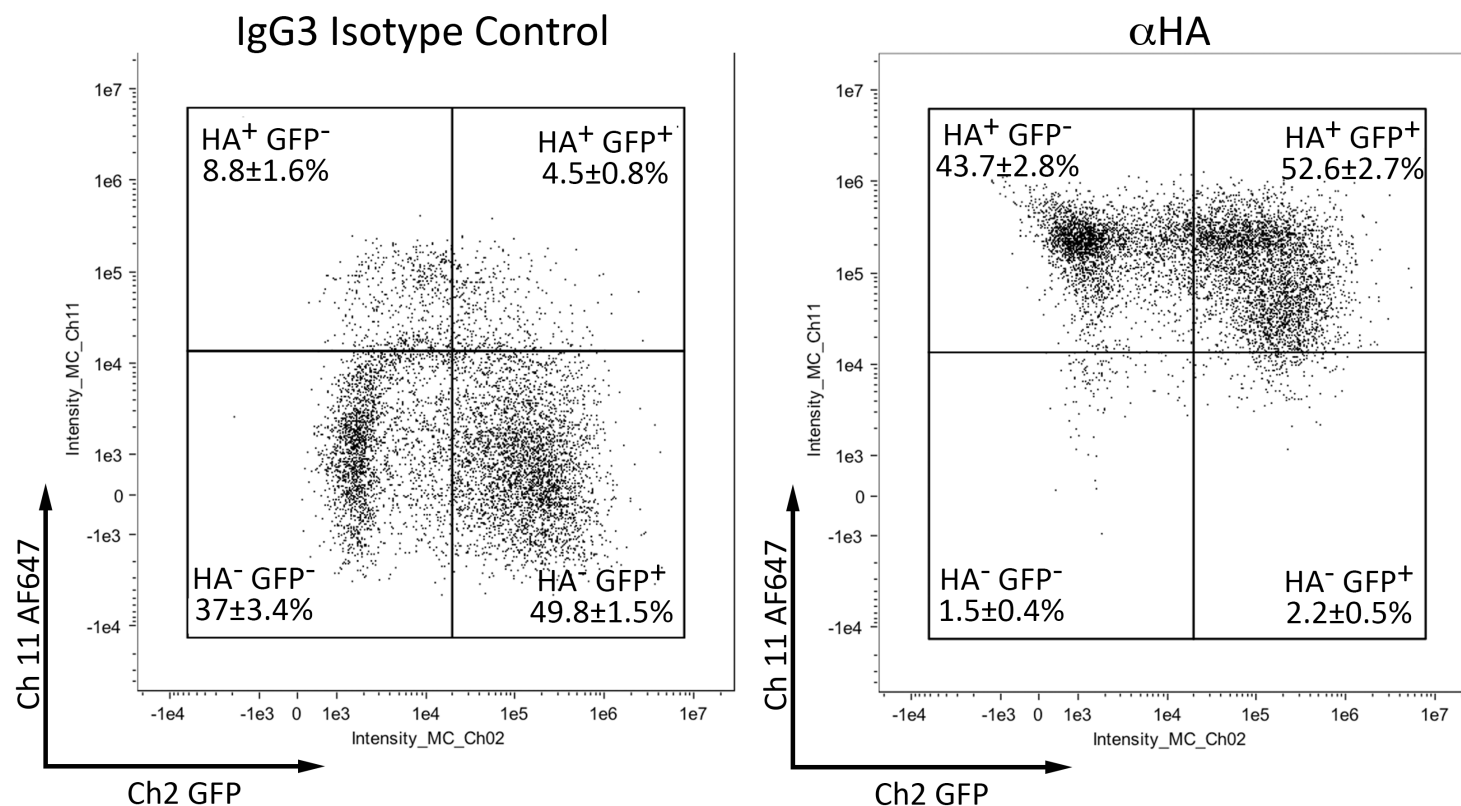


Figure A 4. Imaging flow cytometry-based co-expression analysis of 2xFYVE-GFP and IpLITR 1.1b/ITAM in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) were transiently co-transfected with 2xFYVE-GFP for 24 hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g of IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g R α M AF647. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

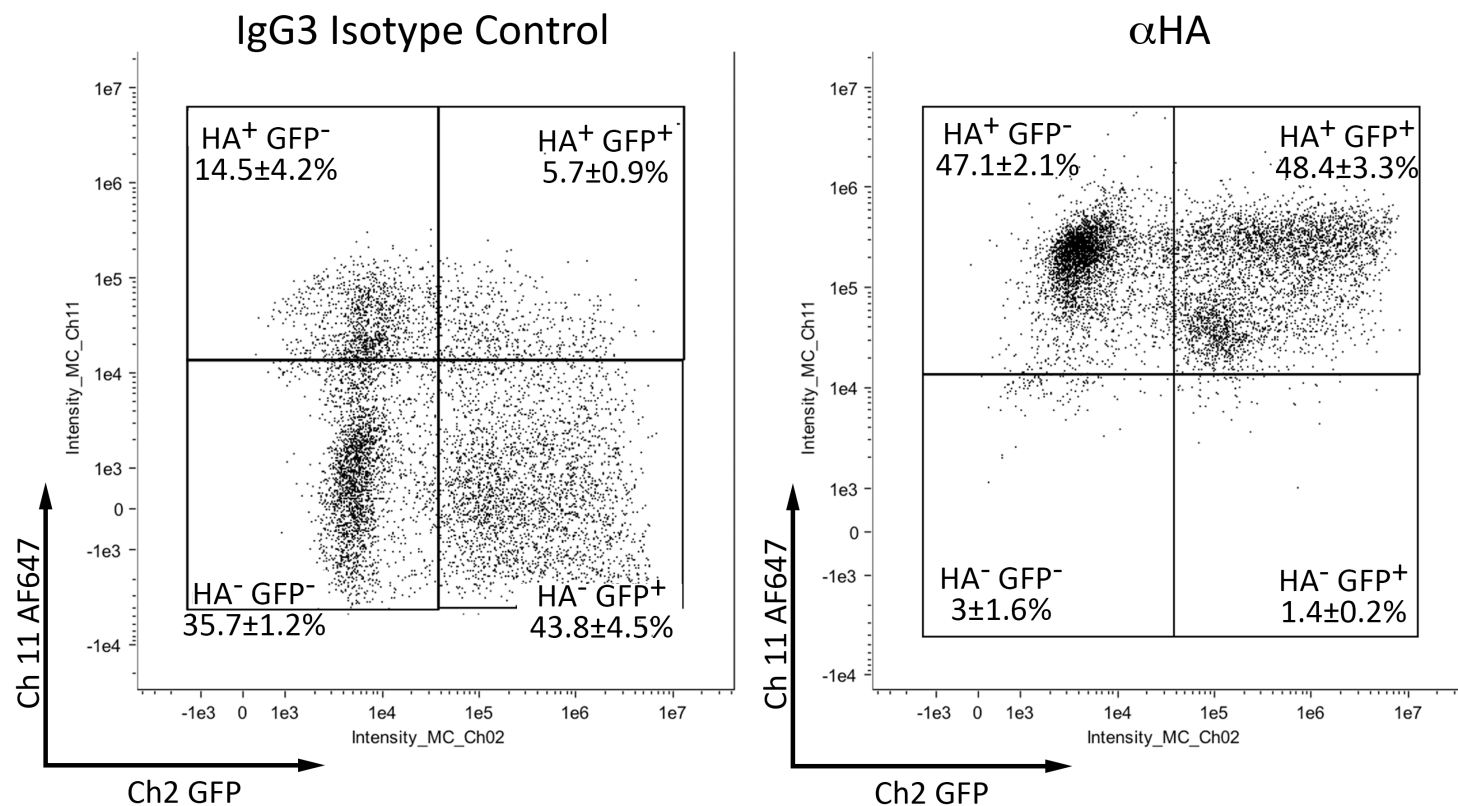


Figure A 5. Imaging flow cytometry-based co-expression analysis of Syk-GFP and IpLITR 1.1b/ITAM in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/ITAM ($\sim 3 \times 10^5$ cells) were transiently co-transfected with Syk-GFP for 24 hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g of IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g R α M AF647. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

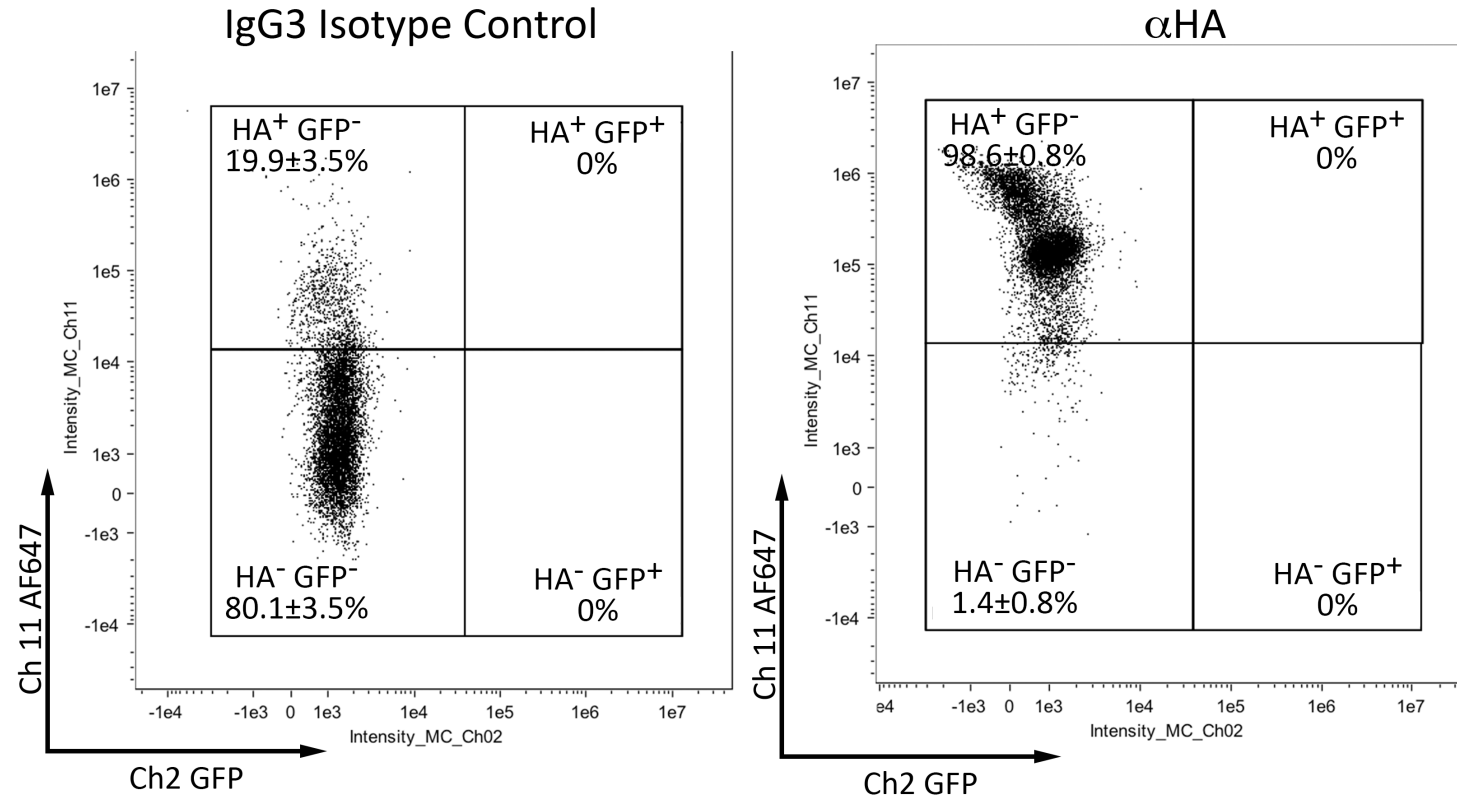


Figure A 6. Representative imaging flow-cytometry based dot plots of IpLITR 1.1b/WT stable expression in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/WT ($\sim 3 \times 10^5$ cells) surface stained for IpLITR co-expression with 0.1 μ g of IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g R α M AF647. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

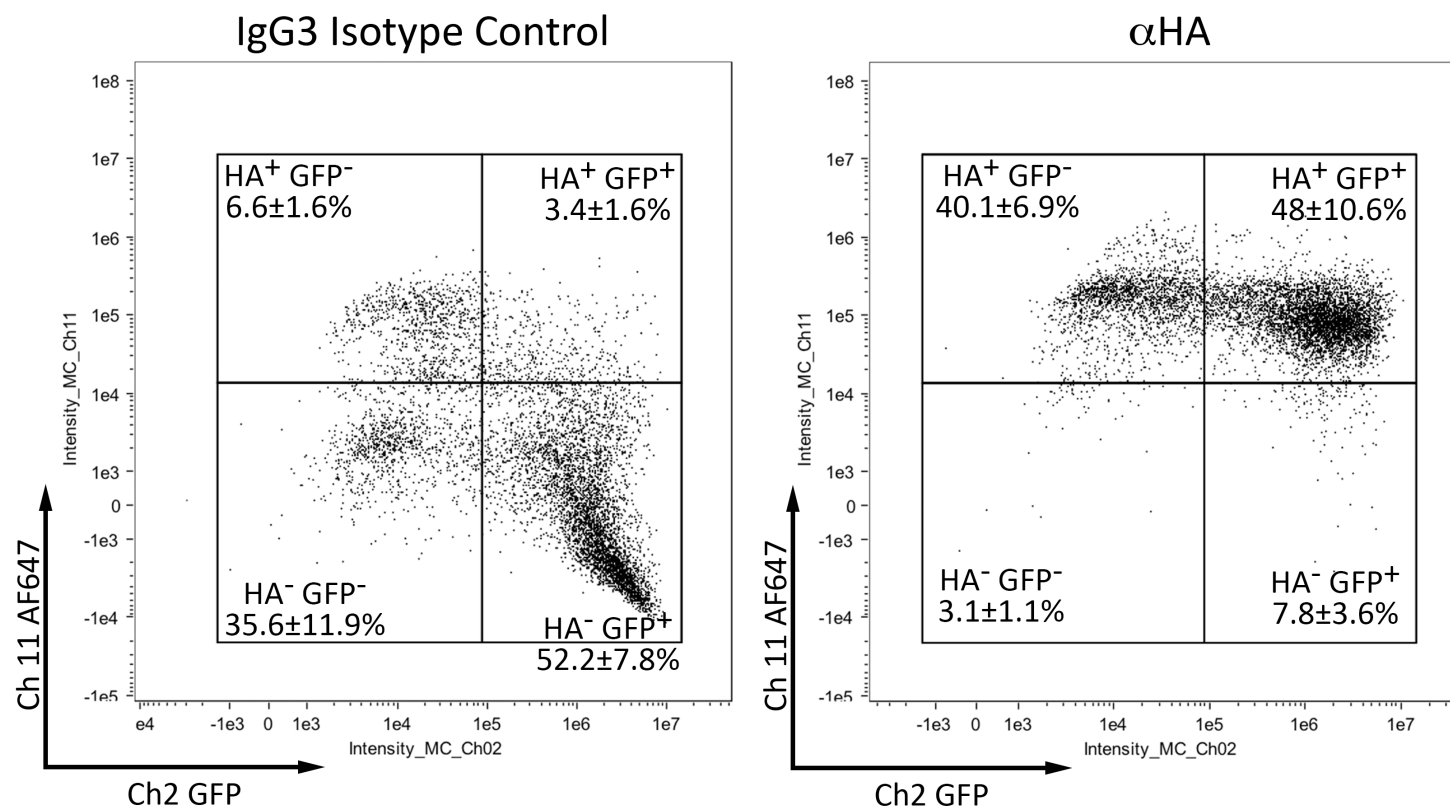


Figure A 7. Imaging flow cytometry-based co-expression analysis of Lifeact-GFP and IpLITR 1.1b/WT in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/WT ($\sim 3 \times 10^5$ cells) were transiently co-transfected with Lifeact-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g R α M AF647. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

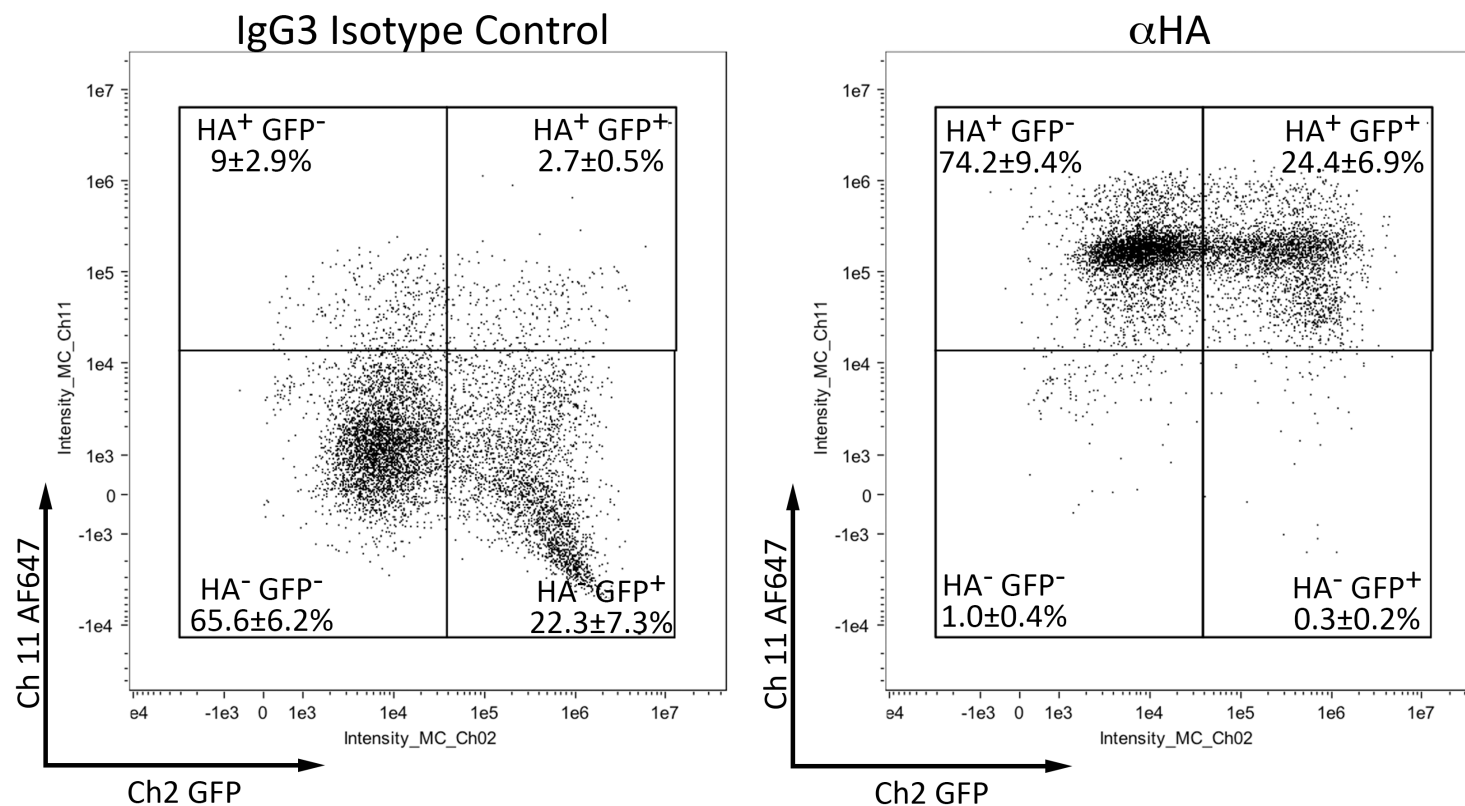


Figure A 8. Imaging flow cytometry-based co-expression analysis of PH-PLC δ_1 -GFP and IpLITR 1.1b/WT in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/WT ($\sim 3 \times 10^5$ cells) were transiently co-transfected with PH-PLC δ_1 -GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g of IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g R α M AF647. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.

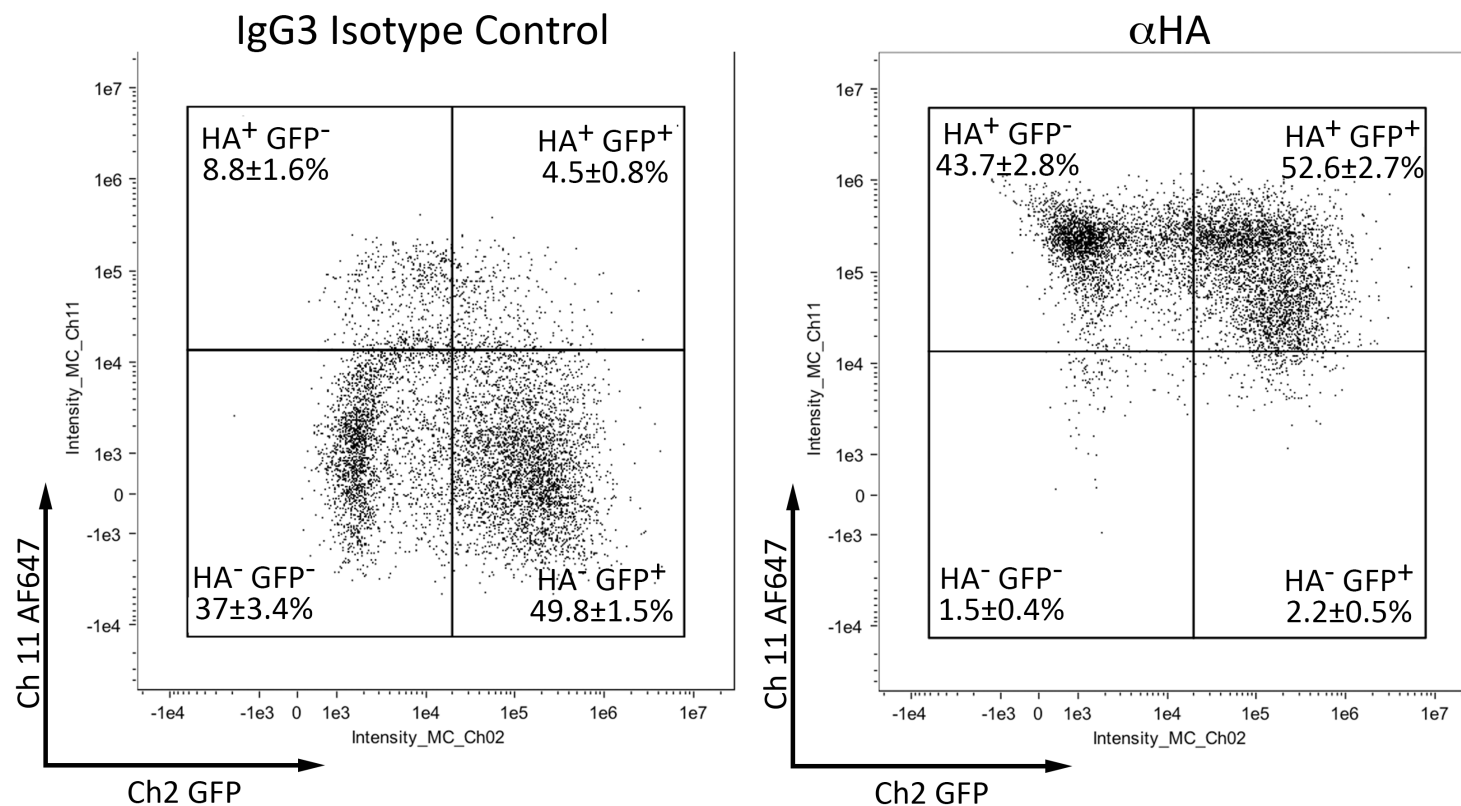


Figure A 9. Imaging flow cytometry-based co-expression analysis of 2xFYVE-GFP and IpLITR 1.1b/WT in AD293 cells. AD293 cells stably expressing IpLITR 1.1b/WT ($\sim 3 \times 10^5$ cells) were transiently co-transfected with 2xFYVE-GFP for twenty-four hours then harvested and surface stained for IpLITR co-expression with 0.1 μ g of IgG3 isotype control or α HA mAb followed by staining with 0.5 μ g RαM AF647. Axes represent intensity of fluorescence in Channel 11 (AF647) and Channel 2 (GFP). Mean percent $\pm$ SEM of cells gated from three independent experiments shown.