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The Role of Membrane Lipid Microdomains (Rafts) in FcγRIIA Effector Functions.

by

Joshua A. Vieth

Submitted to the Graduate Faculty in partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Science

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An Abstract of

The Role of Membrane Lipid Microdomains (Rafts) in FcγRIIA Effector Functions.

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Immunoglobulin G (IgG) dependent effector activity is an important factor in host defense and autoimmune diseases. Both macrophages and neutrophils express a family of Fcγ receptors (FcγR) which recognize both monomeric and complexed IgG. One member of this family, FcγRIIA, is a transmembrane glycoprotein which mediates binding and internalization of large IgG-coated targets (phagocytosis) and small IgG-containing complexes (IC) (endocytosis). Phagocytosis differs from endocytosis in the requirement for actin versus clathrin and the dependency on specific kinases and phosphatases. FcγRIIA is known to translocate into lipids rafts upon binding IgG-containing targets.

We hypothesize that lipid rafts participate to different extents in the initial binding and internalization of IgG containing complexes by leukocytes. Observations using FcγRIIA transfected CHO cells indicate that disruption of lipid rafts with 8mM methyl-β-cyclodextrin (MβCD) nearly abolishes binding of large IgG-coated 4.5μm polystyrene beads (26% binding versus untreated control). Furthermore, disruption of lipid rafts with 8mM MβCD subsequent to binding inhibited phagocytosis of these targets by 74%.

Interestingly, identical experiments with 1.5μm beads displayed a similar loss of binding to their larger counterparts following MβCD treatment (34% of control), though no significant loss in internalization. The binding and internalization of small heat-aggregated IgG displayed significant, though muted losses in comparison to the large targets (binding 82% of control, internalization 74% of control). Finally, FRAP experiments were conducted to examine the role of lipid rafts in FcγRIIA mobility in the membrane. Results indicate that while there is not a significant effect on normal movement through the membrane, upon ligation, there is a significant lipid raft-associated effect on both the rate of movement and fraction of mobile FcγRIIA in the membrane (33% loss in recovery rate, 2.5-fold increase in immobile fraction).

Our current observations suggest that differences between phagocytosis and endocytosis may arise as early as the initial stages of ligand recognition. This is the first observance of a size-related dependency on lipid rafts for FcγRIIA function, and may signal an important avenue of research in both the understanding of the mechanisms involved and the potential for specific modulation of the Ab-dependent immune and inflammatory mechanisms.

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List of Abbreviations

Ab.....Antibody

CHO .....Chinese Hamster Ovary Cells
CHO-IIA .....Chinese Hamster Ovary Cells transfected with human FcγRIIA
CHO-IIAGFP.....Chinese Hamster Ovary Cells transfected with FcγRIIA-GFP
CR2 .....Complement Receptor 2
CR3 .....Complement Receptor 3
CTX-B.....Cholera Toxin B
CytoD.....Cytochalasin D

DAG.....Diacylglycerol
DOPC.....DioleoylPC
DRM .....Detergent Resistant Membrane

EA .....IgG-opsonized erythrocytes

FcR.....Fc Receptor
FcαR .....Fc Receptor for IgA
FcεR .....Fc Receptor for IgE
FcγR .....Fc Receptor for IgG
FITC.....Fluorescein Isothiocyanate
FITC-haIgG.....FITC-conjugated haIgG
FRAP.....Fluorescence Recovery after Photobleaching

HaIgG.....Heat-Aggregated IgG Complexes
H_{131}.....High-responder FcγRIIA isoform

IC.....Immune complex
Ig .....Immunoglobulin
IgA .....Immunoglobulin A
IgE.....Immunoglobulin E
IgG .....Immunoglobulin G
IP_3.....Inositol triphosphate
ITAM .....Immunoreceptor Tyrosine-based Activation Motif
ITIM.....Immunoreceptor Tyrosine-based Inhibitory Motif

kDa.....Kilodalton

LAT.....Linker for activation of T cells
L_o.....Liquid ordered

MβCD .....Methyl-β-cyclodextin

OB .....IgG-Opsonized polystyrene beads
OB1.5 .....1.5μm-diameter OB
OB4.5 .....4.5μm-diameter OB

PE.....Phycoerythrin
PI3K.....Phosphoinositide 3-kinase
PLC .....Phospholipase C
PKC.....Protein kinase C

ROS.....Reactive Oxygen Species
R_{131}.....Low-responder FcγRIIA isoform

SIC .....Small Immune Complex
SLE .....Systemic Lupus Erythematosus
SM.....Sphingomyelin
SNARE .....Soluble NSF Attachment Protein

WASp.....Wiskott-Aldrich Syndrome protein

Chapter 1

Introduction and Literature Review

1.1 Introduction/Synopsis

The mammalian immune system has developed various means of defense against foreign pathogens. One of the most important of these, phagocytosis, involves the cellular internalization of particles and their sequestering into a phagosome. Recognition of a particle for internalization is the primary step in phagocytosis requiring receptor-ligand interactions. Phagocytic cells of the immune system express a variety of receptors specific for this purpose. For example, innate recognition by mannose receptors of non-self sugar moieties and scavenger receptor recognition of specific pathogen recognition motifs and low-density lipoprotein allow phagocytes to detect and degrade pathogens.

Another receptor found on phagocytes, the Fc receptor (FcR) is able to recognize the Fc portion of immunoglobulin (Ig) in the context of pathogens opsonized with antibody (Gavin, Barnes et al. 1998; Joshi, Butchar et al. 2006). The recognition of the appropriate Ig triggers signaling cascades through the clustering of FcRs on the cell surface. This accelerates the engulfment process and can lead to the generation of microbicidal oxygen and nitrogen species (Tamoto and Koyama 1980; Imamichi and Koyama 1990; Ganesan, Joshi et al. 2006; Trivedi, Zhang et al. 2006). FcRs are

designated by the class of Ig they bind. For example, FcγRs recognize and bind IgG, while FcαR binds IgA, and FcεR binds IgE (Ravetch and Kinet 1991; Hulett and Hogarth 1994).

Three classes of FcR are responsible for binding IgG: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), each of which can be further differentiated by structure (Gessner, Heiken et al. 1998). Both activating (FcγRI, FcγRIIA, FcγRIII) and inhibitory (FcγRIIB) FcγRs participate in phagocytosis of IgG-coated particles, allowing for strict self-regulation among the FcR classes through cytoplasmic activation and inhibition motifs (Cambier 1995; Van den Herik-Oudijk, Ter Bekke et al. 1995). FcγRIIA is a 40 kDa transmembrane receptor found on neutrophils, monocytes, macrophages, dendritic cells and platelets (Stuart, Trounstine et al. 1987; Hibbs, Bonadonna et al. 1988; Stengelin, Stamenkovic et al. 1988). FcγRIIA can initiate phagocytosis of large IgG-coated targets or endocytosis of small IgG containing immune complexes. Most signaling is initiated by the immunoreceptor tyrosine based activation motif (ITAM) sequence located in the cytoplasmic domain. The ITAM of FcγRIIA is slightly different than traditional ITAM sequences due to the extra intervening residues between the dual YxxL motifs (Y-M-T-L- 12aa -Y-L-T-L) and is, therefore, referred to as an ITAM-like region (Van den Herik-Oudijk, Capel et al. 1995).

It was recently shown that specific domains of the receptor are differentially involved in phagocytosis and endocytosis (Booth, Kim et al. 2002; Tse, Furuya et al. 2003; Huang, Barreda et al. 2006). The residues in the ITAM-like sequence are specific for various FcγRIIA-mediated processes. For instance, phosphorylation of the tyrosine residues is required for phagocytic signaling and secretion (Indik, Mitchell et al. 1993;

Mitchell, Huang et al. 1994; Indik, Park et al. 1995). However, the first leucine residue has been shown to be necessary for the endocytic mechanisms of FcγRIIA, while the L-T-L motif is required for phagolysosome fusion (Indik, Mitchell et al. 1993; Mitchell, Huang et al. 1994; Indik, Park et al. 1995; Worth, Mayo-Bond et al. 2001; Booth, Kim et al. 2002; Worth, Kim et al. 2003).

Activation of FcγRIIA by IgG-coated pathogens results in lipid raft localization and cross-linking of receptors. FcγRIIA then is phosphorylated on tyrosine residues in the ITAM-like sequence by members of the Src family of tyrosine kinases (Flaswinkel, Barner et al. 1995; Hoessli, Ilangumaran et al. 2000; Kono, Suzuki et al. 2002; Cuschieri 2004). Phosphorylation of the ITAM like region creates two SH2 (Src Homology 2) binding sites, together being able to bind spleen tyrosine kinase (Syk) (Ghazizadeh, Bolen et al. 1995). This signaling leads to reorganization of actin filaments, driving the formation of a phagosome and the initiation of signaling events which result in the release of pro-inflammatory mediators such as cytokines and reactive oxygen species and the destruction of pathogens (Hunter, Kamoun et al. 1994; Greenberg 1999; Joshi, Butchar et al. 2006).

In addition to phagocytosis, FcγRIIA can also mediate the uptake of small immune complexes composed of IgG through endocytic clathrin-mediated mechanisms (Ukkonen, Lewis et al. 1986; Engelhardt, Gorczytza et al. 1991). Mutational analysis has revealed that the YMTL sequence of FcγRIIA is important for internalization of IgG complexes. Furthermore, this process has recently been shown to require ubiquitylation, which is not required for phagocytosis of large targets (Booth, Kim et al. 2002).

Residues outside of the ITAM have also been shown to be necessary for FcγRIIA activity. Recent studies have elucidated functionality in FcR translocation to lipid rafts. Recruitment of FcγRIIA to lipid rafts has been shown to be dependent on the palmitoylation of a juxtamembrane cysteine (cysteine 208) or on transmembrane residues (Barnes, Powell et al. 2006; Garcia-Garcia, Brown et al. 2007). Studies with FcγRIIB2 have suggested a role for lipid rafts in the temporal regulation of functions associated with endocytic mechanisms (Mousavi, Sporstol et al. 2007). While the actual translocation to lipid rafts is well documented, the functional significance on binding leading to phagocytosis or endocytosis is still a matter of much speculation.

1.2 Phagocyte Effector Functions

The observation that transparent sea star larvae contained cells capable of ingesting foreign particles was the first description of phagocytosis as a potential defense mechanism [reviewed in (Hirsch 1959)]. Phagocytes are now known to play a central role in both the regulation of an immune response against pathogen, as well as in the general maintenance of body tissue. Phagocytosis has been shown to be necessary for the removal of apoptotic cells, defense against pathogen invasion, and as a source of nutrition of unicellular organisms (Vogel, Thilo et al. 1980; Gordon, Crocker et al. 1986; Hopkinson-Woolley, Hughes et al. 1994; Hoffmann, Kafatos et al. 1999). While most cells display forms of internalization mechanisms, the professional phagocytes in mammalian systems are monocytes, macrophages, neutrophils, dendritic cells and mast cells (Ernst and Stendahl 2006).

A wide range of cell-surface receptors are present on phagocytes to facilitate the internalization of pathogens. Mannose receptors were among the first phagocytic receptors described on macrophages, and are capable of recognizing mannosyl- and fucosyl-containing neoglycoproteins (Stahl, Rodman et al. 1978; Ezekowitz, Sastry et al. 1990; Taylor, Conary et al. 1990). Overexpression of the mannose receptor in Cos7 cells displayed their ability to recognize yeast, certain bacteria and fungi including *Pneumocystis carinii* (Ezekowitz, Sastry et al. 1990; Ezekowitz, Williams et al. 1991). Likewise, scavenger receptors are multi-ligand receptors capable of binding a wide range of pathogens and modified self-ligands (Janeway 1989; Gordon 2002).

Other phagocytic receptors require the opsonization of an object for recognition. Opsonization coats a target allowing for generic receptors to recognize and mediate uptake of diverse ligands (Ezekowitz, Sim et al. 1984). Activation of the complement cascade through classical or alternative pathways produces surface-bound C3bi, which can be phagocytosed by receptors such as complement receptor 3 (CR3) (Ross, Cain et al. 1985; Ross and Medof 1985). Mannose-binding lectin (MBL) has also proven to be an important opsonin for the recognition of *Staphylococcus aureus* and apoptotic cells (Shi, Takahashi et al. 2004; Stuart, Takahashi et al. 2005). MBL-ligand complexes can activate the complement cascade through association with MASP-2, allowing for creation of the complement-associated opsonins C3b and iC3b, which are recognized by complement receptors, leading to phagocytosis of the target (Thiel, Vorup-Jensen et al. 1997). Another important opsonin is immunoglobulin (Ig). Ig-opsonization of a target allows for recognition by FcR which will be discussed in detail in the next section of this chapter.

Following recognition and binding by a phagocyte-bound receptor, internalization is initiated with the specific mode of internalization varying according to the size and nature of the target itself (Conner and Schmid 2003). Most of the current knowledge of the machinery necessary for the internalization of a target by a phagocyte derives from studies conducted using mammalian Fc and complement receptors which pinpoint necessities for Rho GTPases, nucleotide exchange factors, Syk, actin, and myosin (Caron and Hall 1998; Olazabal, Caron et al. 2002; Colucci-Guyon, Niedergang et al. 2005; Hall, Gakidis et al. 2006; Shi, Tohyama et al. 2006). Much less is known about the internalization mechanisms of the other phagocytic receptors. Recent studies involving RNAi screening strategies have implicated much of the machinery utilized in Fc and complement receptors as responsible for internalization in *Drosophila melanogaster*, which lacks both receptors, indicating that the machinery utilized in phagocytosis may be universal (Ramet, Manfruelli et al. 2002; Agaisse, Burrack et al. 2005; Stuart, Deng et al. 2005).

Along with internalization of a target, phagocytosis-associated response mechanisms include the production of reactive oxygen species (ROS), which assist in bacterial killing and the triggering of inflammatory responses (Babior 1984). This is accomplished through receptor-mediated release of diacylglycerol (DAG) and inositol triphosphate (IP_3), which through calcium signaling, activate protein kinase C (PKC) and trigger the NADPH oxidase, resulting in the release of ROS, including H_2O_2 (Joseph, Thomas et al. 1984; Qualliotine-Mann, Agwu et al. 1993; Griendling, Sorescu et al. 2000; Kanai, Liu et al. 2001). In addition to direct bactericidal properties, this process has been observed to be important for signaling in phagocytes. One of the first signaling pathways

observed to be activated by H_2O_2 was NF-κB (Kaul and Forman 1996). In fact, ROS production is able to induce the translocation of NF-κB to the nucleus regardless of the activation state of the phagocyte itself (Schreck, Rieber et al. 1991). In addition, H_2O_2 has been observed to activate the ERK signaling pathway, and increased production of ROS has been shown to correlate with increased ERK activation (Abe, Kartha et al. 1998; Irani and Goldschmidt-Clermont 1998). Furthermore, JNK signaling has been shown to be activated by ROS production, possibly through direct association with its inhibitor, glutathione S-transferase Pi (Adler, Yin et al. 1999).

1.3 Fc Receptor Biology

Fc receptors (FcR) are able to bridge the gap between innate and adaptive immunity by recognizing particles opsonized by antibodies (Ab) (Gavin, Barnes et al. 1998; Joshi, Butchar et al. 2006). The FcR, which recognize the Fc domain of Ig are expressed on all cells of the immune system. They are capable of existing as membrane receptors, or as soluble molecules which are the product of alternative splicing or proteolysis (Daeron 1997). High affinity FcR bind monomeric antibodies while low-affinity FcR bind aggregated Ig or Ig-containing complexes (Ravetch and Kinet 1991)

Interaction of FcR with Ig has been implicated in various important immune processes including phagocytosis, Ab-dependent cytotoxicity, degranulation, cytokine production, antigen presentation and regulation of Ab production (Daeron 1997). It has been suggested that FcR may act as link between adaptive immune responses through binding of Ig, and innate immune responses through their ability to associate with serum

amyloid P and C-reactive protein bound to pathogens (Mortensen and Duszkiewicz 1977; Stein, Edberg et al. 2000; Mold, Gresham et al. 2001; Chi, Tridandapani et al. 2002). This class of receptors can be divided according to whether they contain an activating domain and promote phagocytosis and Ab-dependent cytotoxicity, or an inhibitory domain which regulates the extent of the activating response (Ravetch 1997; Unkeless and Jin 1997; Ravetch and Clynes 1998). Regulation of cellular responses to FcR ligation is controlled by the ratio of these opposing receptors on a given effector cell (Ravetch and Kinet 1991).

Under normal conditions, the inhibitory receptors dominate a response though higher expression or higher affinity for ligand (Kalergis and Ravetch 2002; Nimmerjahn and Ravetch 2006). As a regulatory mechanism, the termination of an immune response through FcR is accomplished by signaling through binding and coaggregation of inhibitory receptors, and not through the actual loss of activating signals (Billadeau and Leibson 2002). Therefore, co-expression of both activating and inhibitory FcR on cells of hematopoietic lineage determines the threshold for cellular activation. Evidence exists that soluble FcR released into the blood is capable of modulating immune responses, such as in the case of soluble FcγRIIB, which is capable of suppressing an antibody response by preventing the binding of target to cell-bound FcR (Takai 2005).

With only a few exceptions, signaling through FcR is achieved through association with FcRγ-chain subunits, which contain a cytoplasmic ITAM signaling domain (Cambier 1995; Carlsson, Candeias et al. 1995; Paolini, Renard et al. 1995). Experiments involving a knock-out of the γ-chain in mice established it as an imported part of the signaling required for macrophage responses to Ig, such as phagocytosis and

Ab-mediated cellular toxicity, as well as the initiation of an Arthrus reaction, despite the mice exhibiting a normal complement system (Sylvestre and Ravetch 1994; Takai, Li et al. 1994). These findings established an important role for FcR in immune and inflammatory signaling, which was further illustrated by data from experiments involving the inhibition of Syk, an important part of the FcR signaling pathway, and the subsequent restoration of platelet numbers in individuals suffering from immune-associated thrombocytopenia (Podolanczuk, Lazarus et al. 2009).

Fc receptors are named according to the type of Ig that they bind. For instance, FcγR recognizes and binds IgG, FcαR binds IgA and FcεR binds IgE (Ravetch and Kinet 1991; Hulett and Hogarth 1994). The ability of an FcR to recognize a particular Ig directly coincides with the cell type on which it is predominantly expressed. FcγR are principally found on neutrophils, macrophages and monocytes and can initiate phagocytosis of IgG coated pathogens, while FcεR are found on eosinophils, as well as basophils and mast cells, where their activation triggers the release of histamine from intracellular granules (Dombrowicz, Flamand et al. 1993; Takai, Li et al. 1994). The recognition of the appropriate Ig triggers signaling cascades through the clustering of FcR on the cell surface. This accelerates the engulfment process and generation of microbicidal oxygen species (Tamoto and Koyama 1980; Imamichi and Koyama 1990; Ganesan, Joshi et al. 2006; Trivedi, Zhang et al. 2006).

Immunoglobulin A is the most prominent antibody at mucosal surfaces, and the second most prevalent Ab in human serum (Macpherson, Gatto et al. 2000). This makes the FcαR an important factor in the immune response. The sole FcαR, FcαRI (CD89) is a 55-75 kDa transmembrane receptor found on monocytes and neutrophils and a 70-100

kDa receptor on eosinophils, primarily due to heavy glycosylation (Morton, van Egmond et al. 1996). It is a low-affinity receptor (~10^{-6} M) and, therefore, binds more readily to complexed IgA than monomeric IgA (Wines, Hulett et al. 1999; Bruhns, Iannascoli et al. 2009). Expression of FcαRI on the plasma membrane is relatively high, estimated to be 57,000 per monocyte and 66,000 per neutrophil (Monteiro, Cooper et al. 1992).

Interestingly, FcαRI is distantly related to other FcR receptors, as it is the only member of the family encoded on human chromosome 19, while genes for FcγR and FcεR are located on chromosome 1 (Kremer, Kalatzis et al. 1992; Wines, Hulett et al. 1999). Unlike other FcR, binding of FcαRI to its IgA ligand occurs in the EC1 domain, not the EC2 domain (Wines, Hulett et al. 1999; Morton and Brandtzaeg 2001). Upon cross-linking of IgA bound to FcαRI, the receptor moves into sphingolipid- and cholesterol-rich microdomains of the plasma membrane or "Lipid Rafts," where it is able to associate with two FcRγ-chains (Pfefferkorn and Yeaman 1994; Lang, Shen et al. 1999; Lang, Chen et al. 2002). Phosphorylation of tyrosine residues of the γ-chain by the Src kinase Lyn initiates signaling activity and the recruitment of Syk to the ITAM and through various adapter molecules recruits Sos, eventually activating the Ras, Raf1-MEK-MAP and PI-3 Kinase pathways, leading to FcαRI effector functions (Park, Izadi et al. 1999).

The second type of FcR, FcεR, binds to IgE. Two isoforms exist, (1) FcεRI, which has a high affinity for IgE monomers, and (2) FcεRII which binds IgE complexes with low affinity (Plaut, Pierce et al. 1989; Yodoi, Hosoda et al. 1989). Immunoglobulin E antibodies are associated with immune protection from parasites as well as being

integral in allergic and asthmatic reactions (Kinet 1999; Turner and Kinet 1999). While both receptors recognize and bind IgE, they vary greatly in both structure and function.

FcεRI, the high-affinity FcεR, is found on mast cells and basophils, where binding of antigen to receptor-bound IgE initiates the release of inflammatory mediators and cytokines (Plaut, Pierce et al. 1989). Compared to other FcR, the receptor has the highest affinity for its ligand, IgE, with a binding constant of 10^{-9} to 10^{-10} M (Metzger 1992; Daeron 1997; Kinet 1999). Interestingly, the serum concentration of IgE is lowest of all Ab, usually only present at 50-300μg/ml. This is due to most of the Ab being present in the cell-bound state, ligated to the high-affinity receptor. In fact, levels of IgE and FcεRI expression correlate, with a decrease in overall IgE initiating down-regulation of receptor expression (MacGlashan, Bochner et al. 1997; Saini, MacGlashan et al. 1999; Chang 2000). Binding of antigen to receptor-associated IgE initiates cross-linking of the receptor leads to immediate phosphorylation of the receptor and signaling through the ITAM domains of the the FcRγ-chain homodimer, thus initiating the rapid release of histamine and the production of leukotrienes and cytokines which contribute to the inflammatory cascade (Turner and Kinet 1999).

Alternatively, FcεRII (CD23) binds with low affinity and can be found on B cells, monocytes and eosinophils (Capron, Grangette et al. 1989; Delespesse, Hofstetter et al. 1989; Yodoi, Hosoda et al. 1989). FcεRII is the only FcR which does not belong to the super-Ig family, and two forms of FcεRII exist, denominated FcεRIIa and FcεRIIb, and differ by five amino acids in the cytoplasmic domain as a result of alternative splicing (Yokota, Kikutani et al. 1988). FcεRIIa is constitutively expressed on activated B cells, while FcεRIIb expression is induced on mast cells, basophils, eosinophils and

Langerhans cells (Yokota, Kikutani et al. 1988). Additionally, FcεRIIb is found on T cells, monocytes, macrophages, and platelets (Delespesse, Suter et al. 1991; Abdelilah, Bouchaib et al. 1998). While both isoforms of FcεRII bind with low affinity (10^{-7} M) it has been shown that FcεRIIa is associated with the internalization of IgE-coated particles while FcεRIIb is associated with the internalization of IgE complexes (Yokota, Yukawa et al. 1992). Because FcεRII does not associate with a FcRγ-chain or contain any intrinsic signaling domains, recognition (through the C-term integrin recognition site) of complement receptor 2 (CR2, CD21) allows CR2 to act as a counter-receptor for FcεRII, enabling cytoplasmic signaling following ligation of FcεRII (Aubry, Pochon et al. 1992; Pochon, Graber et al. 1992). Another unique property of FcεRII is a susceptibility to cleavage at its α-helical stalk by membrane-associated metalloproteinases, with the subsequent soluble FcεRII exhibiting mitogenic, cytokine and IgE regulatory properties (Delespesse, Sarfati et al. 1992; Mossalayi, Arock et al. 1992; Bonnefoy, Lecoanet-Henchoz et al. 1997; Meng, McFall et al. 2007).

IgG is the most abundant immunoglobulin in the human body, existing at concentrations of up to 10mg/ml in normal sera, making FcγR an important link between innate and adaptive immunity (Fahey and Lawrence 1963; Stiehm and Fudenberg 1966; van der Giessen, Rossouw et al. 1975). FcγR can be divided into three classes: (1) FcγRI (CD64), (2) FcγRII (CD32) and (3) FcγRIII (CD16), each of which can then be further broken down by structure (Gessner, Heiken et al. 1998). FcγR are encoded by at least eight genes found on chromosome 1, with individual isoforms generated through alternative splicing (Ravetch and Kinet 1991; Hogarth, Hulett et al. 1992). Both activating and inhibitory FcR are used to drive phagocytic mechanisms, allowing for

strict self-regulation among the FcR classes through cytoplasmic activation and inhibitory motifs (Cambier 1995; Van den Herik-Oudijk, Ter Bekke et al. 1995). As with FcαR and FcεR1, phosphorylation of cytoplasmic tyrosines occurs upon FcγR activation and is required for internalization of ligand (Huang, Indik et al. 1992; Scholl, Ahern et al. 1992; Kiener, Rankin et al. 1993).

FcγRI is the only FcγR that is confined to a single cell lineage, as it is only found on monocytes/macrophages (Ravetch and Kinet 1991; Hogarth, Hulett et al. 1992; Schreiber, Rossman et al. 1992; van de Winkel and Capel 1993; McKenzie and Schreiber 1994). FcγRI binds monomeric IgG with high affinity (~10^{-7} M), and binds IgG subclasses with an order of: IgG3>IgG1>IgG4, while not binding IgG2 (van de Winkel and Capel 1993; Bruhns, Iannascoli et al. 2009). As with other FcR, phagocytic signaling through FcγRI occurs by association with the FcRγ-chain subunit, though calcium signaling through its cytoplasmic domain can still occur in the absence of the γ-chain (Indik, Chien et al. 1991; Indik, Chien et al. 1992; Indik, Hunter et al. 1994).

FcγRIII is a 50-80 kDa glycoprotein low-affinity receptor encoded by two genes. One variant is FcγRIIIA, a transmembrane receptor which contains two extracellular Ig-like domains and binds with a low binding affinity (~10^{-6} M) which binds IgG subclasses with an order of IgG3>IgG2>IgG4>IgG1. FcγRIIIB, is linked to the outer region of the plasma membrane through a glycosyl phosphatidylinositol (GPI) anchor, also has low binding affinity (~10^{-6} M) and binds IgG subclasses in the order IgG1>IgG3, with no IgG2 or IgG4 binding (Huizinga, van der Schoot et al. 1988; Selvaraj, Rosse et al. 1988; Bruhns, Iannascoli et al. 2009). FcγRIIIA is found on NK cells and macrophages, while FcγRIIIB is primarily found on neutrophils (Salmon, Millard et al. 1995; Gessner, Heiken

et al. 1998). The transmembrane α-chain of FcγRIIIA requires association with the FcRγ-chain for both receptor expression and phagocytic signaling (Wirthmueller, Kurosaki et al. 1992; Park, Isaacs et al. 1993; Park, Murray et al. 1993). FcγRIIIB does not associcate with the FcRγ-chain subunit, nor does it contain intrinsic signaling motifs, and has not been shown to trigger signaling for phagocytosis (Fridman, Bonnerot et al. 1992; Wirthmueller, Kurosaki et al. 1992). Evidence suggests that FcγRIIIB is able to signal through extracellular association with other receptors such as FcγRII and complement receptors (Brown, Bohnsack et al. 1988; Graham, Gresham et al. 1989; Petty and Todd 1993).

Isoforms of FcγRII are generated from the expression of three genes, (1) FcγRIIA, (2) FcγRIIB and (3) FcγRIIC, with alternative splicing giving rise to two individual variants of FcγRIIB (Brooks, Qiu et al. 1989; Ravetch and Kinet 1991; Hogarth, Hulett et al. 1992; Schreiber, Rossman et al. 1992; van de Winkel and Capel 1993; McKenzie and Schreiber 1994). All FcγRII receptors have low binding affinities, though FcγRIIA ($\sim 10^{-6}$ M, binding order of IgG3>IgG1>IgG2>IgG4) has higher overall binding affinity for IgG than FcγRIIB and FcγRIIC (both: $\sim 10^{-5}$ M, binding order of IgG1>IgG4>IgG3>IgG2) (Bruhns, Iannascoli et al. 2009). It is interesting to note that binding affinity for IgG2 by FcγRIIA is two- to four-fold higher than the other two variants, and that collectively, FcγRII along with FcγRIIIA, are the only FcγR capable of binding IgG2 (Warmerdam, van de Winkel et al. 1991; Parren, Warmerdam et al. 1992; Bruhns, Iannascoli et al. 2009). FcγRII is unique in that it does not require the association of the FcRγ-chain for signaling, as it contains a cytoplasmic ITAM motif (FcγRIIA) or immunoreceptor

tyrosine inhibitory motif (ITIM) (FcγRIIB) (Huang, Indik et al. 1992; Indik, Pan et al. 1994; Mitchell, Huang et al. 1994). FcγRIIC is found on NK cells and is the result of an unequal crossover event, exhibiting the extracellular portion of FcγRIIB and the cytoplasmic portion of FcγRIIA (Warmerdam, Nabben et al. 1993).

1.4 FcγRIIA Internalization Mechanisms

FcγRIIA is a 40kDa transmembrane receptor found on neutrophils, monocytes, macrophages, dendritic cells and platelets (Rosales 2005). It is a low affinity receptor (~10^{-6} M), and binds IgG subtypes in the order IgG3>IgG1>IgG2>IgG4; however, it maintains the highest affinity for IgG2 out of all of the FcγR (Bruhns, Iannascoli et al. 2009). Two alleles of the gene for FcγRIIA encode two variants which differ at residue 131 and are denominated high-responder (H_{131}) and low-responder (R_{131}) (Warmerdam, van de Winkel et al. 1990). The H_{131} allotype exhibits a higher affinity for IgG2 (~$4.5x10^{-5}$ M) than R_{131} (~$8x10^{-4}$ M) (Bruhns, Iannascoli et al. 2009). The H_{131} and R_{131} alleles are differentially expressed in Caucasians, Japanese and Chinese individuals, with Japanese and Chinese individuals having a much higher prevalence of homozygosity for the high-responding H_{131} (61 and 50%, respectively) than Caucasians (23%) (Osborne, Chacko et al. 1994). Evidence suggests that homozygosity for the R_{131} variant is associated with increased susceptibility to bacterial infections as well as the development of autoimmune diseases such as systemic lupus erythamatosus (SLE) (Dijstelbloem, Bijl et al. 2000; Tan 2000; Yee, Phan et al. 2000; Moens, Van Hoeyveld et al. 2006). Heterozygous individuals exhibit similar levels of resistance to bacterial infections and

autoimmune diseases as is observed in those homozygous for H_{131}, possibly due to observations that H_{131}-expressing FcγRIIA outcompetes R_{131}-expressing FcγRIIA when they simultaneously compete for the same ligand (Shashidharamurthy, Zhang et al. 2009).

Activation of FcγRIIA results in the reorganization of actin filaments (Greenberg 1999). This drives the formation of a phagosome and the initiation of signaling events which result in the killing of pathogens and the release of pro-inflammatory mediators such as cytokines and reactive oxygen species (Hunter, Kamoun et al. 1994; Joshi, Butchar et al. 2006). Phagocytosis of large IgG-opsonized targets is dependent on the assembly of F-actin structures at the site of particle uptake. The process is similar to events at the leading edge of a migrating cell. Both processes are actin-mediated and involve adhesion, receptor clustering, tyrosine phosphorylation and force generation (Aderem and Underhill 1999).

FcγR are known to associate with complement receptors during phagocytosis and oxidant production, and recent evidence suggests a cooperative role between FcγRIIA, FcγRIIIB, and CR3 (Ehlenberger and Nussenzweig 1977; Sehgal, Zhang et al. 1993; Zhou and Brown 1994; Petty and Todd 1996; Worth, Mayo-Bond et al. 1996). It is fairly well established that GPI-linked FcγRIIIB co-caps with CR3, that CR3 is able to restrain the lateral diffusion of FcγRIIIB through the membrane, and that CR3 is necessary for FcγRIIIB-mediated phagocytic signaling (Graham, Gresham et al. 1989; Zhou, Poo et al. 1992; Poo, Krauss et al. 1995). Similar experiments with FcγRIIA indicated that not only are CR3 and FcγRII found in close proximity to one another in the membrane, but also that phagocytic signaling from FcγRIIA lacking a cytoplasmic domain can be restored

through association with CR3 (Annenkov, Ortlepp et al. 1996; Worth, Mayo-Bond et al. 1996). This CR3-rescued signaling did not entirely replace the lost FcγRIIA signal, as phagosomes were unable to fuse with lysosomes (Worth, Mayo-Bond et al. 2001).

Signaling by FcγRIIA occurs through a cytoplasmic ITAM-like domain. Unlike traditional ITAM sequences, the ITAM-like motif of FcγRIIA contains two Src homology domains separated by 12 amino acids instead of the typical 7 observed in the FcRγ-chain (Worth 2004). The residues in the sequence (Y-M-T-L- 12aa - Y-L-T-L) are specific for FcγRIIA-mediated processes. Signaling is initiated following the cross-linking of receptors and phosphorylation of the tyrosine residues in the ITAM-like sequence by Src Family tyrosines (Flaswinkel, Barner et al. 1995; Cooney, Phee et al. 2001). This creates two SH2 (Src Homology 2) binding sites capable of binding Syk tyrosine kinase (Agarwal, Salem et al. 1993; Ghazizadeh, Bolen et al. 1995). Syk contains two SH2 binding sites and is recruited to phosphorylated ITAM tyrosines (Greenberg, Chang et al. 1994; Jouvin, Adamczewski et al. 1994; Ghazizadeh, Bolen et al. 1995; Indik, Park et al. 1995; Greenberg, Chang et al. 1996). The increased spacing between the tyrosine motifs of the FcγRIIA ITAM does not affect Syk binding, in fact, a gap of 3 amino acids to 12 amino acids has been shown to be capable of being bound by Syk (Indik, Park et al. 1995). Mutation of either tyrosine residue results in 80% disruption of phagocytosis, while mutation of both tyrosine residues disrupts phagocytosis completely (Indik, Mitchell et al. 1993; Park, Murray et al. 1993; Daeron, Malbec et al. 1994; Darby, Geahlen et al. 1994; Mitchell, Huang et al. 1994; Van den Herik-Oudijk, Capel et al. 1995; Strzelecka, Kwiatkowska et al. 1997). It has been shown that Syk signaling is necessary for FcR phagocytosis and FcγR-triggered ROS

production, as both of these are dramatically reduced in its absence, and phagocytosis can be increased following the co-expression of FcγR and Syk in COS-1 cells (Indik, Park et al. 1995; Matsuda, Park et al. 1996; Kiefer, Brumell et al. 1998). Other portions of the ITAM have been shown to be necessary for FcγRIIA functions, as the first leucine residue has been shown to be necessary for the endocytic mechanisms of FcγRIIA, while the L-T-L motif is required for the signaling of phagolysosome fusion (Worth, Mayo-Bond et al. 2001; Worth, Kim et al. 2003).

Through an unknown adaptor molecule, Syk is able to bind the GTPases Rac and CDC42, both of which have been shown to be necessary for actin rearrangement and the formation of a phagocytic cup, the first step in target internalization (Hackam, Rotstein et al. 1997; Caron and Hall 1998; Chimini and Chavrier 2000). CDC42 accumulates at the phagocytic cup and associates with Wiskott-Aldrich Syndrome protein (WASp), which is capable of binding to both CDC42 and Rac (Castellano, Montcourrier et al. 1999). Following this binding, WASp is able to interact with the Arp2/3 complex which mediates the actin nucleation, formation of pseudopods and the formation of the phagocytic cup itself (Welch, Iwamatsu et al. 1997; Machesky and Way 1998).

Lipids and lipid kinases also play an important role in signaling associated with the FcγRIIA-mediated phagocytosis. It has been shown that phosphatidylinositol 3-kinase (PI3K) accumulates at the phagocytic cup, and activated PI3K can be found in the membrane within seconds of FcγR cross-linking (Vossebeld, Homburg et al. 1997; Marshall, Booth et al. 2001; Araki, Hatae et al. 2003). PI3K is thought be involved in the closing-off of the plasma membrane and creation of the phagosome (Cox, Tseng et al. 1999). Similarly, the PI3K inhibitor wortmannin has been shown to allow pseudopod

formation but restrict the closure of the vesicle, thus inhibiting phagocytosis (Araki, Johnson et al. 1996). Like PI3K, phospholipase C (PLC) also accumulates at the phagocytic cup (Azzoni, Kamoun et al. 1992; Liao, Shin et al. 1992; Shen, Lin et al. 1994). PLC is able to hydrolyze two important phagocytic signaling molecules, DAG and IP_3 and appears to be necessary for phagocytosis, as inhibition of PLC disrupts phagocytosis (Botelho, Teruel et al. 2000).

The creation of DAG allows for it to transiently activate protein kinase C (PKC) (Nishizuka 1984). Along with PI3K, PKC interacts with myosin to initiate the retraction through ERK activation of myosin light-chain kinase (Mansfield, Shayman et al. 2000; Kamm and Stull 2001). The phosphorylated myosin is able to then associate with actin, stimulating phagosome entry into the cell (Ryder, Niederman et al. 1982; Chavrier 2002; Diakonova, Bokoch et al. 2002; Olazabal, Caron et al. 2002; Araki, Hatae et al. 2003). Following retraction into the cell, the phagosomal vesicle is formed by the "pinching off" of the opening at the plasma membrane by the cytoskeleton molecule dynamin, which is stimulated by PI3K (Araki, Johnson et al. 1996; Gold, Underhill et al. 1999).

The IP_3 produced by PLC is able to ligate the IP_3 receptor on the endoplasmic reticulum allowing for the release of calcium (Mignery, Sudhof et al. 1989; De Camilli, Takei et al. 1990; Mignery and Sudhof 1990). It has been shown that calcium is released from phagosomes following FcγRIIA-mediated phagocytosis and that cytoplasmic calcium concentrations are highest near the phagosome or phagocytic cup (Sawyer, Sullivan et al. 1985; Lundqvist-Gustafsson, Gustafsson et al. 2000). Calcium has been observed to encircle phagosomes in FcγRIIA-transfected CHO cells, but this can be abolished through disruption of the L-T-L signaling motif of the FcγRIIA ITAM, which

also prevents the fusion of the phagosome to lysosomes (Kindzelskii and Petty 2003; Worth, Kim et al. 2003). This suggests that IP_3-released calcium may play in important role in phagolysome fusion.

Following internalization, a series of mechanisms are activated including the recruitment of lysosomes, targeting and recruitment of major histocompatability complex molecules (MHC) and recruitment of various proteins and signaling molecules which associate with the phagosome as it matures (Ramachandra, Noss et al. 1999). Soluble NSF attachment receptor proteins (SNARE) are involved in the fusion of membranes, and are recruited to mediate the closing of the phagosome and eventual fusion with lysosomes (Hay and Scheller 1997; McNew, Parlati et al. 2000; Luzio, Pryor et al. 2005).

Specific modes of internalization through FcγRIIA-mediated processes depend on the target itself. FcγRIIA is able to mediate the uptake of small immune complexes (SIC) (Ukkonen, Lewis et al. 1986; Engelhardt, Gorczytza et al. 1991). Studies in cells bearing a heat-sensitive mutation in the E1 ubiquitin-activating enzyme demonstrate that endocytosis of small immune complexes requires ubiquitylation, while phagocytosis of large targets did not (Mero, Zhang et al. 2006). The same study also reported that FcγRIIA-mediated endocytosis is not Src-dependent. Contrary to phagocytic signaling, endocytosis of soluble immune complexes by FcR has been shown to be dependent on clathrin and not the actin-based internalization machinery, but interestingly, it does not require the classical AP-2 adaptor protein for clathrin association (Ukkonen, Lewis et al. 1986; Mero, Zhang et al. 2006). While relatively little is know about the mechanism by which FcγRIIA endocytoses small targets the leucine located in the Y-M-T-L motif of the ITAM has been shown to be required for endocytosis by FcγRIIA (Booth, Kim et al.

2002). In contrast phagocytosis, cytoplasmic lysine residues were demonstrated to be necessary for endocytosis, possibly as ubiquitylation targets, and proteosome inhibition likewise only affected endocytic mechanisms (Booth, Kim et al. 2002)

1.5 Lipid Raft Biology

The development of the fluid mosaic model represents the first widely-accepted effort to depict the nature of two-dimensional organization of lipids and proteins in the plasma membrane (Singer and Nicolson 1972). This model depicts a homogeneous bilayer of lipids interspersed with proteins effectively void of any organization outside of protein-protein interactions. The observation that glycosphingolipids cluster in the Golgi apparatus prior to being delivered to the apical membrane of polarized epithelial cells brought into question whether a degree of lipid organization existed, specifically in the absence of any known soluble factor capable of effecting the lateral distribution of membrane lipids and led to the development of the "Lipid Raft" Hypothesis (van Meer, Gumbiner et al. 1986; Simons and van Meer 1988; van Meer and Sprong 2004). Further studies provided evidence of a propensity for hydrogen binding between glycosphingolipids and possible self-association *in vitro*, suggesting the possibility of membrane domains forming as a product of the physical properties of the lipids themselves (Simons and van Meer 1988). Completing the initial definition of lipid rafts, Brown and Rose reported the existence of sphingolipid and GPI-anchered protein enriched membrane domains which were Triton X-100 insoluble at low temperatures and floated to rest in low-density areas of cell fractionations following density gradient

centrifugation (Brown and Rose 1992). This observed detergent-insolubility also was found to be cholesterol-dependent and enriched for liquid-ordered (Lo) phase membrane components (Schroeder, London et al. 1994).

Many theories exist as to the exact nature of the associations present in lipid rafts and detergent resistant membrane domains (DRM). Cholesterol is estimated to account for 30-40% of the total plasma membrane lipid fraction (Yeagle 1985). It has been shown that cholesterol is capable of forming Lo domains through association with saturated acyl chains of lipids or sphingolipid head groups (Brown 1998; McConnell and Radhakrishnan 2003). The packing of cholesterol with saturated acyl chains and sphingolipids is more favorable entropically than with unsaturated acyl chain (Elliott, Szleifer et al. 2006). There also may be an "umbrella effect" in that cholesterol segregates into regions of the membrane with strongly hydrated large head groups, allowing for protection of the sterol rings from the surrounding aqueous environment (Huang and Feigenson 1999). Additionally, studies of the association between cholesterol, dioleoylPC (DOPC) and sphingomyelin (SM) suggest that cholesterol may reside at the interface between DOPC- and SM-enriched areas, with the saturated acyl chains of SM associating with the smooth α-face of cholesterol and the unsaturated DOPC acyl chains associating with protruding methyl groups of the rougher cholesterol β-face (Pandit, Jakobsson et al. 2004).

The association of membrane proteins with lipid rafts adds another level of complexity to membrane organization and segregation. Most transmembrane proteins are excluded from lipid rafts, though some membrane proteins such as the Na^+K^+-ATPase and influenza HA protein preferentially associate with lipid rafts by being anchored

through either a lipid anchor or a transmembrane domain capable of binding raft-associated lipids (Deschenes, Resh et al. 1990; Simons and Ikonen 1997; Anderson and Jacobson 2002). It also has been suggested that changes in membrane thickness due to cholesterol accumulation may play a role in the ability of certain proteins to associate with lipid rafts due to limits in the length of their transmembrane region (Bretscher and Munro 1993). The "wetting" of proteins, or stabilization of a sterically favorable lipid environment surrounding a transmembrane protein, can create a lipid shell capable of interacting with surrounding spingolipid binding motifs and may play a role in the creation of heterogeneic lipid raft domains (Mouritsen and Bloom 1993; Allende and Proia 2002; Fantini 2003; Akimov, Frolov et al. 2008).

An important function suggested for lipid raft microdomains is the regulation of cell signaling (Baird, Sheets et al. 1999; Resh 1999; Ritchie, Iino et al. 2003). In fact, cholesterol has been shown to play an important role in membrane-regulated processes (Yeagle 1985). The tightly packed lipid environment in rafts leads to interactions with saturated acyl chains of GPI-anchored proteins (Brown 1998). Crucial signaling tyrosine kinases such as Lck, Lyn and Fyn are constitutively found associated with lipid rafts of immune cells (Simons and Ikonen 1997). Furthemore, it has been shown that lipid raft domains are essential for the function of these signaling molecules to interact with their targets (Sheets, Holowka et al. 1999). For example, ligation of the T-cell receptor initiates a translocation into lipid rafts, where it can associate with raft-associated Lck and the linker for activation of T cells (LAT) (Montixi, Langlet et al. 1998). The functions of both Lck and LAT require association with lipid rafts (Kabouridis, Magee et al. 1997; Stulnig, Berger et al. 1998; Zhang, Trible et al. 1998; Lin, Weiss et al. 1999).

Ample evidence also exists for the necessity of lipid rafts for the association of Lyn and FcεRI and subsequent phagocytic signaling (Field, Holowka et al. 1995; Field, Holowka et al. 1997). Recent evidence suggests the recruitment of ligated FcγRIIA to lipid rafts which act as a platform for signal transduction (Hoessli, Ilangumaran et al. 2000; Kono, Suzuki et al. 2002; Cuschieri 2004). Recruitment to rafts has been shown to be dependent on the palmitoylation of a juxtamembrane or transmembrane cysteine located on FcγRIIA (Barnes, Powell et al. 2006; Garcia-Garcia, Brown et al. 2007).

Methyl-β-Cyclodextrin (MβCD) has been shown to be capable of depleting isolated or intact cell membranes of cholesterol, and that removal of cholesterol by MβCD elicits changes in cell function (Irie, Fukunaga et al. 1992; Kilsdonk, Yancey et al. 1995; Choi, Chin et al. 2004). Due to its relatively low toxicity, MβCD is considered to be a potential candidate for drug delivery as well as an effective tool for the study of cholesterol and lipid rafts and their role in the membrane (Hartel, Diehl et al. 1998; Grosse, Bressolle et al. 1999).

Based on this knowledge, we sought to elucidate whether lipid rafts play a role in FcγRIIA-mediated binding and internalization. To accomplish this, we performed experiments whereby utilized cholesterol depletion to disrupt lipid rafts, and observed the subsequent effect on the ability of the receptor to bind, endocytose, or phagocytose targets of varying size. In order to further examine to interaction between FcγRIIA and lipid rafts in the membrane, we also performed Fluorescence Recovery After Photobleaching (FRAP) experiments to measure receptor mobility in the presence and absence of rafts.

Chapter 2

Materials and Methods

2.1 Cell Culture

Chinese hamster ovary (CHO) cells expressing human FcγRIIA (CHO-IIA) were generated as previously described and maintained in Ham's F-12 (Biowhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO) and expression was maintained by selection in G-418 (HyClone, Logan, UT)(Worth, Mayo-Bond et al. 2001).

FcγRIIA-GFP (IIA-GFP) was constructed by swapping the FcγRIIA cDNA sequence from pCDNA3.1 to pEGFP-N1 by HindIII and SacII flanking sites. Following ligation using a Liga-Fast kit (Promega, Fitchburg, WI), competent *Escherichia coli* were transformed and grown overnight in LB plates containing kanamycin (25μg/ml). Individual colonies were selected, and analyzed for insertion of FcγRIIA sequence. Plasmids were purified using Qiagen miniprep (Qiagen, Germantown, MD) then transfected into CHO cells using FuGene6 (Roche, Basel, Switzerland). FcγRIIA-GFP positive cells were selected by neomycin resistance, enriched by fluorescence sorting and individual clones were selected by limited dilution (CHO-IIAGFP).

2.2 Measurement of Cellular Cholesterol

Total cellular cholesterol was measured following Methyl-β Cyclodextrin (MβCD) treatment either using an Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA) or labeling with Filipin complex from *Streptomyces filipinensis* (Sigma, St. Louis, MO). Filipin staining was performed at a working concentration of 0.5mg/ml on ice for 30 min, and then washed away. The amount of remaining filipin bound to cholesterol was determined fluorometrically (355nm excitation, 460nm emission). Amplex Red cholesterol measurement was performed according to protocol provided by the manufacturer. Both assays were read using a FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany).

2.3 Lipid Raft Disruption

Disruption of lipid rafts by plasma-membrane cholesterol depletion was accomplished through treatment with a range of MβCD concentrations (0mM, 1mM, 2mM, 5mM and 8mM) (Sigma, St. Louis, MO). To test the effect of lipid raft disruption on binding, cells were pre-treated with MβCD for 45 min at 37^{o}C and binding experiments were performed with opsonized beads or heat-aggregated IgG in the presence of MβCD. To determine the effect of lipid raft disruption on internalization, binding in the absence of MβCD occurred on ice, followed by the removal of unbound target with cold PBS washes and then incubation with MβCD for 45 min on ice to disrupt lipid rafts. Internalization was initiated by warming the cells to 37^{o}C .

2.4 Membrane Fractionation and Western Blot

CHO-IIA-MH cells were treated with MβCD (5mM) for 30 min then exposed to opsonized targets or heat-aggregated IgG for an additional 30 min. Cells were washed twice with cold PBS to remove unbound targets and incubated in TNE buffer (0.05% TX-100 and protease inhibitor) for 30 min on ice for lysis. Lysates were suspended to a final concentration of 40% sucrose, loaded onto a sucrose step gradient (10-80%) and centrifuged overnight at 38k RPM in a Beckman SW41 rotor. Fractions of 1ml were collected, sucrose was removed by MeOH/Chloroform precipitation and protein loaded onto an SDS-PAGE gel. Following gel electrophoresis, samples were transferred to a PVDF membrane and blotted with an anti-MYC FcγRIIA antibody (Santa Cruz, Santa Cruz, CA). Factions were compared for the presence of FcγRIIA following exposure to either target or MβCD drug treatment. Samples were blotted for GM1 using cholera toxin B (Sigma, St. Louis, MO) to confirm raft presence. Quantitation was performed using ImageJ software to determine pixel intensity for the blot image.

2.5 IgG Coated Targets

Opsonized latex beads (OB) were used to investigate FcγRIIA mediated activities. 4.5μm polystyrene beads (OB4.5) or 1.5μm polystyrene beads (OB1.5) (Polysciences, Warrington, PA) were opsonized by incubation in a 10mg/ml solution of human IgG (MP, Aurora, OH) for 1 hr at 37^{o}C, followed by two consecutive washes in PBS at 10,000 xg for 10 min. For the fractionation experiments, opsonized sheep erythrocytes (EA) were created by opsonizing sheep red blood cells (Alsever; Rockland Scientific,

Gilbertsville, PA) with the highest subaglutinating concentration of rabbit anti-sheep IgG (Rockland Scientific). Opsonization was accomplished through incubation of the SRBC with antibody for 30 min in a 37^{o}C water-bath, and then washes with ice-cold buffered saline (containing calcium and magnesium) to remove excess antibody. Targets were added to effector cells (CHO-IIA) at a ratio of 10:1 (OB4.5 or EA) or 30:1 (OB1.5) targets to cells. The larger ratio for OB1.5 accounts for lower binding that the larger targets, possibly due to less "settling" of the targets of a smaller size. Binding was controlled for similar numbers of beads bounds per cell for each ratio. Binding occurred for 45 min on ice, and non-bound targets were removed with buffered saline washes. For internalization assays, six-well plates were floated on the surface of a 37^{o}C water bath and allowed to internalize for 30 min. Following internalization, the cells were returned to the ice, cold PBS was added to each well, and non-internalized beads were labeled with goat anti-human IgG $F(ab')_2$ fragments conjugated with phycoerythrin (Jackson Immunoresearch, Gilbertsville, PA) in a 7.5μg/ml solution for 20 min. Cells were washed again with ice-cold buffered saline and fixed in 2% paraformaldehyde. Binding and phagocytosis were assessed by counting the number of bound beads per cell as well and the number internalized as evidenced by lack of staining with the secondary antibody. Each experiment was repeated in triplicate at least three times, assessing ≥300 cells for each test sample.

2.6 Heat-aggregated IgG Complexes (haIgG).

To simulate small immune complexes, 10mg/ml FITC-conjugated human IgG (Sigma, St. Louis, MO) was complexed by aggregation at 62^{o}C for 20 min. Large aggregates were cleared by centrifugation (10,000 xg for 10 minutes). The IgG complexes (concentration 100μg/ml) were allowed to bind to cells for 45 min on ice. Excess IgG was removed by washing twice with PBS (containing calcium and magnesium). Internalization assays were performed by floating six-well plates containing the cells on the surface of a 37^{o}C water bath and allowed to internalize for 30 min. Following internalization, the cells were returned to the ice and non-internalized IgG complexes were labeled with a 2.5μg/ml solution of goat anti-human IgG $F(ab')_2$ fragments conjugated with phycoerythrin (PE) (Jackson Immunoresearch, Gilbertsville, PA) for 20 min. Excess secondary antibody was removed through buffered saline washes and the cells were detached and fixed in 2% paraformaldehyde overnight. The samples were filtered through 100μm nylon mesh and analyzed using a BD-FACSCalibur (Becton Dickinson, San Jose, CA) and using Cell Quest software. Percent total binding and internalization were calculated by comparing the Mean Fluorescence Intensity (MFI) of either FITC (total cell-associated immune complex, binding value) or PE (external immune complex only, internalization value) at each concentration of MβCD and normalizing to untreated control samples. Each experiment was repeated at least three times, and 10,000 cellular events were assessed through flow cytometry for each test sample.

2.7 Fluorescence Microscopy

CHO-IIA or CHO-IIAGFP cells were grown on coverslips, which then were mounted in phosphate buffered saline and placed on an Axiovert 200 fluorescence microscope (Carl Zeiss, Thornwood, NY) utilizing mercury illumination. Cells were visualized using differential interference (brighfield) contrast or epifluorescence microscopy. Optical filters for fluorescein excitation and emission were 480DF22 and 530DF30, respectively (Chroma, Rockingham, VT). Images were observed using an Orca ER-AG (Hamamatsu, Japan) CCD camera connected to a Dell Optiplex 620 Workstation (Round Rock, TX). Metamorph software (Molecular Devices, Downingtown, PA) was used to acquire and process images.

2.8 Fluorescence Recovery after Photobleaching (FRAP)

Experiments were performed using a TCS SP5 multi-photon laser scanning confocal microscope by Leica (Wetzlar, Germany). In each experiment, a portion of the IIA-GFP cell membrane was targeted for exposure to a high-intensity 488nm laser. This photobleached the GFP in that membrane section, and fluorescence recovery indicated the migration of receptors from other parts of the membrane into that area. The recovery of fluorescence was analyzed using LEICA software for both the rate of return (Tau) and the percentage/fraction of membrane-localized FcγRIIA-GFP that was mobile.

2.9 Statistics

Significance values determined by Student's t-test for the binding and internalization studies, and by ANOVA analysis for the FRAP studies. Significance was determined as $p<0.05$ being significant and $p<0.005$ being very significant.

Chapter 3

The Role of Lipid Rafts in FcγRIIA Binding and Internalization

To ascertain the role lipid rafts play in FcγRIIA-mediated internalization mechanisms, we have investigated the necessity for such membrane domains in the binding and internalization of IgG coated targets and heat-aggregated IgG-complexes. By disrupting lipid rafts and observing FcγRIIA mediated binding and internalization, we report that a necessity for lipid rafts in either the binding or internalization of a target is related to target size, and appears to be independent of established mechanical delineations between phagocytic and endocytic processes.

3.1 Methyl β-Cyclodextrin (MβCD) is an Effective Cholesterol-depleting Agent

In order to ascertain the role played by lipid rafts in FcγRIIA activity, it was first necessary to identify a suitable cholesterol-depleting agent with which to disrupt the raft microdomains. Methyl β-cyclodextrin has been well-characterized in regard to its ability to sequester cholesterol from biological membranes, particularly from sphingolipid and cholesterol-enriched areas of the membrane such as lipid rafts (Pitha, Irie et al. 1988).

Initial experiments displayed no significant effect on the viability of CHO-IIA cells following treatment with concentrations of up to 8mM MβCD for 45 minutes (Figure 3.1). Measurement of cellular cholesterol by either filipin staining of membrane cholesterol or amplex red fluorometric reactions each displayed that treatment with 8mM MβCD for 45 minutes reduced total cellular cholesterol by 44%, similar to previous studies (Figure 3.2) (Romanenko, Fang et al. 2004).

The expression of FcγRIIA was assessed before and after treatment with MβCD to determine any effect on receptor expression. Labeling with FcγRIIA-specific IV.3 $F(ab')_2$ fragments showed no change in expression when analyzed by flow cytometry (Figures 3.3 and 3.4). Together, these data confirm MβCD is capable of disrupting lipid rafts without affecting cellular viability or expression of the receptor in our model system.

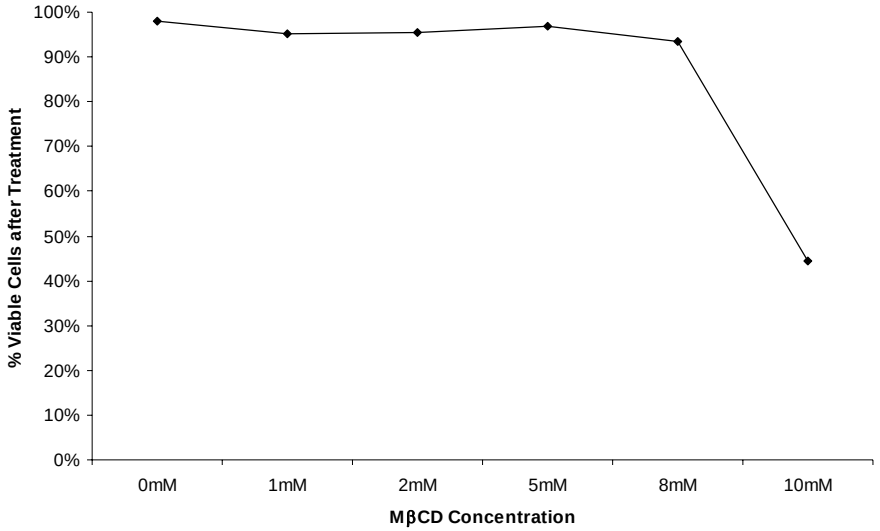

Figure 3.1 Treatment with up to 8mM MβCD does not affect cellular viability in CHO-IIA cells. Viability was assessed by trypan-blue staining following treatment with varying concentrations of MβCD, and then observation under light microscopy. Cells remained viable through treatment with 8mM MβCD.

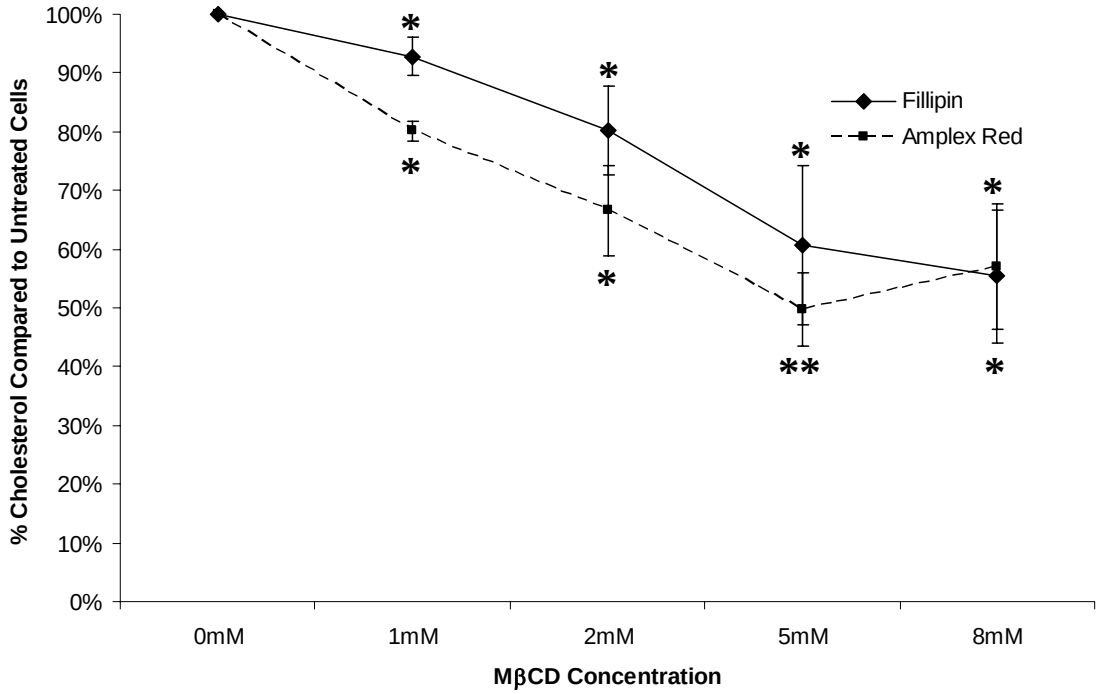

Figure 3.2 Assessment of MβCD-mediated depletion of cellular cholesterol by fillipin staining and Amplex Red. Measurement of cellular cholesterol by filipin staining or amplex red fluorometric analysis show a 44% reduction in total cellular cholesterol following treatment with 8mM MβCD. Data representative of one experiment performed in triplicate. Three indivual experiments were performed on three separate days. Statistical significance was determined by the student's t test and defined as * $p < 0.05$ and ** $p < 0.005$.

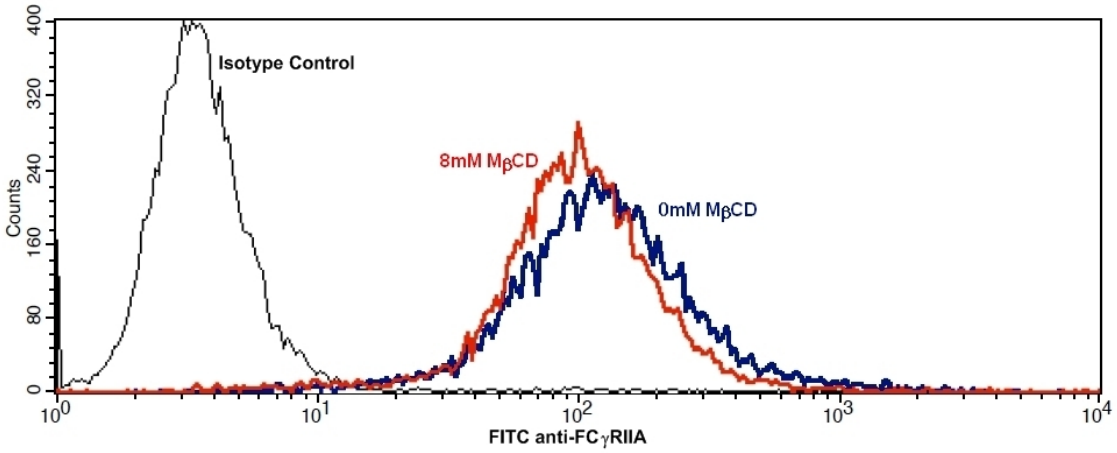

Figure 3.3 Flow-cytometric analysis of FcγRIIA expression following treatment of CHO-IIA cells with 8mM MβCD. Representative histogram of flow cytometry analysis of labeling of FcγRIIA with or without MβCD treatment.

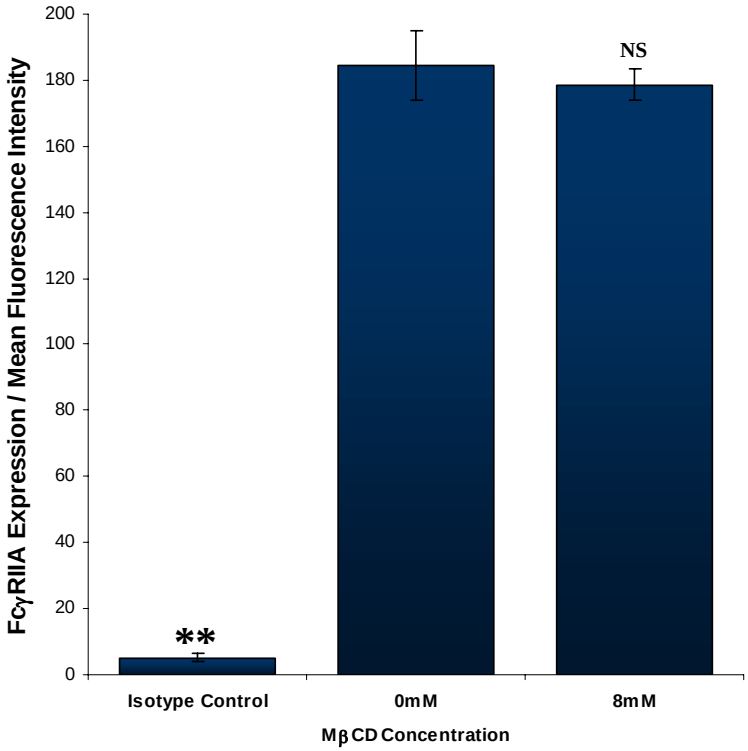

Figure 3.4 Quantification of flow-cytometric analysis of FcγRIIA expression following treatment of CHO-IIA cells with 8mM MβCD. No significant difference in FcγRIIA expression following MβCD treatment. Data are representative of one experiment performed in triplicated. Three individual experiments were repeated on three separate days. Data was compared to 0mM (untreated) control. Statistical significance was determined by the student's t test and defined as ** $p < 0.005$.

3.2 FcγRIIA Translocates to Lipid Rafts in Response to Ligand Binding

In order to determine the level of association between FcγRIIA and lipid rafts during effector mechanisms, cell fractionation experiments were performed to track FcγRIIA following binding in CHO-IIA cells. The presence of lipid rafts in the lower-density fractions was determined by cholera toxin b staining, which is specific for ganglioside M1, a sphingolipid consitutively found in lipid rafts (Figure 3.5, row 1, lanes 6-8) [reviewed in (Barenholz 2004; Ostrom and Liu 2007; Zidovetzki and Levitan

2007)]. Initially, unligated FcγRIIA is evenly dispersed throughout non-raft layers following fractionation by a sucrose gradient. These data are consistent with fluorescence microscopy experiments showing that FcγRIIA is evenly distributed across cell membranes (not shown). Exposure to IgG-opsonized sheep erythrocytes (EA) causes a shift in FcγRIIA fraction localization to less-dense fractions normally associated with plasma-membrane lipid rafts (Figure 3.5, row 3, lanes 6-8, and Figure 3.6). This suggests that FcγRIIA translocates to lipid domains following binding of EA. Translocation of FcγRIIA to lipid rafts is almost completely halted upon treatment with 5mM MβCD (Figure 3.5, row 4, and Figure 3.6).

To explore whether this lipid raft co-localization is standard for different FcγRIIA functions, human IgG was heat-aggregated into small IgG complexes (haIgG) and exposed to CHO-IIA cells. Similar fractionation experiments were conducted and a similar proportion of FcγRIIA translocated into lipd rafts (Figure 3.5, rows 5 and 6, and Figure 3.6). This Ha-IgG translocation also was inhibited by 5mM MβCD exposure. Therefore, FcγRIIA translocates into rafts upon binding both large (EA) and small (haIgG) targets.

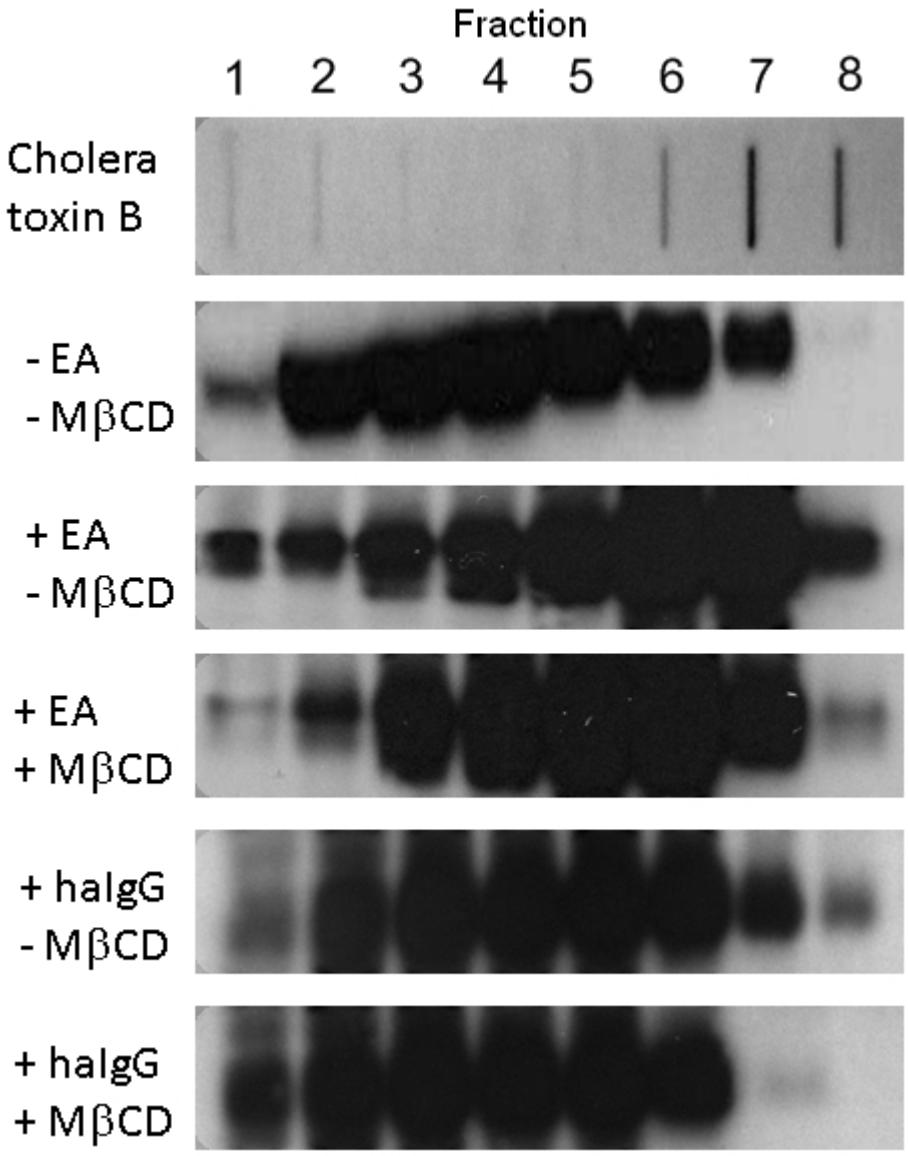

Figure 3.5 FcγRIIA translocates to cell fraction densities associated with lipid rafts upon ligation. Localization of his-tagged FcγRIIA following activation by IgG-opsonized erythrocytes (EA) or heat-aggregated IgG (haIgG). The highest-density fractions were loaded into lane 1, and the lowest-density fractions were loaded into lane 2. Activation of the receptor initiates translocation into less-dense areas of the cell lysate, normally associated with lipid rafts (lanes 6-8). Disruption of membrane cholesterol with 5mM MβCD (signified by "+MβCD") inhibits some of this translocation. Samples were blotted for GM1 using cholera toxin B (Sigma, St. Louis, MO) to confirm rafts in lanes 6-8.

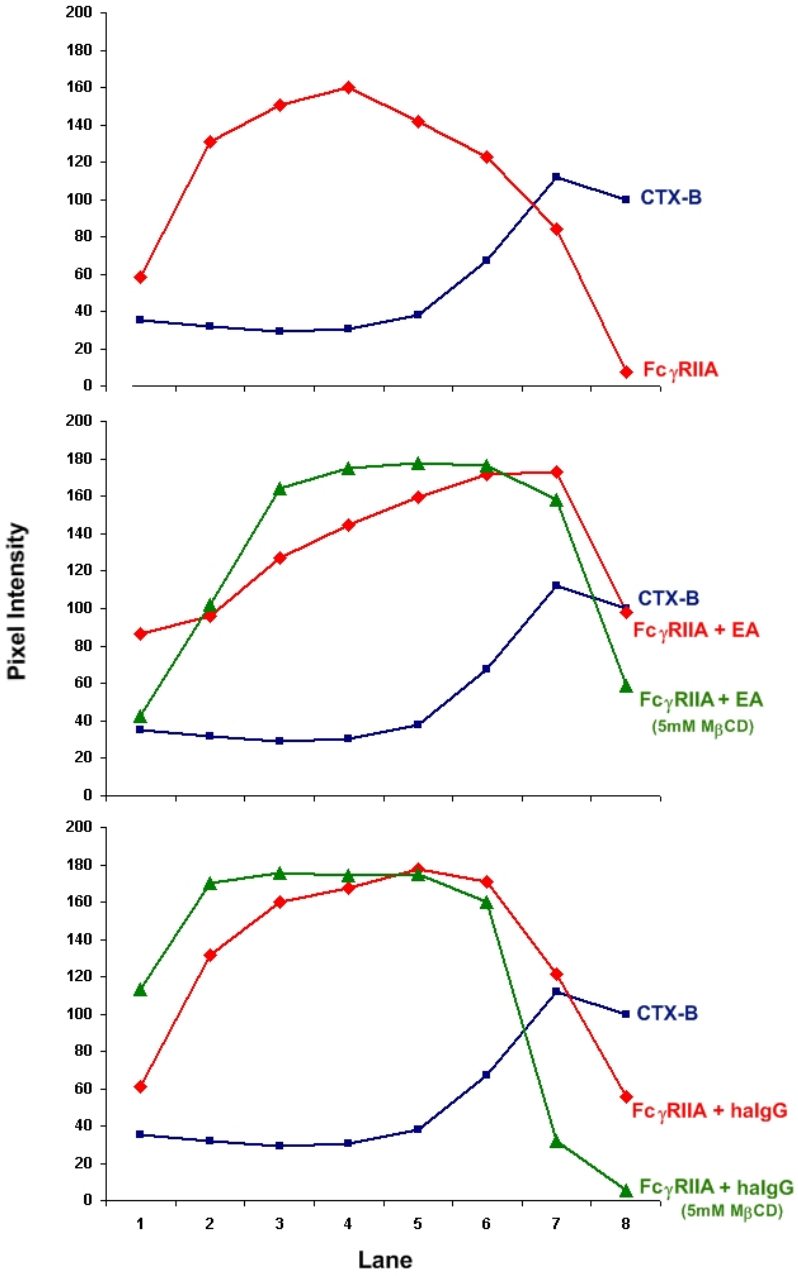

Figure 3.6 Quantification of FcγRIIA translocation to cell fraction densities associated with lipid rafts upon ligation. Quantification of the blot data in Figure 3.5 was performed by analyzing pixel intensities of the blot image using ImageJ software. The blue line indicates Cholera Toxin B (CTX-B) staining of lipid raft-associated GM1, and can be found primarily in the lower density fractions (lanes 6-8, all three panels). Following addition of EA (middle panel) or haIgG (bottom panel), a higher amount of FcγRIIA is observed in the less-dense cell fractions (red lines). Pre-treatment with 5mM MβCD (green lines, bottom two panels) partially disrupts this shift.

3.3 FcγRIIA is More Reliant on Lipid Rafts for Opsonized Target Binding than for Binding of Small Immune Complexes

To investigate potential differences in FcγRIIA function, binding assays using IgG-opsonized beads with diameters of either 1.5 micrometers (OB1.5) or 4.5 micrometers (OB4.5) and heat-aggregated immune complexes (HaIgG) (~50nm) were performed. In order to measure the effect of lipid raft disruption on FcγRIIA binding, CHO-IIA cells were pretreated with varying concentrations of MβCD for 45 min at 37^{o}C. To evaluate the dependency of FcγRIIA binding on lipid rafts, cells were treated with MβCD, cooled to 0^{o}C and exposed to either OB or HaIgG for 45 min at which time excess target was removed by washing the cells with an isotonic phosphate-buffered saline solution. Microscopic observations (Figure. 3.7) revealed a gradual decrease of bound OB4.5 as MβCD concentrations increased. Following treatment with 8mM MβCD, binding of OB4.5 and OB1.5 was reduced by roughly 74% and 66% respectively (Figure 3.8). Pre-treating cells with the anti-FcγRIIA mAb IV.3 blocked binding of both targets, demonstrating that the effects observed are FcγRIIA-mediated (Figure 3.8).

Because we observed translocation of FcγRIIA to lipid rafts following not only the binding of large opsonized targets, but alsofollowing binding of small immune complexes as well, we assessed the necessity for lipid rafts in the binding of these smaller targets. We utilized heat-aggregated IgG complexes (haIgG) consisting of two-six individual antibody molecules and assessed binding in the presence or absence of 8mM MβCD.

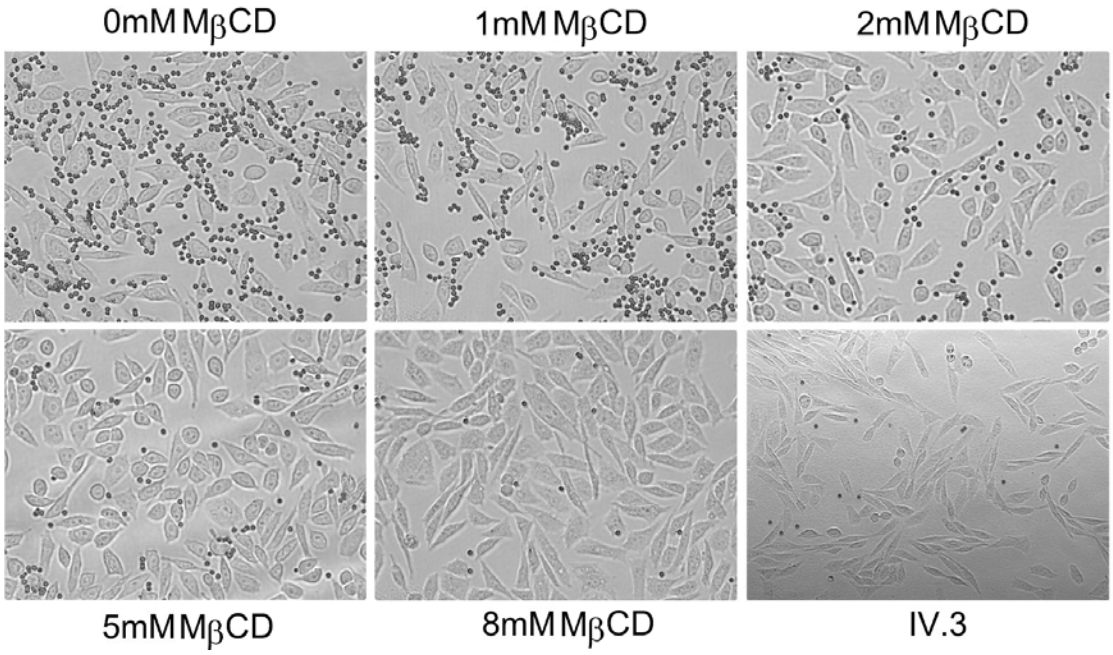

Figure 3.7 Binding of OB4.5 by FcγRIIA is disrupted in a concentration-dependent manner following treatment with MβCD. Binding of OB4.5 by CHO-IIA cells is attenuated following pre-treatment for 45 minutes with higher concentrations of MβCD. Individual micrographs were observed for the number of OB4.5 (small round dots) bound per cell. Parental CHO cells not transfected with FcγRIIA displayed no binding of target. Blocking with IV.3 mAb completely disrupted binding, verifying that the results are FcγRIIA-specific.

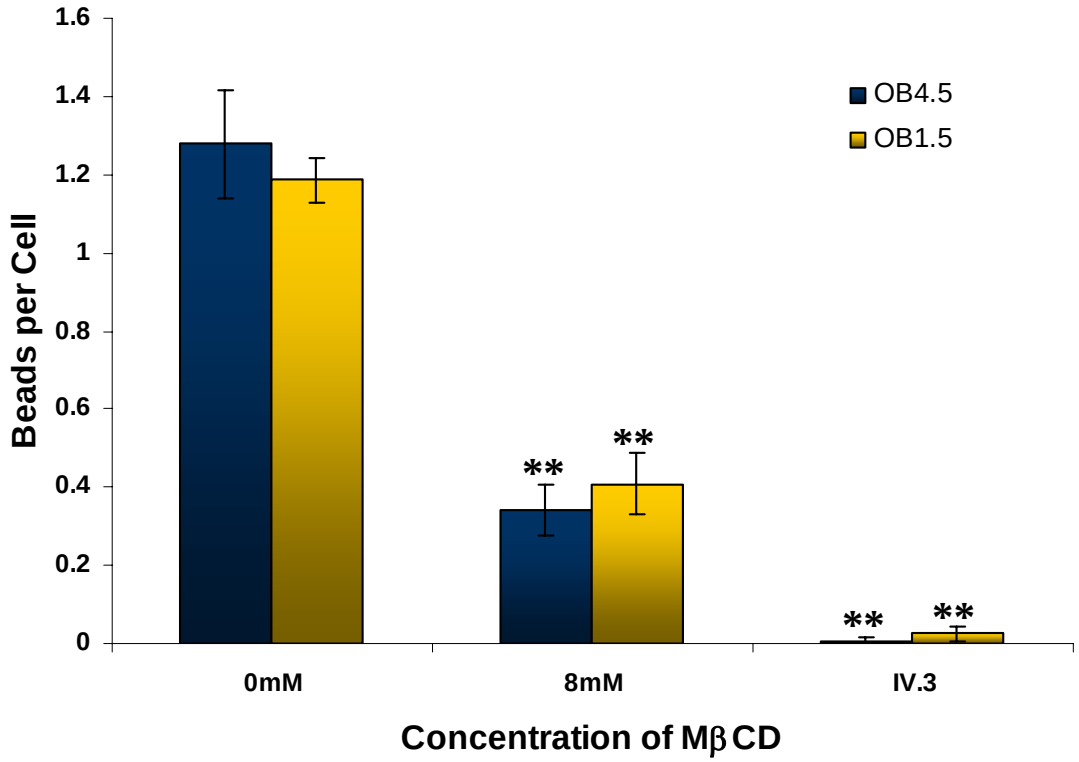

Figure 3.8 Comparison of the necessity for lipid rafts in the binding of OB4.5 and OB1.5 by CHO-IIA cells. Data shown compare number of beads bound per cell according to MβCD concentration. Similar decreases were observed for both targets, suggesting both rely on lipid rafts for binding. Blocking with IV.3 mAb verified receptor specificity. 300 cells were analyzed per experiment in triplicate. Data are representative of one experiment performed in triplicate, and were repeated on three separate days. Significance was obtained for each bead size compared to the 0mM control, and determined by the student's t test, defined as ** $p < 0.005$.

Binding of FITC-labeled haIgG (FITC-haIgG) was verified by fluorescence microscopy and a slight decrease in binding was observed following a pre-treatment with 8mM MβCD (Figure 3.9). As before, specificity for FcγRIIA was verified using a blocking IV.3 mAb. Quantification of bound FITC-haIgG was performed using flow cytometry (Figure 3.10). The intensity of FITC fluorescence was compared as an indicator of bound FITC-haIgG in the presence and absence of MβCD. In contrast to OB4.5 and OB1.5, binding of FITC-haIgG was diminished by only 18% following pre-treatment with 8mM MβCD (Figure 3.11).

Based on these data, it is evident that FcγRIIA-mediated binding of larger opsonized targets is significantly more dependent on lipid rafts than that for small IgG complexes. This variation in raft requirement suggests distinct differences in receptor function at the initial binding stages of phagocyte effector activity. To further examine the role of lipid rafts in FcγRIIA function, we next examined the effect of lipid raft disruption on different modes of internalization.

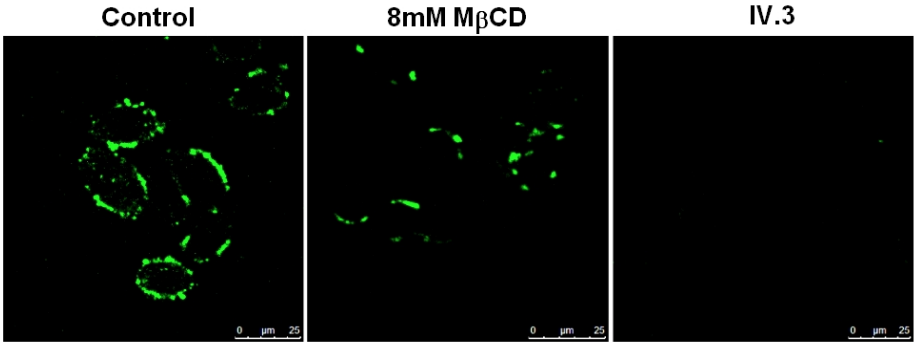

Figure 3.9 Fluorescence microscopy analysis of binding of FITC-haIgG to CHO-IIA cells in response to 8mM MβCD. A decrease in FITC fluorescence is observed in samples treated with 8mM MbCD prior to binding. Blocking IV.3 mAb was used to verify receptor specificity. (63x objective lens)

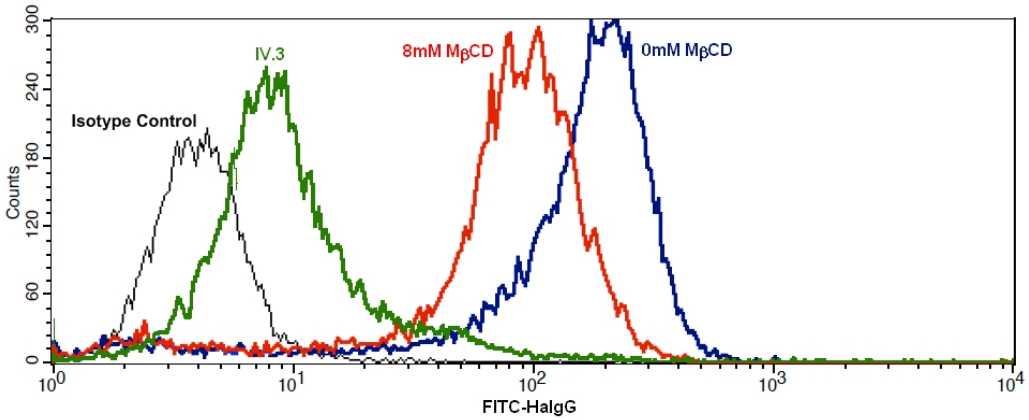

Figure 3.10 Flow-cytometric analysis of FITC-haIgG binding by CHO-IIA cells following MβCD treatment. The representative histogram shows a slight decrease in FITC intensity, suggesting a decrease in the amount of FITC-haIgG bound in response to pre-treatment with 8mM MβCD. Blocking with IV.3 mAb diminished FITC signal levels to near-negative control levels.

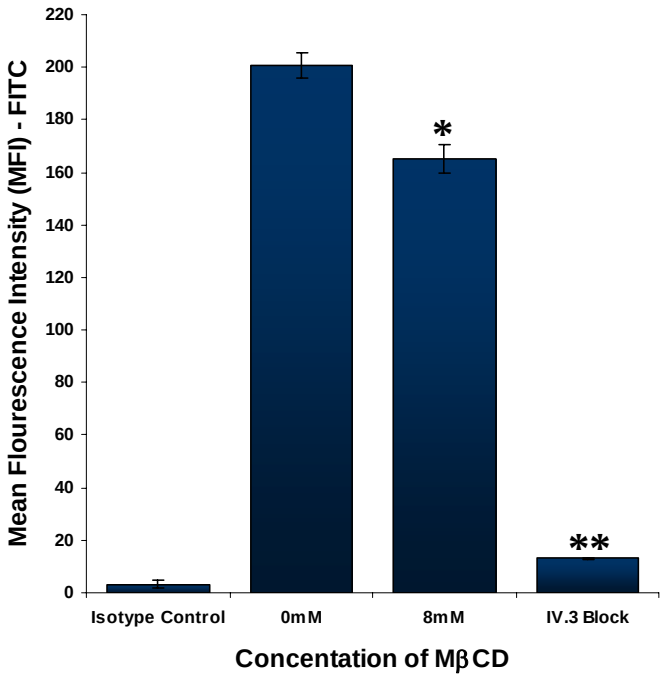

Figure 3.11 Quantification of flow-cytometric analysis of FITC-haIgG binding by CHO-IIA cells following MβCD pre-treatment. Treatment with 8mM MβCD for 45 minutes elicited a slight, though significant decrease in haIgG binding by CHO-IIA cells. IV.3 mAb was used to veryify receptor specificity. 10,000 cells were analyzed for each condition. Data are representative of one experiment performed in triplicate and were repeated on three separate days. Statistical significance was determined by the student's t test and defined as * $p < 0.05$ and ** $p < 0.005$.

3.4 Internalization of Large Targets by FcγRIIA is More Reliant on Lipid Rafts than Internalization of Small Targets

To elucidate the differences in lipid raft requirement related to FcγRIIA function, internalization assays were performed using OB4.5 and OB1.5 as targets. Binding was allowed to occur on ice prior to MβCD treatment and samples were evaluated by microscopy to be certain that displacement of targets did not occur. Following binding, phosphate-buffered saline washes were performed (0^{o}C) to remove any unbound target. CHO-IIA cells were treated with variable concentrations of MβCD on ice for 45 min, and the samples then were heated to 37^{o}C for 30 min to trigger internalization. Phagocytic function was arrested by returning the cells to ice. In order to discriminate between internal and external OB, phycoerythrin (PE) tagged secondary antibody against OB (Figure 3.12) was employed. Fluorescence microscopy was performed and the total number of internalized OB was determined by subtracting the PE-labeled (external) targets from the total OB associated with the cell.

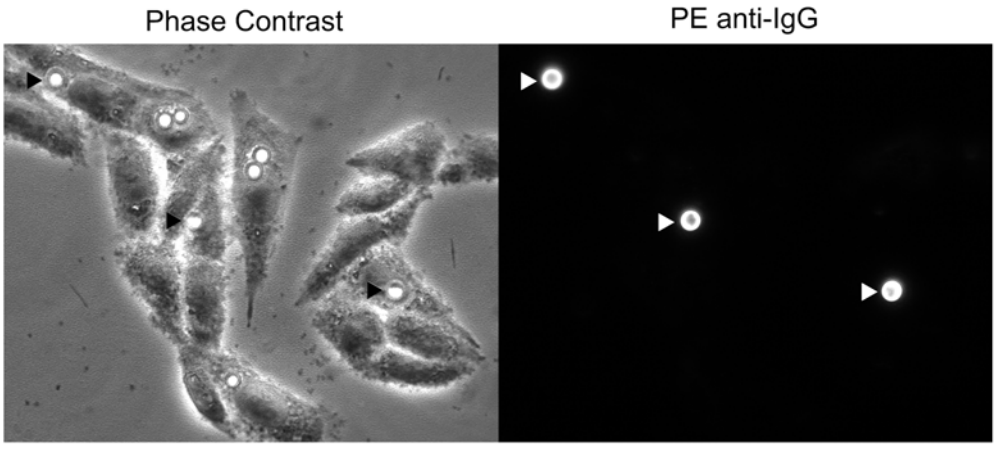

Figure 3.12 Representative micrographs depicting the strategy for discriminating between internal and external opsonized beads. A secondary PE-labeled anti-IgG Ab was used to label non-internalized OB4.5 following internalization (white arrows). (100X objective lens)

A significant decrease was observed in the ability of FcγRIIA to internalize OB4.5 following disruption of lipid rafts (55% loss of internalization following treatment with 8mM MβCD) (Figure 3.13). Interestingly, only a minimal, non-significant decrease was observed in the ability of FcγRIIA to internalize OB1.5 following MβCD treatment (8% loss) (Figure 3.13). As the number of OB per cell was controlled between the two bead targets, the remaining difference in lipid raft dependency seems to be a function of the size of the target. As a control, cells also were pre-treated with 1 micromolar cytochalasin-D (CytoD), a known inhibitor of actin-dependent phagocytosis. Internalization of OB1.5, while not reliant on lipid rafts, appears to remain actin dependant, suggesting a previously unseen actin-dependent, but lipid raft-independent mode of internalization somewhere between FcγRIIA-mediated phagocytosis and endocytosis (Figure 3.13).

To test whether lipid raft dependency for FcγRIIA-mediated internalization is a size-dependent phenomenon, we proceeded with analyzing the necessity of lipid rafts for the internalization of haIgG by CHO-IIA cells. To measure haIgG internalization we employed both fluorescence microscopy and flow cytometry, using two colors to monitor both the haIgG and its relative location. As opposed to the binding assay, higher PE signals suggested a decrease in internalization efficiency (Figure 3.14). FITC-labeled immune complexes were used to verify that MβCD treatment did not affect the total complex bound to each cell, as well as to observe the overall localization of internalized and non-internalized haIgG simultaneously (Figure 3.15).

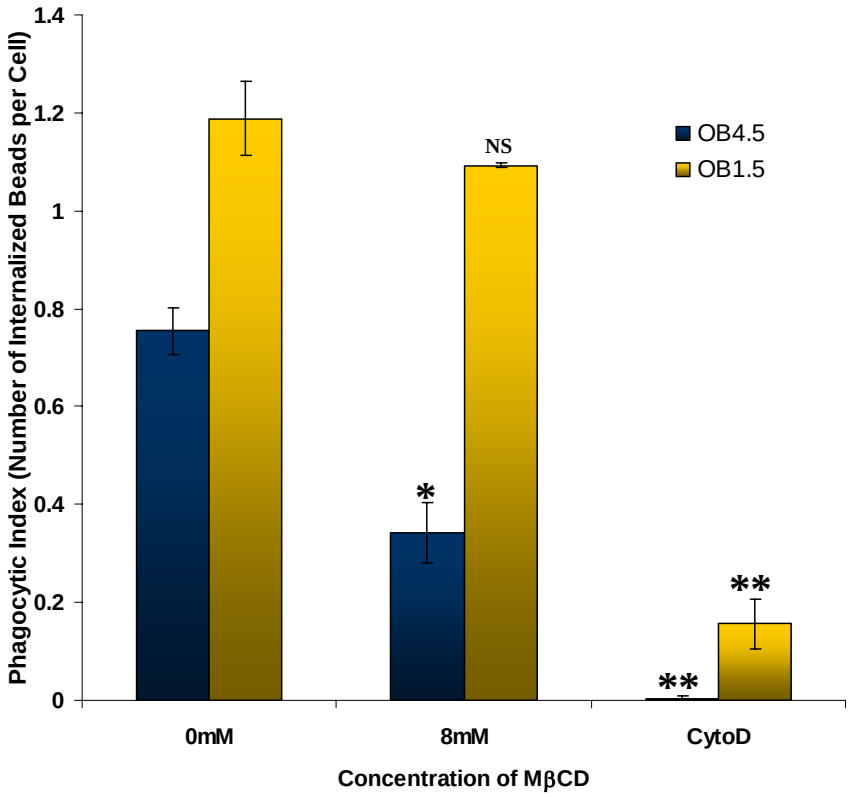

Figure 3.13 Comparison of the ability of CHO-IIA cells to internalize OB4.5 and OB1.5 following disruption of lipid rafts with 8mM MβCD. Data represented display phagocytic index (PI), or the number of internalized targets per cell. A significant decrease was observed in OB4.5, but not OB1.5 internalization, indicating a difference in lipid raft requirement. Cytochalisin-D (CytoD) was used as an actin-dependent phagocytosis control. 300 cells were analyzed for each condition. Data are representative of one experiment performed in triplicate, which was repeated on three separate days. Statistical significance was determined by the student's t test and defined as * $p < 0.05$ and ** $p < 0.005$.

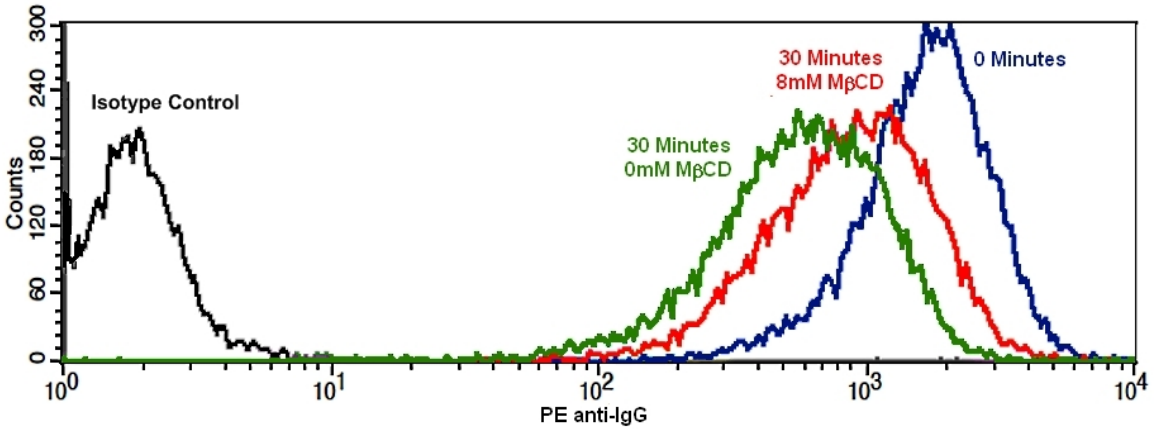

Figure 3.14 Flow-cytometric analysis of haIgG internalization by CHO-IIA following treatment with 8mM MβCD. The representative histogram shows relative PE intensity following labeling of non-internalized haIgG on CHO-IIA cells. A higher PE signal indicates more external haIgG, thus signifying less internalization. This is evidenced by the 0 minute control kept on ice displaying no internalization, or a high PE signal. The drop in PE signal following internalization in the absence of MβCD (green line) is slightly attenuated by pre-treatment with 8mM MβCD (red line), suggesting less internalization.

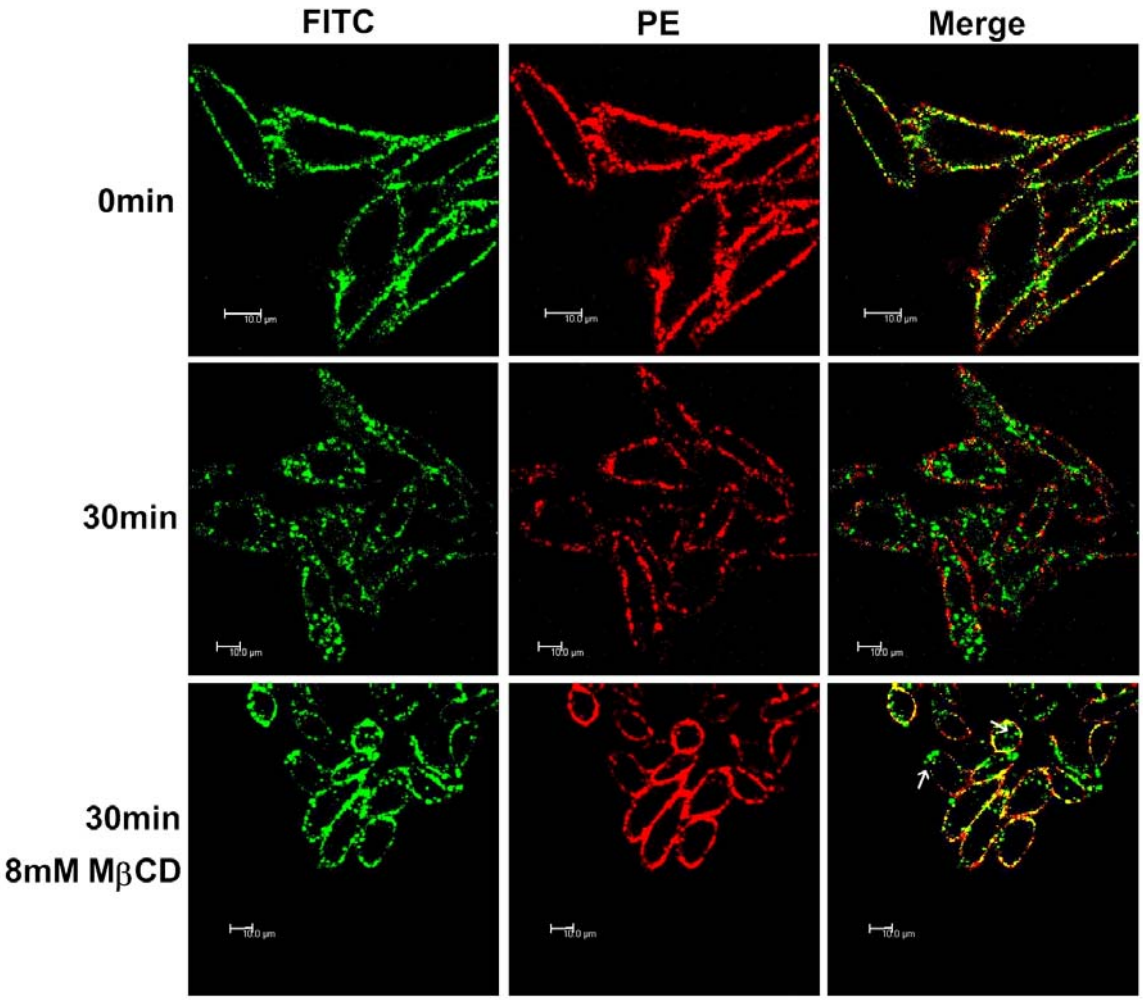

Figure 3.15 Fluorescence micrographs depicting the localization of haIgG during internalization by CHO-IIA cells following treatment with MβCD. The top row represents untreated cells kept on ice, signifying no internalization as evidenced by the colocalization of the FITC-haIgG and secondary PE anti-IgG in the merged panel. 30 minutes of internalization clearly depicts almost all of the FITC-haIgG inside of the cell, and very little colocalization with the external PE signal (row 2). Pre-treatment with 8mM MbCD (row 3) disrupts some of the internalization and a return of colocalization with the PE signal, though an increased amount of internalization is still occurring as evidence by the numerous endosomes containing haIgG (white arrows). (63X objective lens)

Quantification of the flow cytometry data displayed a minimal reliance on lipid rafts for FcγRIIA-mediated internalization of haIgG, with pre-treatment of 8mM MβCD attenuating haIgG internalization by only 25% (Figure 3.16). Pre-treatment with 1 micromolar CytoD did not decrease internalization of haIgG, and pre-treatment with 17% sucrose, which inhibits clathrin-mediated endocytosis, almost completely abrogated internalization, verifying that FcγRIIA internalization of haIgG is an endocytic process (Figure 3.17). Interestingly, treatment with CytoD decreased the level of overall bound haIgG, suggesting a role for actin in the binding of small targets by FcγRIIA, perhaps suggesting that binding of immune complex by FcγRIIA is an active process requiring association with the cytoskeleton. This is in accordance with recent studies showing that binding of target by FγRIIA is an active process, requiring receptor clustering and phosphorylation, as well as association with actin and the Cbl ubiquitin-ligase (Sobota, Strzelecka-Kiliszek et al. 2005; Dale, Traum et al. 2009). Altogether, we have shown that both the smaller-diameter beads (OB1.5) and haIgG are not as reliant on lipid rafts for internalization as the larger opsonized beads (OB4.5). These results suggest that lipid raft necessity for internalization of target by FcγRIIA has more to do with the size of the target than its composition.

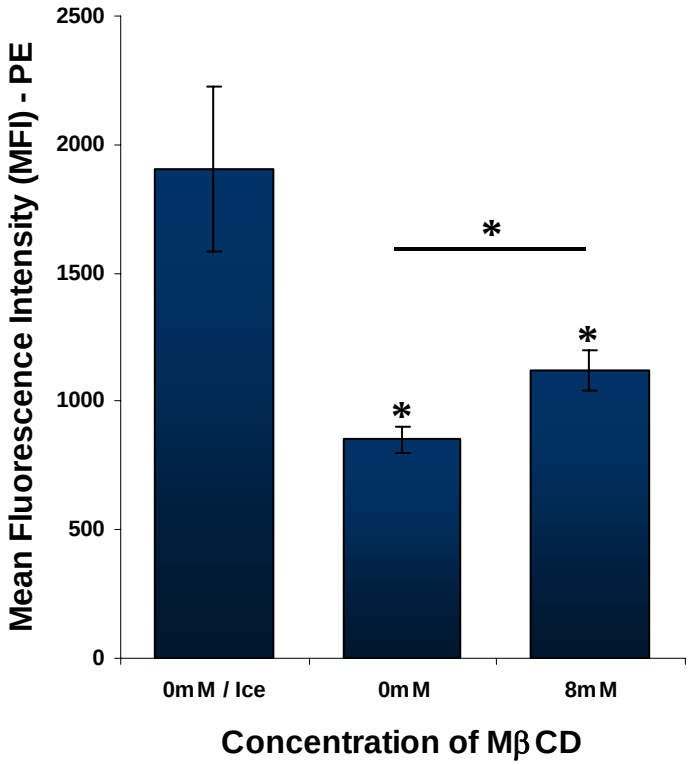

Figure 3.16 Quantification of flow-cytometric analysis of haIgG internalization by CHO-IIA cells. Internalization of FITC-haIgG by CHO-IIA cells is only minimally affected by disruption of lipid rafts with 8mM MβCD. As a higher PE signal indicates more external haIgG, the decrease observed in the 0mM MβCD sample equals the total amount of internalization of target. Treatments with both 0mM and 8mM MβCD elicited significant decreases in PE signal, suggesting that lipid rafts are not necessary for internalization of haIgG by FcγRIIA, though a signifant difference was observed between the 0mM and 8mM MβCD samples, signifying that disruption of lipid rafts does affect endocytosis by FcγRIIA, but to a limited extent. 10,000 cells were analyzed for each condition. Data are representative of one experiment performed in triplicate which was repeated on three separate days. Statistical significance was determined by the student's t test, both between the binding (ice) control and each sample, and between the samples themselves. Significance was defined as * $p < 0.05$.

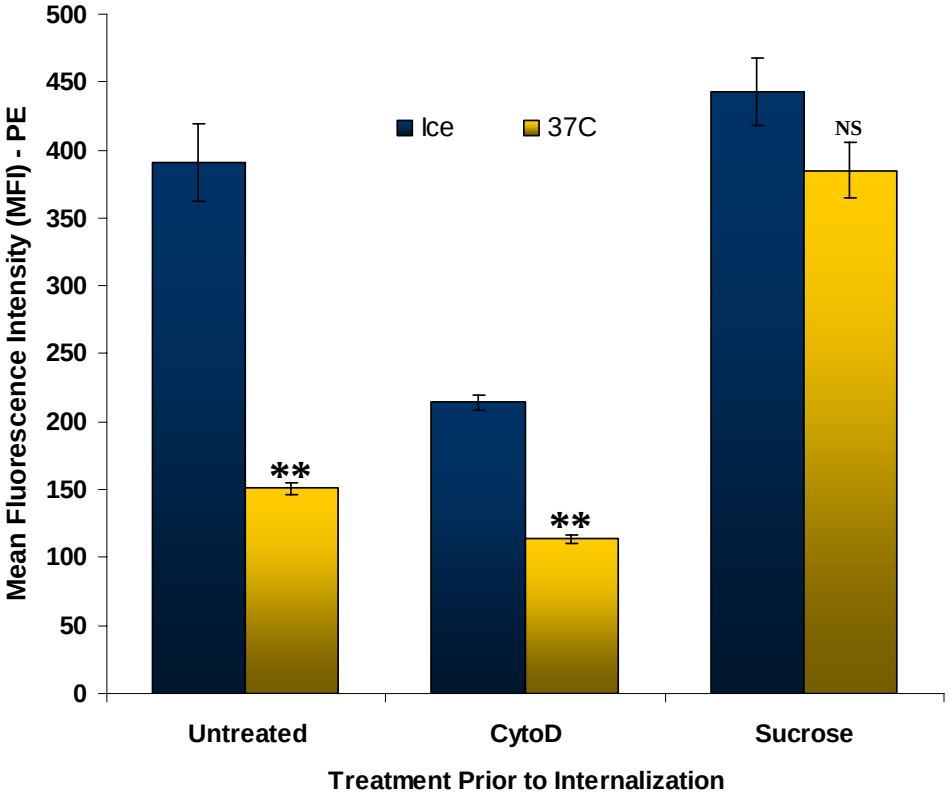

Figure 3.17 Quantitative verification of endocytic mechanisms for internalization of haIgG by CHO-IIA cells. Pre-treatment with 1 micromolar CytoD or 17% Sucrose confirms that internalization of haIgG by FcγRIIA is an actin-independent, but clathrin-coated pit-dependent endocytic mechanism. Disruption of actin by CytoD exhibits no deacrease on the level of internalization, while 17% sucrose almost completely disrupts the ability of FcγRIIA to internalize haIgG. 10,000 cells were analyzed for each condition. Data are representative of one experiment performed in triplicate, which was repeated on three separate days. Statistical significance was determined by the student's t test, comparing each treatment between a binding control kept on ice, and a sample allowed to internalize haIgG at 37^{o}C. Significance was defined as ** $p < 0.005$.

Chapter 4

The Role of Lipid Rafts in FcγRIIA Membrane Mobility

We have demonstrated that lipid rafts play more of a role in the binding and internalization of large targets than small targets, and hypothesize that lipid rafts function to control movement of FcγRIIA through the membrane, allowing for accumulation points both for the quantity of FcγRIIA needed to achieve a strong avidity interaction as well as platforms for the signaling molecules necessary for downstream activity. To test this hypothesis, we analyzed the association of FcγRIIA and lipid rafts in the membrane, and the subsequent effect of lipid rafts on the mobility of FcγRIIA in the plasma membrane.

4.1 Fluorescence Recovery after Photobleaching (FRAP) as a Means to Study FcγRIIA Mobility

Fluorescence Recovery after Photobleaching (FRAP) allows for the quantification of the movement of a fluorescently-tagged protein through a biological membrane or space. By photobleaching the fluorophores in a particular area, the rate of signal return can be equated to the movement both those bleached molecules leaving the area and of

other, non-photobleached proteins moving into that area. Axelrod and colleagues (with later refinements by Soumpasis) developed a model for the quantification of molecular mobility according to fluorescence recovery, which remains the basis of FRAP analysis (Axelrod, Koppel et al. 1976; Soumpasis 1983). This model for two-dimensional diffusion through an infinite plane allows the fitting of a non-linear curve to fluorescence intensity values as the area of interest recovers from photobleaching. From this curve, it is possible to extract both the extent and speed of recovery. The extent of recovery is indicative of the availability for movement by a protein of interested, or the percent immobile fraction. The speed of recovery can be represented by a time constant, tau, equal to roughly 63% of the plateau or asymptomatic value of the recovery curve.

A CHO cell line transfected with green fluorescence protein (GFP) -labeled human FcγRIIA (CHO-IIAGFP) was developed. This allowed for the examination of the effect of exposure to 8mM MβCD, haIgG or both on the ability of the receptor to move through the plasma membrane (Figure 4.1). Data were analyzed in the form of a recovery curve, allowing us to examine both the rate of movement and the percent of FcγRIIA that is mobile in the membrane (Figure 4.2).

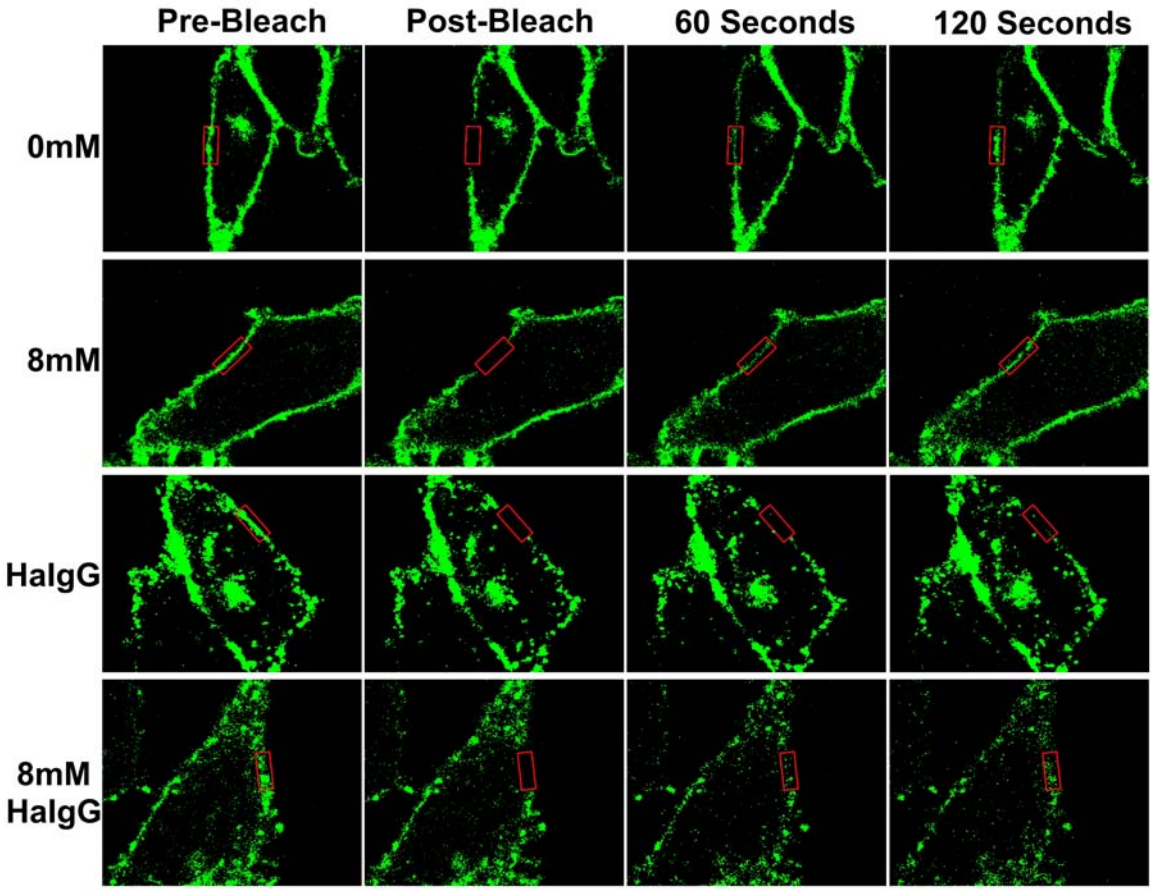

Figure 4.1 Images from Fluorescence Recovery after Photobleaching (FRAP) experiments. Images were recorded at 63X magnification prior to bleaching (column 1), just after bleaching (column 2), 60 seconds post-bleach (column 3) and 120 seconds post-bleach (column 4). CHO-IIAGFP cells were treated with 8mM MβCD (row 2), haIgG (row3), or treated with haIgG and then 8mM MβCD (row 4). Interestingly, ligation of FcγRIIA with haIgG displayed a decreased recovery compared to the other samples, and disruption of lipid rafts with 8mM MβCD abrogated this decrease.

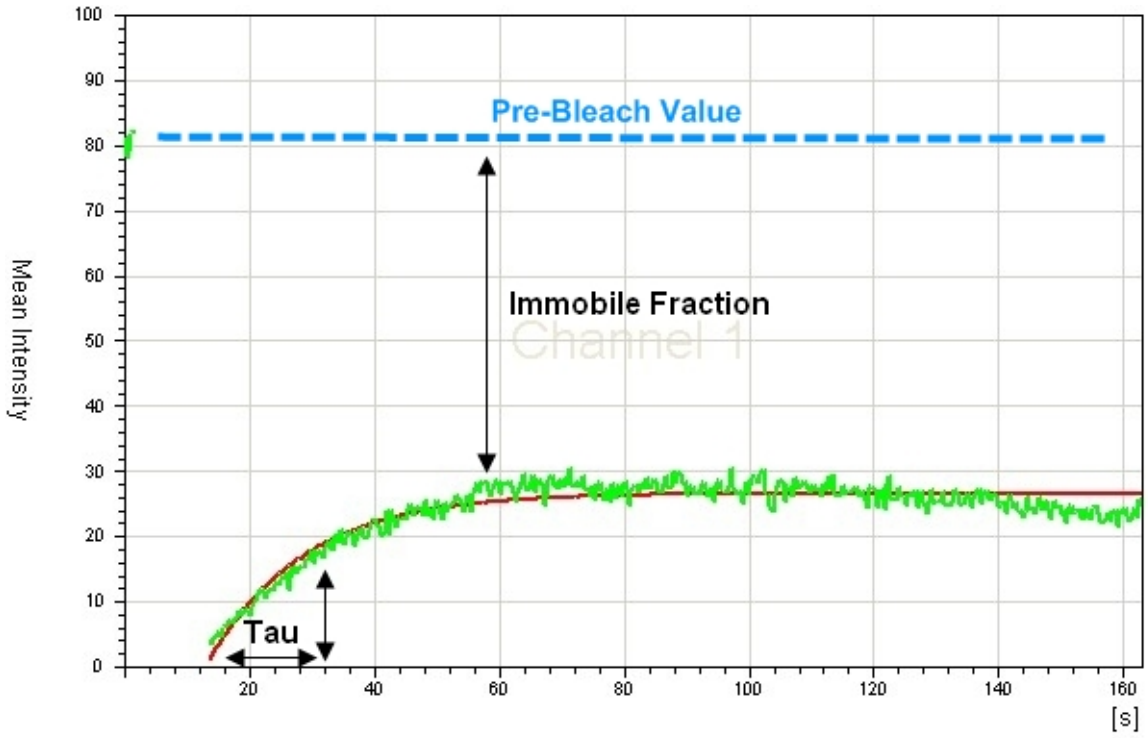

Figure 4.2 Analysis of FRAP recovery curves. The recovery curve resists of a pre-bleach value, then the measure of fluorescence intensity following photobleaching of a region of interest. The resulting values can be fit to a non-linear curve. Tau values are calculated as time in seconds at which 63% total recovery is reached, giving an indication of the rate of movement of FcγRIIA through the membrane. Immobile fractions are calculated as the difference between the pre-bleach and post-bleach values, and indicate the percentage of receptor unable to migrate into the bleached area.

4.2 Lipid Rafts Affect the Rate of FcγRIIA Movement Through the Membrane Following Ligation

We first examined the rate at which the receptor moved through the membrane by comparing the tau value of each recovery curve. Tau represents a 63% return to the eventual plateau value of the curve in seconds. A significant decrease in the rate of movement through the membrane was observed following ligation of FcγRIIA with haIgG (Figure 4.3). Interestingly, addition of MβCD did not significantly effect the ability of unligated receptors to move through the membrane, suggesting that under normal conditions FcγRIIA either freely diffuses in and out of lipid rafts or travels around them with ease. The decrease in tau following ligation of the receptor with haIgG can be abrogated by following ligation with 8mM MβCD, as exhibited by there being no significant difference between the untreated control cells and cells ligated with haIgG then post-treated with 8mM MβCD. This indicates that lipid rafts may play a role in the rate of FcγRIIA movement through the membrane following ligation. Additionally, no significant difference was observed between ligated cells in the presence or absence of MβCD, even though no significant difference exists between untreated control cells and those post-treated with MβCD following ligation. This suggests that only a partial return of the rate of movement occurs when lipid rafts are disrupted after ligation of the receptor.

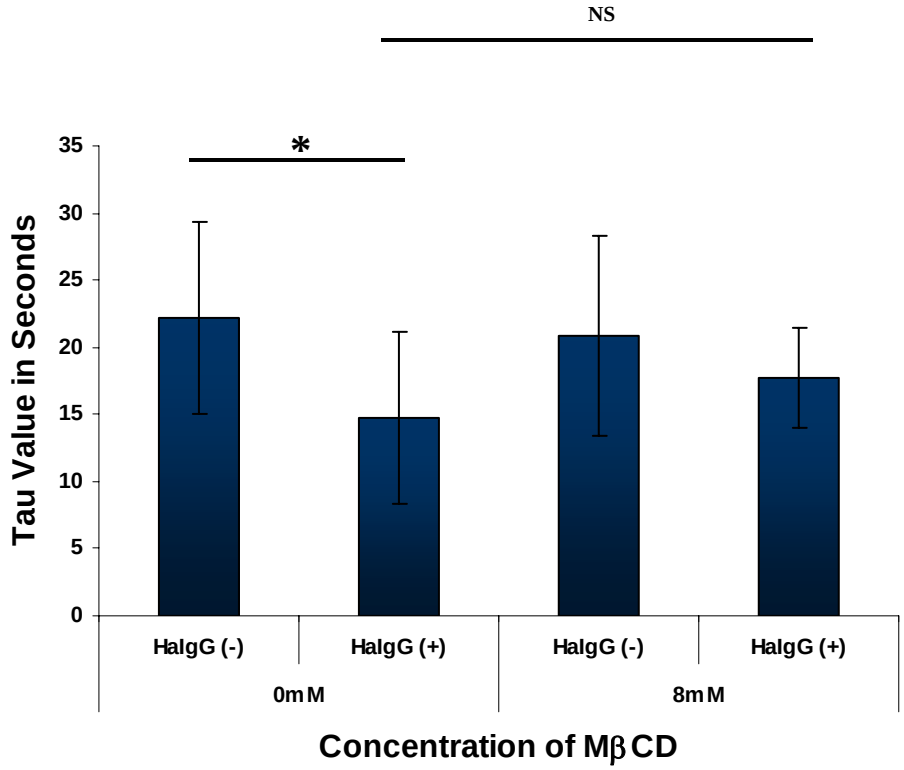

Figure 4.3 The mobility of FcγRIIA through the plasma membrane as reported by tau values of recovery curves. Tau represents the time, in seconds, that it takes a recovery curve to reach 63% of its plateau height. A slight, though significant decrease was observed in the tau values reported upon ligation of FcγRIIA with haIgG. Treatment with 8mM MβCD had no significant effect on the rate of movement of FcγRIIA through the plasma membrane; though post-treatement with haIgG following ligation of the receptor with 8mM MβCD diminished the ligation-associated decrease suggested that upon ligation, the decrease in mobility is due to association with lipid rafts. N= 8 individual experiments for each data point, and statistical significance was determined by ANOVA. Significance was defined as * $p < 0.05$.

4.3 Association with Lipid Rafts Affects the Mobile Fraction of FcγRIIA in the Membrane in Response to Receptor Ligation

We next decided to look at the total percent of receptor that is mobile in the membrane, which may be a possible indicator of the nature of the association between FcγRIIA and lipid rafts in the membrane. Analysis of the immobile fraction accounts for the inability of a recovery curve to reach the maximum pre-photobleach level due to a decrease in the available fluorophore-tagged proteins capable of moving into that area (Figure 4.2). Remarkably, the percentage of immobile FcγRIIA in the membrane rose from 55.4% to 89.4% following ligation (Figure 4.4). Treatment with 8mM MβCD after ligation returned to the immobile fraction down to normal levels, verifying that the increase in the immobile fraction is due to association with lipid rafts. This suggests that upon ligation, almost 90% of FcγRIIA present in the membrane is immobilized due to association with lipid rafts. Interestingly, treatment with MβCD without ligation did not significantly affect the immobile fraction.

These results suggest that association with lipid rafts serves to stabilize FcγRIIA in the membrane, perhaps allowing for increased localized signaling and the formation of a strong avidity interaction to target for binding. Furthermore, as receptors ligated within the same area of the membrane will presumably associate with the same or adjacent lipid rafts, lipid rafts may assist in producing the signals necessary for endocytosis and phagocytosis.

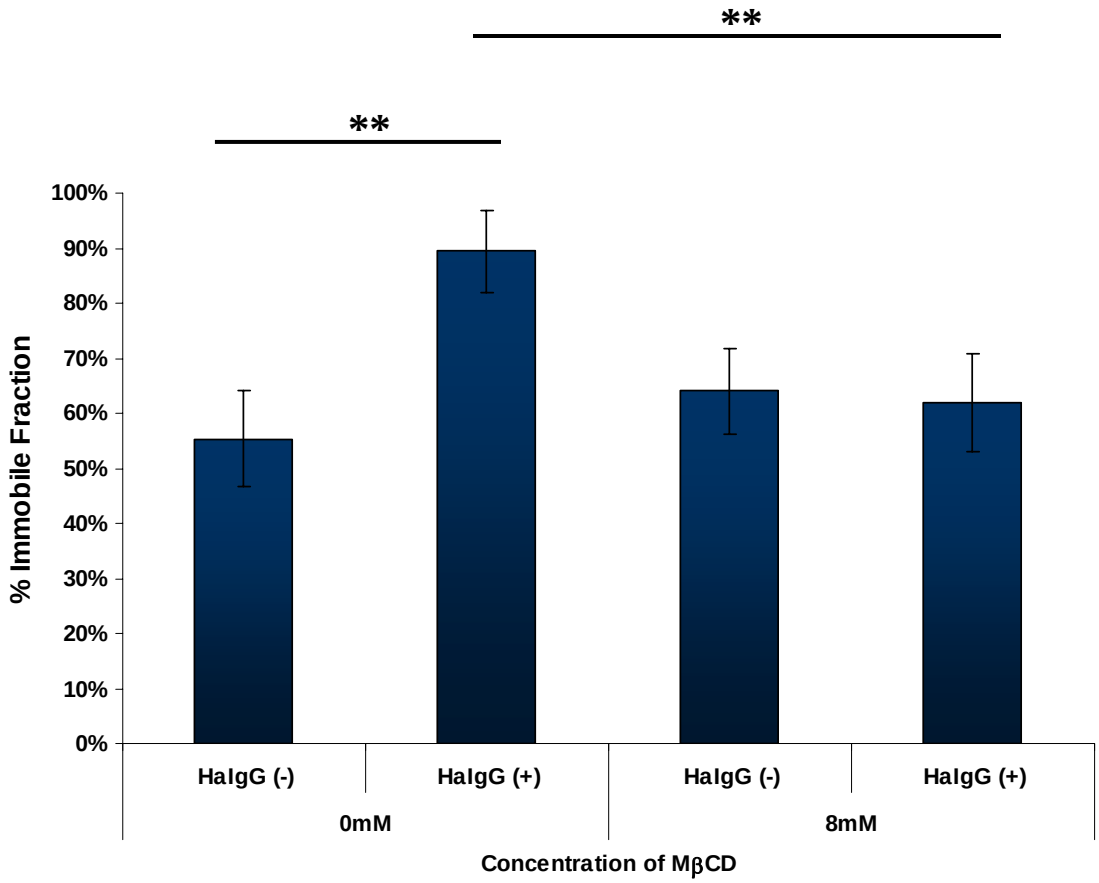

Figure 4.4 The mobility of FcγRIIA through the plasma membrane as reported by the immobile fraction of FcγRIIA according to recovery curves. The immobile fraction is defined as the population of receptor in the membrane unable to move into the bleached area, as represented by the difference between the initial fluorescence value and that of the plateau following recovery. A significant increase was observed in the immobile fraction upon ligation of FcγRIIA with haIgG. Treatment with 8mM MβCD had no significant effect on the rate of movement of FcγRIIA through the plasma membrane; though post-treatement with 8mM MβCD following ligation of the receptor with haIgG diminished the ligation-associated increase, again suggesting that upon ligation, the decrease in mobility is due to association with lipid rafts. N= 8 individual experiments for each data point, and statistical significance was determined by the ANOVA. Significance was defined as ** $p < 0.005$.

Chapter 5

Conclusions and Discussion

We have shown that FcγRIIA associates with lipid rafts upon ligand binding. This association appears to be necessary for both the binding and internalization of large OB4.5. Binding and internalization of small haIgG complexes was demonstrated to be much less dependent on lipid rafts by comparison. Interestingly, binding of the intermediately-sized OB1.5 required lipid raft association, though internalization appears unaffected following lipid raft disruption. Through FRAP analysis, we have shown that lipid rafts play a role in both the mobility and rate of movement of FcγRIIA through the membrane. Taken together, these results indicated that lipid rafts play a major role in the function of FcγRIIA.

The FRAP experiments demonstrate that ligation of FcγRIIA by haIgG both decreases the rate of movement of the receptor through the membrane, as well as the percent of the receptor population that is mobile. Under normal conditions, roughly 50% of FcγRIIA molecules in the plasma membrane are mobile. Following ligation, the mobile population drops to under 10%. We were able to almost abolish completely the decrease through treatment with 8mM MβCD, suggesting that this immobilization is due to association with lipid rafts. Interestingly, the 10% of FcγRIIA which remained mobile

post-ligation exhibited a roughly 33% loss in the rate of movement through the membrane. Two possible explanations for this phenomenon would be either transient association with lipid rafts, or the added complexity of traversing a membrane riddled with increasingly larger and more complex lipid raft domains. These experiments lend further validation to the idea of lipid rafts serving as a stable platform from which to recruit the signaling molecules and machinery necessary to facilitate FcγRIIA effector functions, and also suggest that lipid rafts play a large role in the trafficking and compartmentalizing of the receptor prior to the initiation of internalization.

The binding and internalization assays suggest varying roles for FcγRIIA function based on the size of the target itself. While the differences between phagocytic and endocytic mechanisms are well-characterized (Walters and Papadimitriou 1978; Allen and Aderem 1996; Mukherjee, Ghosh et al. 1997; Booth, Kim et al. 2002), there is little data suggesting the rationale behind initiation of either of these processes. The necessity for lipid rafts suggests they may contribute to the direction and ability of FcRs to facilitate the engulfment of particles of drastically different sizes. While being crucial for phagocytosis of large particles (OB4.5), lipid rafts appear to be less essential for FcγRIIA mediated endocytosis, or of the actin-dependent internalization of intermediate targets (OB1.5). This suggests unique mechanisms of internalization depending on the size of the target.

Is is well-documented that FcγRs utilize detergent resistant microdomains or lipid rafts to mediate effector activities (Kono, Suzuki et al. 2002; Korzeniowski, Kwiatkowska et al. 2003; Kwiatkowska, Frey et al. 2003; Abdel Shakor, Kwiatkowska et al. 2004; Strzelecka-Kiliszek, Korzeniowski et al. 2004; Hinkovska-Galcheva, Boxer et

al. 2005; Mansfield, Hinkovska-Galcheva et al. 2005). Numerous laboratories have investigated the essential components of the receptor to translocate to lipid rafts including a cytoplasmic cysteine residue (C208A) and within the transmembrane domain (A224S) (Barnes, Powell et al. 2006; Garcia-Garcia, Brown et al. 2007). Consistent with both of these studies, we have shown that lipid rafts are essential for receptor mediated events prior to signaling.

Eloquent studies by Grinstein et al. have suggested that many proteins are targeted to the plasma membrane through interaction of the cationic C-2 domain with the negatively charged inner leaflet of the plasma membrane. This negative charge is largely contributed to by phosphatidylserine (Yeung, Terebiznik et al. 2006; Yeung, Gilbert et al. 2008). Additional studies claim that PS prefers liquid-ordered domains which are commonly referred to as lipid rafts (Bakht, Pathak et al. 2007). These two observations suggest that lipid rafts, composed of PS on the inner leaflet, attract C-2 containing src family members and thus contribute to the lipid raft dependency of phagocytic signaling. These observations support our current findings that FcγRIIA phagocytosis of large targets is dependent on lipid rafts, presumably due to the availability of src family members.

A role for lipid rafts in T cell receptor (TCR) signaling has been well-documented, and is based upon observations of the necessity for the src kinase Lck in signaling, and its localization to lipid domains (Shenoy-Scaria, Gauen et al. 1993; Rodgers, Crise et al. 1994; Kabouridis, Magee et al. 1997). Observations that lipid raft domains are small entities, usually less than 20nm, and evidence that they can be observed in large aggregates of up to 200nm, lead to examination of the physiological

role of lipid raft aggregates (Pralle, Keller et al. 2000; Zacharias, Violin et al. 2002). It has been demonstrated that larger rafts are likely to be internalized by endocytic mechanisms unless they are stabilized by membrane events such as receptor stimulation, suggesting a potential regulatory mechanism for raft size and maintenance (Tanimura, Nagafuku et al. 2003). Furthermore, it was established that the induction of protein-protein interactions by receptor activation leads to further aggregation and signaling, suggesting that an increase in the size of a lipid raft directly affects the strength or duration of receptor signal (Douglass and Vale 2005).

Activation of platelets also have been revealed to be lipid raft-associated. Early studies suggested that cholesterol content and platelet function were both altered in some dyslipoproteinemias (Carvalho, Colman et al. 1974; Shattil, Bennett et al. 1977; Shastri, Carvalho et al. 1980; Corash, Andersen et al. 1981). Additionally, a correlation was observed between membrane cholesterol content and platelet responses in vitro (Shattil, Anaya-Galindo et al. 1975; Schick and Schick 1985). More recently, it has been shown that activation of platelets through the collagen receptor GpVI occurs through translocation of the receptor to lipid domains and association with the FcRγ-chain (Locke, Chen et al. 2002; Wonerow, Obergfell et al. 2002). This has been shown to be an important platform for the accumulation of Lyn, PIP_3 and PLCγ2 in FcγRIIA-mediated platelet activation (Clements, Lee et al. 1999; Pasquet, Gross et al. 1999; Falet, Barkalow et al. 2000; Watson, Asazuma et al. 2001).

Lipid rafts also have been shown to be enriched for membrane fusion mediators such as SNARE proteins, and SNARE-mediated exocytosis has been shown to be necessary in phagocytosis (Hay and Scheller 1997; Hackam, Rotstein et al. 1998;

McNew, Parlati et al. 2000; Luzio, Pryor et al. 2005). Recent experiments have displayed interactions between membrane domains and the cytoskeleton, as well as a requirement for F-actin in the regulation of FcεRI interaction with lipid rafts and the stimulation of tyrosine phosphorylation (Holowka, Sheets et al. 2000; Chichili and Rodgers 2007). This provides further evidence that a function of lipid rafts in FcγRIIA effector functions is to facilitate the accumulation and recruiting of the necessary signaling molecules and/or machinery for internalization.

Herein, we have demonstrated that in CHO-IIA cells, FcγRIIA translocates to lipid rafts regardless of the size of the ligand bound. That this translocation occurs even in the case of haIgG endocytosis, which we have shown to be only minimally reliant on lipid rafts for binding and internalization, suggests that lipid raft association may serve to regulate the mode of internalization. These results are consistent with previous observations suggesting that internalization of small ligands by FcγRIIA occurs through clathrin coated pits and is independent of ITAM-phosphorylation versus phagocytosis which is actin and ITAM-phosphorylation dependent (Tse, Furuya et al. 2003; Mero, Zhang et al. 2006). Because FcγRIIA-mediated endocytosis does not require ITAM phosphorylation, it may be the general mechanism whereby a target is internalized as long as it is not too large to be supported by clathrin-coated pit formation, either due to clathrin availability or the biophysical properties of clathrin-coated pit formation. In the event of an inability to form a clathrin-coated pit, progression of phagocytic mechanisms could continue the internalization process.

Of particular intrigue are the differences observed between the opsonized beads, OB1.5 and OB4.5, in terms of lipid raft necessity for internalization. While

internalization of both appear to be actin-dependent (veryifying phagocytic mechanisms), the requirement for lipid rafts for the larger beads and not the smaller suggests that the role of lipid rafts in FcγRIIA-mediate phagocytosis may be elucidated from the physical differences between the two. Both sizes of OB were opsonized with a super-saturated solution of IgG, meaning that both should exhibit similar densities of surface-IgG by area. The only difference between the two is diameter, with the surface area of membrane necessary to form a phagosome containing OB4.5 roughly nine-fold higher than that necessary for OB1.5 (surface areas of 63.6μm^2 and 7.1μm^2, respectively). By simple deduction, the internalization of the larger OB4.5 targets would require increased signaling and an increase in actin nucleation for the phagosome to form.

In that respect, lipid rafts may serve to accumulate the level of signaling paraphernalia necessary to mediate a size-appropriate response, such as what is observed in TCR signaling. Therefore, the ability to phagocytose OB1.5, but not OB4.5 following lipid raft disruption could be directly related to the ability to achieve a basal level of signaling through juxtapositional membrane-associated signaling molecules. Phagocytosis of larger targets by FcγRIIA may require a higher level of signaling for the recruitment of the machinery necessary for internalization. The association of lipid rafts with src kinases and F-actin, among a multitude of other signaling molecules, as well as their propensity to aggregate around activated receptors suggests they may play a very important role in this process. In particular, the presence of lipid raft-bound SNARE proteins may play a role in the differences observed for internalization of OB1.5 and OB4.5. SNARE proteins have been shown to be required for phagocytosis as a means of

replacing membrane through exocytic mechanisms, something that becomes more and more important as you create larger phagosomes (Hackam, Rotstein et al. 1998).

Based upon our observations and the material discussed here, we propose that that the modes of FcγRIIA-mediated internalization may overlap and act more in a "leaking threshold" manner than as an "on-off switch" according to size. Instead of distinct mechanisms induced by unknown factors, we suggest a single pathway with multiple options. An analogy would be the difference between sorting balls by placing them into distinct chutes according to diameter, or by rolling them down a board containing holes of increasing size, allowing them to sort themselves.

Following binding of a ligand to FcγRIIA, palmitoylation of the receptor initiates lipid-raft association. Concurrently, both association of the ITAM Leucine with an unknown adaptor to clathrin, and phosphorylation of the ITAM tyrosine residues occurs, allowing for both endocytic and phagocytic signaling, respectively. As we were able to show that internalization of haIgG is only mildly reliant on lipid rafts, it may be that the required signaling molecules are present at adequate levels outside of rafts, but may be concentrated in rafts to bolster signaling. An inability to form a clathrin-coated pit large enough to contain the target would arrest the endocytic mechanism, though continuation of phagocytic signaling would occur. Based upon the differential requirement for lipid rafts between the OB1.5 and OB4.5, induction of phagocytosis occurs as soon as a basal level of signaling is achieved through lipid raft aggregation. In cases of smaller phagocytic targets such as OB1.5, sufficient signaling machinery may be present in the membrane, making accumulation of lipid rafts unnecessary.

Lipid rafts are emerging as an important target for understanding immune signaling, as well as immune dysfunction. For example, it is well-documented that in SLE, aberrant signaling of both the TCR and B cell receptor occurs (Liossis, Kovacs et al. 1996; Liossis, Ding et al. 1998). Recent studies of the TCR in mice prone to SLE suggests disease progression is enhance by increased aggregation of lipid rafts, the consequence of which being both prolonged and amplified TCR signaling (Deng and Tsokos 2008). This, along with our current findings suggest a role for lipid rafts in the induction of signaling, the ability of phagocytic receptors to mediate the binding and internalization of targets of various sizes, and the strength and duration of the downstream signal following aggregation.

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