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Differential requirement of lipid rafts for FcγRIIA mediated effector activities

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Abstract

Immunoglobulin G (IgG) dependent activities are important in host defense and autoimmune diseases. Various cell types including macrophages and neutrophils contribute to pathogen destruction and tissue damage through binding of IgG to Fcγ receptors (FcγR). One member of this family, FcγRIIA, is a transmembrane glycoprotein known to mediate binding and internalization of IgG-containing targets. FcγRIIA has been observed to translocate into lipid rafts upon binding IgG-containing targets. We hypothesize that lipid rafts participate to different extents in binding and internalizing targets of different sizes. We demonstrate that disruption of lipid rafts with 8mM methyl-β-cyclodextrin (MβCD) nearly abolishes binding (91% reduction) and phagocytosis (60% reduction) of large IgG-coated targets. Conversely, binding and internalization of small IgG-complexes is less dependent on lipid rafts (49% and 17% inhibition at 8mM MβCD respectively). These observations suggest that differences between phagocytosis and endocytosis may arise as early as the initial stages of ligand recognition.

Keywords

methyl-β-cyclodextrin; endocytosis; phagocytosis; Fc receptors

Introduction

The mammalian immune system has developed various means of defense from 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.

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Authorship

J.A.V. performed experiments and wrote the manuscript, M.K.K., X.Q.P. performed experiments, A.D.S. contributed crucial reagents, R.G.W. designed the project, analyzed data and edited the manuscript.

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 [1;2]. FcRs are designated by the class of Ig they bind. For example, Fc γ Rs recognize and bind IgG, while Fc α R binds IgA, and Fc ϵ R binds IgE [3;4]. 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 [5;6;7;8].

Three classes of FcR are responsible for binding IgG: Fc γ RI (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16), each of which can be further differentiated by structure [9]. Both activating (Fc γ RI, Fc γ RIIA, Fc γ RIII) and inhibitory (Fc γ RIIB) Fc γ Rs participate in phagocytosis of IgG-coated particles, allowing for strict self-regulation among the FcR classes through cytoplasmic activation and inhibition motifs [10;11]. Fc γ RIIA is a 40 kDa transmembrane receptor found on neutrophils, monocytes, macrophages, dendritic cells and platelets [12]. Fc γ RIIA 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 Fc γ RIIA 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 (reviewed in [12]).

Our laboratory has recently shown that specific domains of the receptor are differentially involved in phagocytosis and endocytosis [13;14;15]. For instance, phosphorylation of the tyrosine residues in the ITAM-like sequence is required for phagocytic signaling. However, the first leucine residue has been shown to be necessary for the endocytic mechanisms of Fc γ RIIA, while the L-T-L motif is required for phagolysosome fusion [16;17;18;19;20].

Activation of Fc γ RIIA by IgG-coated pathogens results in lipid raft localization and cross-linking of receptors. Fc γ RIIA is then phosphorylated on tyrosine residues in the ITAM-like sequence by members of the Src family of tyrosine kinases [21;22;23;24]. Phosphorylation of the ITAM like region creates two SH2 (Src Homology 2) binding sites, together being able to bind spleen tyrosine kinase (Syk) [25]. This signaling leads to reorganization of actin filaments, driving the formation of a phagosome and the initiation of signaling events which result in the destruction of pathogens and the release of pro-inflammatory mediators such as cytokines and reactive oxygen species [2;26;27].

In addition to phagocytosis, Fc γ RIIA can also mediate the uptake of small immune complexes composed of IgG through endocytic (clathrin-dependent) mechanisms [28;29]. Mutational analysis has revealed that the YMTL sequence of Fc γ RIIA is important for internalization of IgG complexes. Furthermore, this process has also recently been shown to require ubiquitylation, which is not required for phagocytosis of large targets [15].

Residues outside of the ITAM have also been shown to be necessary for Fc γ RIIA activity. Recent studies have elucidated functionality in FcR translocation to lipid rafts. Recruitment of Fc γ RIIA to lipid rafts has been shown to be dependent on the palmitoylation of a juxtamembrane cysteine (cysteine 208) or on transmembrane residues [30;31]. Studies with Fc γ RIIB2 have suggested a role for lipid rafts in the temporal regulation of functions associated with endocytic mechanisms [32]. While the actual translocation to lipid rafts and the effect of lipid rafts on binding is well documented, the functional significance leading to phagocytosis or endocytosis is still a matter of much speculation [33].

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 lipid rafts are required for binding and internalization of large IgG coated targets but may inhibit endocytosis of IgG complexes.

Materials & Methods

Cell Culture

Chinese hamster ovary (CHO) cells expressing human FcγRIIA containing a MYC/His epitope tag or CR3 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)[16]. THP-1 (ATCC) cells were grown in RPMI-1640 media supplemented with 10% FCS, streptomycin and leucine per ATCC recommendations. THP-1 cell differentiation was induced by the addition of 100nM phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis MO) for three days at 37°C. Surface expression and blocking of FcγRIIA was accomplished using anti-FcγRII mAb IV.3 or isotype matched control antibodies at 2.5μg/ml.

Measurement of cellular cholesterol

Total cellular cholesterol was measured following 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 minutes, then washed away and remaining filipin bound to cholesterol was read (355nm excitation, 460nm emission) using a FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany).

Lipid Raft Disruption

Disruption of lipid rafts by plasma-membrane cholesterol depletion was accomplished through treatment with a range of Methyl-β Cyclodextrin (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 minutes at 37°C and binding experiments were performed with opsonized beads or heat-aggregated IgG. To determine the effect of lipid raft disruption on internalization, binding in the absence of MβCD occurred on ice, followed by incubation with MβCD for 45 minutes on ice prior to warming cells to 37°C for stimulation of internalization.

Membrane fractionation and Western Blot

FcγRIIA expressing CHO cells were treated with MβCD (5mM) for 30 minutes then exposed to opsonized targets or heat-aggregated IgG for an additional 30 minutes. 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 minutes on ice for lysis. Lysates were processed as previously described[23;34]. Briefly, lysates were suspended in a final concentration of 40% sucrose, loaded onto a sucrose step gradient (10–80%) and centrifuged overnight at 38k RPM using a Beckman SW41 rotor. 1ml fractions 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) or with horseradish peroxidase conjugated Cholera toxin B (Invitrogen) which recognizes the lipid raft associated sphingolipid, GM-1. Fractions were compared for the presence of FcγRIIA following exposure to either target or

M β CD drug treatment. Samples were blotted for GM1 using cholera toxin B (Sigma, St. Louis, MO) to confirm rafts in lanes 7–9. Blots were imaged and quantitated on an Omega 12iC (Ultra-Lum, Claremont, CA) using UltraQuant 6.0 software (Ultra-Lum).

IgG coated targets

Opsonized latex beads were used to investigate Fc γ RIIA mediated phagocytosis. 4.5 μ m polystyrene beads (OB) (Polysciences, Warrington, PA) were opsonized by incubation in a 10mg/ml solution of human IgG (MP, Aurora, OH) for 2 hours at room temperature. OBs were added to effector cells (CHO-IIA or THP-1) at a ratio of 10:1 targets to cells. Binding was allowed for 45 minutes 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°C water bath and allowed to internalize for 30 minutes. 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 7.5 μ g/ml solution for 20 minutes. 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 as the number internalized determined by lack of staining with the secondary antibody. Percent total binding and internalization are calculated by comparing the number of beads bound or internalized per cell in untreated samples with treated samples. Each experiment was repeated at least three times, assessing 300+ cells for each test sample.

Heat-aggregated IgG Complex

Solutions of 10mg/ml FITC-conjugated human IgG (Sigma, St. Louis, MO) were complexed by aggregation at 62°C for 20 minutes. Large aggregates were cleared by centrifugation (10,000xg for 10 minutes). The remaining soluble complexes have been reported to contain a mixture of IgG aggregates of 2 to 6 molecules per complex[35]. Assuming the size of individual IgG molecules to be ~12nm in length along the heavy chain, the maximum size aggregate could not exceed 72nm.

The IgG-complexes (concentration 100 μ g/ml) were allowed to bind to cells for 45 minutes on ice. Excess IgG-complex 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°C water bath and allowed to internalize for 30 minutes. Following internalization, the cells were returned to ice and non-internalized IgG complexes were labeled with a 2.5 μ g/ml solution of goat anti-human IgG F(ab')₂ fragments conjugated with phycoerythrin (PE) (Jackson Immunoresearch, Gilbertsville, PA) for 20 minutes. 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 100 μ m nylon mesh and analyzed on a BD-FACSCalibur (Becton Dickinson, San Jose, CA) using Cell Quest software.

Percent binding and internalization are 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 M β CD and normalizing to untreated control samples. Each experiment was repeated at least three times, and 10,000 cellular events were assessed for each test sample.

Fluorescence Microscopy

After treatments were complete, coverslips (No 1, 25mm, Corning Life Sciences, Lowell, MA) were mounted on glass microscope slides (Corning Life Sciences) 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 contrast or fluorescence. 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.

Confocal Microscopy

Treated samples were mounted on glass coverslips and imaged on an inverted Leica SP5 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany). Excitation was accomplished using laser lines at 488nm for FITC and 561nm for TRITC and fluorescence emission was collected at 530nm and 580nm respectively. Acquisition and processing of confocal images was accomplished using Leica Applications Suite Advanced Fluorescence (LAS AF) v. 2.0.2 software (Leica Microsystems).

Statistics

Significance values were determined by Students t-test.

Results

FcγRIIA translocates to lipid rafts in response to target binding

In order to determine the level to which lipid rafts play a role in FcγRIIA-mediated binding and internalization of targets, cell fractionation experiments were performed to determine the membrane raft localization of FcγRIIA following binding in Chinese hamster ovary cells expressing human FcγRIIA (CHO-IIA). To verify the cell fractionation procedure, slot blots were performed on individual fractions using HRP-conjugated cholera toxin B which binds the sphingolipid GM1, known to be enriched in lipid rafts. As demonstrated in Figure 1A, row 1, GM1 is found primarily in fractions 6–9.

In non-stimulated cells, FcγRIIA is evenly dispersed throughout non-raft layers following fractionation by a sucrose gradient (Figure 1A (row 2), Figure 1B (top)). These data are consistent with fluorescence microscopy experiments showing that FcγRIIA is evenly distributed across cell membranes. Exposure to IgG-opsonized sheep erythrocytes (EA) induces FcγRIIA to translocate to cell fractions associated with lipid rafts, as evidenced by the increased presence in fractions 6–9 (Figure 1A (row 3), Figure 1B (middle)). Translocation of FcγRIIA to lipid rafts is reduced by ~48% upon treatment with the cholesterol chelator MβCD which disrupts lipid raft domains by sequestering cholesterol and other lipophiles from the plasma membrane (Figure 1A (row 4), Figure 1B (middle)) (reviewed in [36; 37; 38]). Because MβCD has been shown to dissolve lipid rafts by chelating membrane cholesterol, total cellular cholesterol was measured by filipin staining. As shown in Figure 1C, exposing cells to 8mM MβCD depleted nearly 50% of the total plasma membrane cholesterol content while not affecting cell viability. There is also no effect on the surface expression of FcγRIIA following treatment with 8mM MβCD (Figure 1D and 1E).

To explore whether this lipid raft co-localization is standard for different FcγRIIA functions, human IgG was heat-aggregated into small IgG-complexes (ha-IgG) and exposed to CHO-IIA cells. Similar fractionation experiments were conducted and a similar proportion of FcγRIIA translocated into lipid rafts (Figure 1A (rows 5 and 6), Figure 1B (bottom)). Ha-IgG translocation was also inhibited by 5mM MβCD exposure demonstrating that FcγRIIA translocates into cholesterol rich microdomains (lipid rafts) upon binding both large (EA) and small (IC) targets.

FcγRIIA binding is inhibited by MβCD

To investigate potential differences in FcγRIIA function, binding assays using IgG-opsonized beads (OB) and FITC-tagged heat-aggregated immune complexes (FITC-HaIgG) were performed. In order to measure the effect of lipid raft disruption on FcγRIIA binding, Chinese Hamster Ovary (CHO) cells expressing human FcγRIIA (CHO-IIA) were pretreated with varying concentrations of MβCD for 45 minutes at 37°C. Concentrations of MβCD up to 8mM had no significant effect on cell viability (Fig. 1B). Total cellular cholesterol was reduced by ~50% following treatment with 8mM MβCD, similar to previous studies (Fig. 1B)[39]. To evaluate the dependency of FcγRIIA binding on lipid rafts, cells were treated with MβCD, cooled to 0°C and exposed to either OB or FITC-HaIgG for 45 minutes and then unbound targets were removed by washing the cells with an isotonic phosphate-buffered saline solution. Microscopic observation (Fig. 2A) revealed a gradual decrease of bound OB as MβCD concentrations increased. Likewise, microscopic observations and flow cytometry measured a decrease in FITC-tagged HaIgG binding as MβCD concentration increased (Fig. 2B). Following treatment with 8mM MβCD, binding of OB was reduced by ~90% while FITC-HaIgG binding was only inhibited by ~50% versus control (Figure 2C). Pre-treating cells with anti-FcγRIIA mAb IV.3 blocked binding of both FITC-HaIgG and OB, demonstrating that the effects observed are FcγRIIA-mediated (Figure 2C).

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

Lipid rafts participate in FcγRIIA-mediated internalization

To elucidate the differences in lipid raft requirement related to FcγRIIA function, internalization assays were performed using OB and HaIgG. As binding of both OB and HaIgG are dependent on lipid rafts, binding was allowed to occur on ice prior to MβCD treatment and samples were evaluated by microscopy and flow cytometry to be certain that displacement of targets did not occur. Following binding, CHO-IIA cells were treated with variable concentrations of MβCD on ice for 45 minutes. After phosphate-buffered saline washes (0°C) to remove any unbound target, the samples were then heated to 37°C for 30 minutes to trigger internalization. Phagocytic and endocytic function was arrested by returning the cells to ice. In order to discriminate between internal and external targets, phycoerythrin (PE) tagged secondary antibody against OB (Fig. 3A) or HaIgG (Fig. 3B) 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.

To measure FITC-HaIgG internalization we employed both confocal fluorescence microscopy and flow cytometry, using two colors to monitor both the FITC-HaIgG and its relative location (inside – PE negative vs. outside – PE positive). As opposed to the binding assay, higher PE signals represent less internalization (Figure 3C). FITC-HaIgG complexes were used to verify that MβCD treatment did not result in loss of complex bound to FcγRIIA (Figure 3B).

An even more drastic difference in the necessity for cholesterol-containing lipid rafts was observed for internalization of the two target sizes mediated by FcγRIIA (Figure 3C). Treatment with 2mM MβCD diminished internalization of OB by ~30% while FITC-HaIgG was only inhibited by ~10%. Remarkably, while phagocytosis was inhibited by >60% after 8mM MβCD treatment, endocytosis of FITC-HaIgG was only slightly reduced (<20%) in response to increased MβCD concentrations.

Lipid rafts are not required for phagocytosis by all receptors

To determine if M β CD treatment was responsible for inhibition of phagocytosis due to dissolving lipid rafts or by inhibiting other cellular process responsible for phagocytosis, the experiments were repeated in CHO cells expressing human Complement Receptor 3 (CR3). CR3 is capable of triggering internalization by Type 1 (non-opsonized, lipid raft dependent) or Type 2 (opsonized, lipid raft independent) phagocytosis [40;41;42]. To investigate if M β CD treatment inhibits all forms of phagocytosis, we chose to observe CHO-CR3 cells exposed to human serum-opsonized zymosan particles (SOZ) to initiate Type 2 (Lipid raft independent) CR3-mediated phagocytosis. Binding of SOZ to CR3 was inhibited by M β CD similar to Fc γ RIIA (Figure 4A). However, no effect was observed on the ability of CR3 to internalize targets, suggesting that M β CD is not inhibiting cellular processes leading to phagocytosis but has a more direct effect on lipid raft-dependent processes (Figure 4B). Together, these data suggest that while lipid rafts may play an important role in binding by various receptors, there appears to be a unique role for lipid rafts in Fc γ RIIA-mediated phagocytosis.

Results observed in the CHO-IIA model system are similar in THP-1 human monocyte/macrophage-like cells

In order to test the physiological relevance of these observations using a model system, similar experiments were performed in a model human macrophage cell line (THP-1). THP-1 cells have been used as a model for observing the role of lipid rafts and their association with various surface receptors [43;44;45]. Interestingly, similar results were obtained for binding and internalization as those observed in CHO-IIA cells at each M β CD concentration tested (Figure 5, A and B). Values for loss of binding mimicked the differential lipid raft requirement for large and small targets (OB and FITC-Ha-IgG respectively) as in Fc γ RIIA-expressing CHO cells (Figure 5A). Internalization studies yield a similar trend, though both the loss of phagocytosis of OB and endocytosis of FITC-HAIgG were dampened compared to the CHO-IIA data. Compared to blocking with IV.3, similar levels of inhibition are achieved suggesting that loss of binding and internalization is most likely due to Fc γ RIIA activity. However, additional differences may be due to interference from other Fc γ R such as Fc γ RI and Fc γ RIII. Furthermore, since Fc γ RIIA and CR3 interact and can trigger internalization signals on behalf of one another, these buffeted observations are not surprising [46].

Discussion

Together, these data suggests two very different mechanisms for Fc γ RIIA function based on the size of the target itself. While the differences between phagocytic and endocytic mechanisms are well characterized [15;47;48;49], there is little data suggesting the rationale behind initiation of either of these processes. We propose that the mode of internalization is determined very early in the process of engulfment. 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, lipid rafts appear to be non-essential for Fc γ RIIA mediated endocytosis.

Interestingly, fractionation experiments revealed that ~50% of the total cellular Fc γ RIIA is associated with lipid rafts following exposure of CHO-IIA cells to opsonized erythrocytes (EA). Given this fraction of the receptor exhibited to translocate to lipid rafts, it raises the question of how removal of roughly 50% of cellular cholesterol by 8mM M β CD is capable of nearly abolishing the ability of the receptor to bind phagocytic targets and significantly disrupt its ability to internalize them. Considering that the size of the large targets used in this study is 4.5 μ m in diameter, combined with the average size of our CHO-IIA cells (20 μ m diameter) suggests roughly 5% of the total cell membrane being necessary to form a phagosome around

an individual target. As we added target to the cells at a ratio of 10:1 respectively, and generally observed 3–5 targets per cell bound under normal conditions, the 50% translocation of FcγRIIA to lipid rafts is more than enough to facilitate phagocytosis. This is verified by our findings that the affects of lipid raft disruption on FcγRIIA are concentration-dependent, suggesting that activation of the receptor and translocation to lipid rafts is a localized, not a global event.

FcγRs utilize detergent resistant microdomains or lipid rafts to mediate effector activities [23;33;50;51;52;53;54;55]. Work by numerous laboratories have investigated essential components of the receptor needed to translocate to lipid rafts including a cytoplasmic cysteine residue (C208A) and the transmembrane domain (A224S)[30;31;33]. Consistent with both of these studies, we have shown that lipid rafts are essential for receptor mediated events prior to signaling. For example, we have shown that binding of both small IgG complexes and larger IgG coated targets are inhibited by the addition of MβCD similar to previous reports[33]. This observation suggests that some form of receptor organization needs to be present in order for efficient target binding. However, disruption of lipid rafts after ligand binding drastically inhibits only internalization of IgG coated targets such as erythrocytes or latex beads but not small IgG complexes. These results are consistent with previous observations suggesting that entry of small ligands is through clathrin coated pits versus phagocytosis which is actin dependent[13;56]. Interestingly, lipid rafts do not play a significant role in phagocytosis by all receptors. As shown in this study, binding but not phagocytosis of SOZ by CR3 is dependent on lipid rafts which is consistent with previous finding [41].

Similar observation of discrepancies between FcγRIIA mediated endocytosis and phagocytosis have implicated differential use of kinases[14]. FcγRIIA mediated phagocytosis is triggered by phosphorylation of ITAM tyrosines by members of the src family of tyrosine kinases. Phosphorylation of the ITAM creates a SH2 binding site required for syk binding and activation. Inhibition of this process by mutating ITAM tyrosines, treating cells with the src inhibitors PP1/2 or treatment with the syk inhibitor piceatannol results in loss of phagocytic capacity but has limited if any affect on FcγRIIA triggered endocytosis[14].

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[57;58]. This negative charge is largely contributed to by phosphatidylserine[57;58]. Additional studies claim that PS prefers liquid ordered domains which are commonly referred to as lipid rafts[59]. 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.

Further studies will be needed to determine the molecular mechanism(s) by which lipid rafts participate in discrimination between endocytosis and phagocytosis.

Abbreviations

CR3	complement receptor type 3
EA	IgG coated erythrocyte
FcγRIIA	IgG receptor type 2A
haIgG	heat-aggregated IgG
IC	immune complex
OB	IgG coated latex bead

M β CD methyl- β -cyclodextrin

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Figure 1. FcγRIIA migrates into lipid rafts following activation, and this migration can be attenuated by treatment with MβCD

(A) Blot data from FcγRIIA expressing CHO cells treated with IgG coated erythrocytes (EA) (phagocytosis) or heat aggregated IgG (endocytosis). Detection of the sphingolipid GM-1 by cholera toxin B demonstrates that lipid rafts are located predominantly in fraction 6–8 (Top Row). Cross-linking with EA induces FcγRIIA to migrate into detergent insoluble fractions as shown by a shift in location toward larger numbered fractions (lanes 6–8). Treatment with MβCD inhibits the translocation. (B) The blot in A was quantitated for Cholera Toxin B binding and FcγRIIA expression using UltraQuant software. Band size and density of each lane were determined for normal cells at rest (top), the cross-linking of FcγRIIA with EA (middle), and

the cross-linking of Fc γ RIIA with heat aggregated IgG (bottom). **(C)** Cell viability was assessed by Trypan blue exclusion and plasma membrane cholesterol composition via fluorescent filipin binding. As the concentration of M β CD is increased, the amount of filipin binding decreases nearly 50% while over 95% of cell remain viable. **(D)** Flow cytometry analysis after labeling CHO-IIA cells with anti-Fc γ RIIA mAB IV.3 verifies no loss of receptor expression following treatment with 8mM M β CD. **(E)** Data compiled from 3 experiments analyzing 10,000 cells each by flow cytometry.

Figure 2. Disruption of lipid rafts inhibits binding of both large IgG coated targets and small IgG complexes

(A) Representative micrographs displaying the concentration-dependent loss of binding following MβCD exposure. Small dark objects clustered around the cells are IgG-opsonized beads (OB). Blocking with IV.3 (last panel) verifies observed results are due to FcγRIIA-related activity. (B) (Top) Representative histogram demonstrating a loss in FITC-IgG signal in response to 8mM MβCD treatment, suggesting a loss in the number of FITC-conjugated IgG complexes bound to each cell. Blocking of the receptor with IV.3 completely diminished binding ability. (Bottom) Fluorescence micrographs depicting loss of fluorescence represented by less FITC-conjugated HaIgG bound to the cell surface following 8mM MβCD treatment or blocking with IV.3 mAb. (C) Binding of both IgG opsonized polystyrene beads and IgG complexes are inhibited by MβCD. Percent binding values are normalized to the 0mM control sample and represent percent of control binding after treatment. (Flow Cytometry: N=7; 10,000 cells/sample/condition. Microscopy: N=5, 300+ cells/sample/condition) (* p<0.05 compared to control, ** p<0.005 compared to control)

Figure 3. Disruption of lipid rafts inhibits phagocytosis of IgG opsonized beads but not endocytosis of IgG complexes

(A) CHO-IIA cells internalizing opsonized beads. Following internalization, extracellular beads were labeled with PE-conjugated goat anti-human IgG antibody to discriminate between internalized and non-internalized (right panel) beads (arrows pointing to external beads), with those fluorescing being located on the outside of the cell. **(B)** CHO-IIA cells internalizing FITC-conjugated HaIgG. As before, following internalization, extracellular HaIgG were labeled with PE-conjugated goat anti-human IgG antibody. Row 1 displays binding on ice with no internalization, as can be observed in the merged image, showing near complete colocalization with the secondary Ab. Row 2 exhibits changes in HaIgG location after allowing for internalization, as evidenced by an increased amount on FITC signal inside the cells, a lesser degree of PE staining, and very little colocalization in the merged image. Row 3 displays the effects of 8mM M β CD treatment prior to internalization, exhibiting a slight loss of internalization (increased PE colocalization), though some internalization is still taking place (arrows in merged image, FITC signal inside cell). **(C)** Representative histogram displaying measurement of IgG complex internalization. Non-labeled CHO-IIA cells exhibited no PE signal (grey line). A positive control kept on ice to inhibit internalization is included (bold black line). Samples treated with 8mM M β CD exhibited a higher level of PE labeling than untreated samples following 30 minutes of internalization at 37°C (dashed and dotted lines, respectively). This increase in PE following treatment represents more external IgG following endocytosis, thus signifying a decrease in the total amount of IgG complexes internalized. **(D)** Comparative graph displaying variances in lipid raft dependency for large and small targets. Percent total internalization is calculated as the loss of internalized targets normalized to control. (Flow Cytometry: N=6, 10,000 cells each sample condition. Microscopy: N=5, 300+ cells each sample condition)(* p<0.05 compared to control, ** p<0.005 compared to control)

Figure 4. Lipid rafts participate in binding but not phagocytosis triggered by CR3

(A) Binding of serum-opsonized zymosan particles (OZ) is decreased by lipid raft disruption, similar to what is observed for Fc γ RIIA (N=3, 300+ cells/sample/condition). (B) Internalization of serum opsonized zymosan particles determined by trypan blue staining is not affected by disrupting lipid raft with M β CD (N=3, 300+ cells/sample/condition). (* p<0.05 compared to control, ** p<0.005 compared to control)

Figure 5. Differentiated THP-1 cells display similar characteristics in lipid raft utilization as CHO cells

Dependency on lipid rafts for the binding (A) and internalization (B) of large opsonized beads and small IgG complexes in THP-1 cells mimicked results observed in our model CHO-IIA cells. Binding (Flow Cytometry: N=4, 10,000 cells each sample condition. Microscopy: N=3, 300+ cells/sample/condition). Internalization (Flow Cytometry: N=7, 10,000 cells each sample condition. Microscopy: N=6, 300+ cells/sample/condition). (* p<0.05 compared to control, ** p<0.005 compared to control)