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The Contributions of FC Gamma Receptors and Macropinocytosis to the Internalization, Sorting and Clearance of Antibody Coated Nanovesicles in Macrophages

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THE CONTRIBUTIONS OF FC GAMMA RECEPTORS AND MACROPINOCYTOSIS TO THE INTERNALIZATION, SORTING AND CLEARANCE OF ANTIBODY COATED NANOVESICLES IN MACROPHAGES

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Biochemistry degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I dedicate this dissertation to my mum Cecilia Kusi and to the memory of my father George Kusi.

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CONTENTS

ABBREVIATIONS

CME: Clathrin mediated endocytosis

CRISPR/Cas9: Clustered regularly interspaced short palindromic repeat/CRISPR associated enzyme 9

CSF-1: Colony stimulating factor-1

CSF-1R: Colony stimulating factor receptor-1

Fab domain: Fragment antigen-binding domain

Fc region: Fragment crystallizable region

FcRγ: Fc receptor associated gamma chain

FcγR: Fc gamma receptor

IgG: Immunoglobulin G

ITAM: Immunoreceptor tyrosine-based activation motif

ITIM: Immunoreceptor tyrosine-based inhibitory motif

PIP_2: Phosphoinositol-3,4-bisphosphate

PIP_3: Phosphoinositol-3,4,5-trisphosphate

Syk: Spleen tyrosine kinase

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ABSTRACT

THE CONTRIBUTIONS OF FC GAMMA RECEPTORS AND MACROPINOCYTOSIS TO THE INTERNALIZATION, SORTING AND CLEARANCE OF ANTIBODY COATED NANOVESICLES IN MACROPHAGES

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Macrophages are tissue-resident phagocytes that play critical roles in immune response and tissue homeostasis. They have a tremendous capacity to internalize objects of various sizes ranging from nanoscale viral particles to micron sized bacteria and tumor cells. This phenomenon, termed phagocytosis for the uptake of large particles, or endocytosis for the uptake of small particles, is integral to the immune response in multicellular organisms. Macrophages express FcγRs, which are tyrosine kinase receptors that bind IgG on an opsonized target. Binding of IgG Fc domain to the extracellular domain of an FcγR triggers signaling cascades that coordinate internalization of the opsonized target, generation of reactive oxygen species and release of cytokines.

FcγR mediated phagocytosis of large particles has been characterized in macrophages and dendritic cells, but an understanding of FcγR endocytosis of small particles is limited. Insight into FcγR endocytosis in macrophages will be useful in the design and targeting of therapeutic antibodies for treatment of cancers and viral infections.

Here, ~100nm fluorescent PEGylated liposomes displaying surface biotin antigen and antibiotin IgG2a were used to investigate FcγR trafficking. Internalization of liposomes occurred by FcγR dependent endocytosis with no contributions from macropinocytosis.

Rather, liposomes associated with clathrin on the plasma membrane and within the cytoplasm at early time points, demonstrating that the endocytic process involved the participation of clathrin. Internalized IgG2a-liposome complexes were trafficked to the limiting membrane of macropinosomes where IgG was segregated from cargo liposomes in a pH dependent manner. Contrary to trafficking of CSF-1 in macrophages, segregated IgG and liposomes were excluded from the lumen of macropinosomes and did not undergo immediate intraluminal budding, demonstrating that lysosomes did not immediately destroy the immune complex. Segregated IgG was recycled back to the cell surface where it was capable of phagocytosing new biotinylated SRBCs. Although macropinosomes and liposomes formed a multivesicular object, three-color live cell microscopy showed that fluid phase marker movement did not predict movement of liposomes to the lysosome. Lysosomes rapidly internalized luminal content of macropinosomes via piranhalysis (or squidlysis), but interacted with IgG-liposome complexes by kiss-and-run events resulting in early transition of fluid phase marker and delayed transition of liposome cargo into lysosomal compartments. Thus, we show that the macropinosome has a novel role in organizing antigen-antibody segregation in macrophages.

The contributions of murine FcγRs (I, IIb, and III) and FcRγ to endocytosis of IgG2a-liposomes were delineated using single and multiple receptor knockouts in macrophages. Knockouts were created by means of synthetic gRNAs targeting FcγRs and FcRγ in FLM cells harvested from CRISPR/Cas9 transgenic mice. Analysis of particle uptake showed that FcγRs drive differential internalization of IgG2a-liposomes with binding and uptake of particles heavily dependent on high affinity FcγR I and FcRγ. Knockout of three receptors namely FcγR I, IIb, and III abolished binding and uptake of particles. Analysis

of fluid phase uptake in knockout lines showed that FcγR I, III, IIb&III, FcRγ, and TKO were defective for macropinosome formation, whilst FcγR IIb knockouts produced more than twice the number of macropinosomes compared to parental cells. Together, these demonstrate that activating FcγRs may have a novel role in driving constitutive macropinosome formation attenuated by inhibitory FcγR IIb.

CHAPTER 1

Introduction

1. Introduction and Background

1.1. Macrophages are professional phagocytes critical for immunity

Macrophages are phagocytic cells that differentiate from hematopoietic stem cells (Sheng *et al.*, 2015) requiring the growth factor colony stimulating factor-1 (CSF-1) for development from progenitor stage to matured phagocytic cells (Cypher *et al.*, 2016; Koike *et al.*, 1986; Mossadegh-Keller *et al.*, 2013). CSF-1 binds its cognate receptor colony stimulating factor-1 receptor (CSF-1R) leading to trans autophosphorylation of tyrosine residues in its cytoplasmic tail (Felix *et al.*, 2015; W. Yu *et al.*, 2012). This generates a cascade of signaling events ensure that macrophage signaling, differentiation, growth and survival (W. Yu *et al.*, 2012). Aberrant signaling from CSF-1R and members of its signaling pathway have been associated with cancers and inflammatory disorders (Sossey-Alaoui *et al.*, 2017; Tang *et al.*, 2018).

Macrophages belong to a class of immune cells commonly called professional phagocytes (Rabinovitch, 1995) due to their multifaceted roles and central importance in cellular and adaptive immunity as well as maintenance of tissue homeostasis (Colucci-Guyon *et al.*, 2011; Flannagan *et al.*, 2010; Henson & Hume, 2006). Their ubiquitous presence in tissues and organs underscores their specialized functions in different tissue microenvironments (Gordon & Pluddemann, 2017; Yona *et al.*, 2013). As an important member of the mononuclear phagocytic system, macrophages serve as the primary tissue resident phagocyte, to clear apoptotic cells, tumor cells and foreign targets ranging from nanoscale viral particles to micron sized bacteria and fungi (Bhatia *et al.*, 2011; Chiba *et al.*, 2018; Colucci-Guyon *et al.*, 2011; Lang *et al.*, 2010).

They accomplish this complex task using a combination of different membrane receptors expressed on their surfaces and by non-specific internalization of extracellular milieu (Bruhns, 2012; Peiser & Gordon, 2001). Macrophages express pattern recognition receptors (PRRs) such as SRA1, mannose receptor and dectin1 that bind pathogen associated molecular patterns (PAMPs). Ligation of PRRs can promote transcriptional changes in the macrophage that promote anti-microbial activities known as macrophage activation. Macrophage activation in turn promotes the capture, internalization and destruction of pathogens (Zhou *et al.*, 2015). PAMPs are specific motifs on non-self molecules located on the cell walls of invading pathogens (Janeway, 2013). Moreover, macrophages express a class of membrane receptors called Fc gamma receptors (FcγRs), which mediate internalization of antibody bound targets (Alexander *et al.*, 1978; Sobota *et al.*, 2005). Antibodies are unique molecules that have the ability to bind foreign or pathogenic targets thereby directly inactivating their pathogenic capacities; alternatively, they link bound targets to phagocytic cells like macrophages for clearance (Forthal, 2014). The role of FcγRs and antibodies in mediating the internalization of small antibody bound targets in murine macrophages is the focus of this study. The general structure and function of antibodies and FcγRs have been discussed in section 1.3.

1.2. Functional phenotypes of macrophages influence their phagocytic capacity and immune responses

The phagocytic and non-phagocytic functions of macrophages are influenced by cytokines secreted by macrophages and other cells of the immune and stromal systems (Chung *et al.*, 2006; van der Poel *et al.*, 2010; Zhou *et al.*, 2015). Macrophages are classified into classically activated M1 macrophages or alternatively activated M2 macrophages (Mills *et al.*, 2000; Tarique *et al.*, 2015). Macrophages can be promoted to the M1 phenotype by proinflammatory cytokines such as interferon gamma (IFN-γ), which is secreted by Th1 cells and lipopolysaccharide (LPS) from the cell walls of gram-negative bacteria (Mills *et al.*, 2000). M1 macrophages secrete the acute inflammatory cytokines tissue necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12) and interleukin-23 (IL-23) (Manderson *et al.*, 2007; Murray *et al.*, 2005; Scull *et al.*, 2010). M1 macrophages have high phagocytic capacity, which promotes clearance of bacterial pathogens and antigen presentation during an acute infection. Additionally, M1 macrophages have elevated reactive oxygen and nitric oxide species production mediated by increased expression of the NADPH oxidase and inducible nitric oxide synthase, which promote microbial killing (Mills *et al.*, 2000; Tarique *et al.*, 2015). M2 macrophages (also called alternatively activated macrophages), are promoted by various stimuli including interleukin-4 (IL-4), interleukin-13 (IL-13), interleukin-10 (IL-10), fungi and helminth infections, and exposure to immune complexes (Gerber & Mosser, 2001; Mills *et al.*, 2000; Rodríguez-Sosa *et al.*, 2002; Tarique *et al.*, 2015). Signaling and cytokine production of M2 macrophages drive proliferation, tissue repair and cell matrix production. Unlike their

M1 counterparts, M2 macrophages express low levels of proinflammatory cytokines and are associated with tissue healing whilst M1 macrophages are associated with tissue damage caused by inflammation (Holt *et al.*, 2008; Kambara *et al.*, 2015; Mirza *et al.*, 2009; Trujillo *et al.*, 2008). The mouse macrophages used in this study are not polarized; therefore, they can be regarded as uncommitted (M0) macrophages (Tarique *et al.*, 2015). The rationale behind using M0 macrophages for this study is to understand how immune complexes are trafficked in uncommitted macrophages, since functional differentiation usually occurs after exposure to a stimuli. Moreover, this effort lays the ground work for a more elaborate future study on macrophage immune response and how exposure to various stimuli of different densities influence the commitment to a functional class M1 or M2.

1.3. Macropinosomes are important for phagocyte mediated immune functions

An important, yet poorly understood, feature of macrophages is their ability to perform macropinocytosis. Macropinocytosis occurs when the plasma membrane folds over unto itself, capturing extracellular fluid that in a single membrane bound organelle, called a macropinosome. Macropinosomes are 0.2µm to 5µm in diameter. In macrophages, macropinocytosis is constitutive, but the frequency and size increases in response to specific agonists like CSF-1 and phorbol 12-myristate 13-acetate (PMA) (J. Canton *et al.*, 2016; J. Lou *et al.*, 2014; Wang *et al.*, 2014; Yoshida, Gaeta, *et al.*, 2015).

Macropinocytosis is important for antigen uptake and presentation in phagocytic cells and is thought to be a major route used by immature dendritic cells for bulk uptake of antigen.

During maturation of dendritic cells, macropinocytosis is downregulated as the cells commit to antigen presentation (Federica Sallusto, 1995a; West *et al.*, 2004). The primary role of dendritic cells is to capture antigen and process it for presentation on MHC class II molecules. Antigen presentation is essential for activation of T cells with profound downstream effects on cytokine levels and production of antibodies by B-cells (Dubois *et al.*, 1997; Humeniuk *et al.*, 2017). In macrophages, macropinocytosis enables non-specific antigen uptake and lysosome degradation for antigen presentation on MHC class II. Consequently, disruption of macropinocytosis has been associated with decreased antigen presentation by macrophages (Lim *et al.*, 2012; von Delwig *et al.*, 2006).

In addition to antigen presentation, macropinocytosis is implicated in promoting cell growth and in the case of cancer cells that gain macropinocytic ability, this activity may facilitate tumor progression (Palm *et al.*, 2015). Some cancer cells upregulate macropinocytosis as one of multiple ways to scavenge extracellular materials from the nutrient depleted tumor microenvironment. Internalized protein and lipid degraded in the lysosomes are used for the generation of ATP and synthesis of cellular components thereby enabling cancer cells to thrive (Commisso *et al.*, 2013; Palm *et al.*, 2015). These observations raise the possibility that macrophages use macropinocytosis for similar ends in nutrient poor, inflamed or tissue repair microenvironments. Furthermore, macropinocytosis is a route for internalization of extracellular vesicles (EVs). EVs are membrane bound vesicles responsible for cell-to-cell transport of biomaterials including proteins, RNA and DNA. Beside their physiological functions, EVs have also been associated with cancer metastasis via macropinocytosis. For these reasons,

macropinocytosis has garnered interest as a therapeutic target for some cancers and inflammatory disorders (Costa Verdera *et al.*, 2017; Nakase *et al.*, 2015).

The roles of macropinosomes in growth and development of immune cells, antigen uptake and presentation, and tumor metastasis underscore their significance in immunity. As our understanding of macrophage biology evolves, there is potential for discovery of novel functions of macropinosomes that would be essential in the treatment of diseases. This study is in part, a contribution to the effort of unraveling new roles of macropinosomes in macrophage-mediated immune response.

1.4. Antibodies and Fc gamma receptors

Antibodies are proteins secreted by B cells that bind to antigens found on pathogens or native antigenic targets. There are five main classes of antibodies namely IgA, IgD, IgE, IgG and IgM. They have different functions and enable the immune system to recognize and clear a wide range of antigenic targets whiles maintaining a high degree of specificity (Schroeder & Cavacini, 2010). This study focuses on the interaction of immunoglobulin G (specifically, IgG2a subclass) and how it regulates macrophage-mediated uptake of small antibody bound targets via cognate Fc gamma receptors (FcγRs).

Once bound to the antigen, antibodies promote destruction of the target by phagocytosis (McHeyzer-Williams & McHeyzer-Williams, 2005; Schroeder & Cavacini, 2010). Direct target lysis is called antibody dependent cell-mediated cytotoxicity (ADCC) whilst antibody mediated internalization of targets by phagocytic cells is termed antibody

dependent cell phagocytosis (ADCP) (Gül & van Egmond, 2015; Iannello & Ahmad, 2005).

1.4.1. Immunoglobulin G structure and function

Immunoglobulin G (IgG) belongs to a class of antibodies consisting of two heavy and two light chains (Figure 1.1). Each heavy or light chain contains a variable domain and a constant domain that possesses an amino (NH_2) terminus and carboxyl (COOH) terminus respectively. Generally, antibodies possess an antigen binding region (Fab region) and a fragment crystallizable region (Fc region). The Fab region is made up of one variable and one constant domain from both heavy (CH_1) and light chains (C_L) whilst the Fc region contains two constant domains (CH_2 and CH_3) from the heavy chain linked by a hinge region to the first constant domain (CH_1) in the Fab region. The hinge contains disulfide bonds from conserved cysteine residues that stabilize the structure of IgG. Interchain disulfide bonds linking heavy chains to light chains as well as intradomain disulfide bonds contribute to the overall structural integrity of antibodies. Antigen recognition and binding occurs in the Fv region which is comprised of the variable regions of the light chain (V_L) and heavy chain (V_H). The hinge and Fc regions determine effector functions via binding to Fc receptors (Schroeder & Cavacini, 2010; Vidarsson *et al.*, 2014).

Fc gamma receptors (FcγRs) bind the Fc domain of antibodies to mediate endocytosis or phagocytosis of small and large antibody opsonized targets. Receptor-antibody binding

activates a series of signaling events that result in target engulfment and internalization by phagocytes (Bruhns, 2012).

Antibodies may bind to FcγRs with different affinities; the nature and strength of this interaction influences the efficiency of target internalization and production of inflammatory mediators (Beutier *et al.*, 2017; Gillis *et al.*, 2017). The affinities of mouse IgG isotypes for various FcγRs are show in in the Table 1.

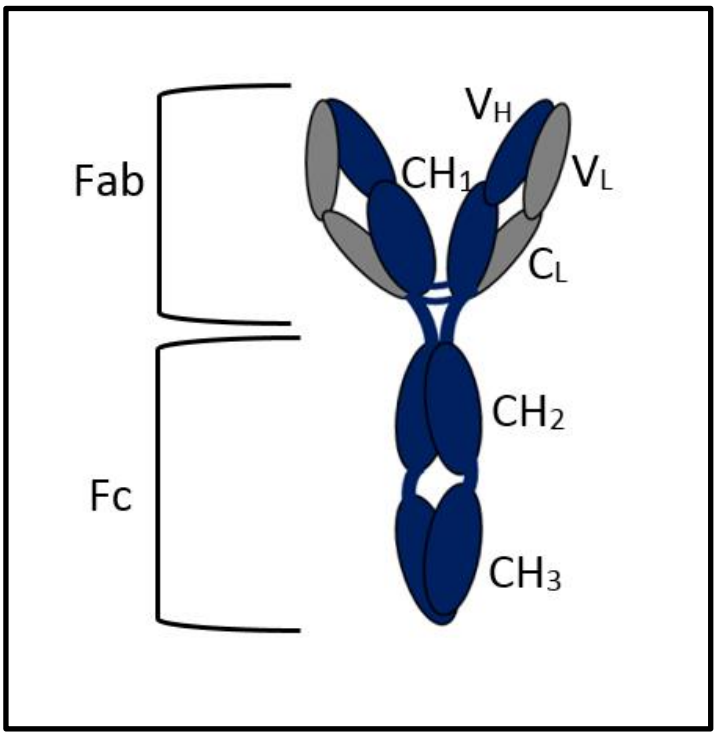

Figure 1. 1 Schematic of immunoglobulin G (IgG) structure. IgG consists of a light chain (L) and a heavy chain (H). Both L and H chains possess a variable region V_L and V_H and one constant region C_L and CH_1 within the antigen binding region called Fab domain. Fc domain is the constant binding region is responsible for interaction with Fc receptors to mediate effector functions of IgG. Fc region consists two constant domains CH1 and CH2 from the heavy chain (H).

Table 1. 1 Association constants (Ka) of mouse IgG subclasses for corresponding FcγRs

Receptor	IgG1	IgG2a	IgG2b	IgG3	Reference
FcγR I	____	1.0×10^8	1.0×10^5	(+)	(Gavin *et al.*, 1998a; Nimmerjahn *et al.*, 2005; Saylor *et al.*, 2010)
FcγR IIb	3.3×10^6	4.2×10^5	2.2×10^6	____	(Nimmerjahn *et al.*, 2005)
FcγR IIII	3.1×10^5	6.8×10^5	6.4×10^5	____	(Nimmerjahn *et al.*, 2005)
FcγR IV	____	2.9×10^7	1.7×10^7	____	(Nimmerjahn *et al.*, 2005)

Table 1. 2 Dissociation constants (Kd) of mouse IgG subclasses for FcγRs.

Receptor	IgG1	IgG2a	IgG2b	IgG3	Reference
FcγR I	____	1.0×10^{-8}	1.0×10^{-5}	(+)	(Gavin *et al.*, 1998a, 1998b; Nimmerjahn *et al.*, 2005)
FcγR IIb	3.0×10^{-7}	2.4×10^{-6}	4.6×10^{-7}	____	(Nimmerjahn *et al.*, 2005)
FcγR IIII	3.2×10^{-6}	1.47×10^{-6}	1.6×10^{-6}	____	(Nimmerjahn *et al.*, 2005)
FcγR IV	____	3.5×10^{-8}	5.9×10^{-8}	____	(Nimmerjahn *et al.*, 2005)

1.4.2. Murine Fc gamma receptor structure and function

Fc gamma receptors (FcγRs) are transmembrane receptor tyrosine kinases that bind the Fc domains of immunoglobulin G (IgG). Binding of IgG molecules by FcγRs enables integration of the adaptive and innate arms of immunity. This is critical for clearance of targets as well as generation of the signals needed for spatiotemporal regulation of immune response. There are four murine FcγRs namely FcγRs I (CD 64), IIb (CD 32b), III (CD 16) and IV (CD 16-2) (Bruhns, 2012).

FcγR I consists an alpha chain that has three extracellular immunoglobulin-like (Ig-like) domains, a transmembrane region and a small intracellular domain as depicted in the schematic diagram in figure 1.2 (Lu *et al.*, 2011). The extracellular domain of the alpha chain is responsible for binding antibodies via their CH_2 domains near the hinge/Fc junction. Signaling from FcγR I requires an associated dimeric gamma FcRγ, that is encoded by the gene *Fcer1g* and is distinct from the "cytokine common γ-chain" (Brandsma *et al.*, 2016). FcRγ contains an immunoreceptor tyrosine-based activation motif (ITAM) bearing conserved tyrosine residues that become phosphorylated upon ligand binding and receptor clustering by Src-family kinases. The phosphorylated tyrosines serve as docking sites for molecules such as Syk that mediate downstream signal transduction (John C Cambier, 1995; S A Johnson, 1995). The mechanism of FcγR activation is described below (section 1.4.3).

Similarly, FcγR III and IV have FcRγ associated with their α-chains. Their alpha chains however contain only two Ig-like domains. FcγR I, III and IV are activating receptors

because they generate signals that promote internalization of opsonized targets (Allen, 1998; Bruhns, 2012; Nimmerjahn *et al.*, 2005).

FcγR IIb is an inhibitory receptor (Getahun & Cambier, 2015). Like FcγR III and IV, FcγR IIb has two Ig-like extracellular domains but unlike activating receptors, it possesses a conserved immunoreceptor tyrosine-based inhibitory motif (ITIM) within the cytoplasmic tail of the α-chain. As the name implies, ITIM has conserved tyrosine residues that become docking sites for signaling proteins such as SHP-1 phosphatase after phosphorylation (Ai *et al.*, 2006; Huang *et al.*, 2003).

FcγRs can be classified as high affinity or low affinity receptors based on binding to IgG (F. Nimmerjahn & J. V. Ravetch, 2005). FcγR I is regarded as a high affinity receptor because it binds IgG2a with a high affinity K_d of $1x10^{-8}$ M but has low affinity for IgG2b with a K_d of $1x10^{-5}$ M (Allen, 1998; Nimmerjahn *et al.*, 2005). FcγRI does not bind IgG1 and there is controversy as to whether it binds IgG3 (Gavin *et al.*, 1998b). FcγR IIb has undetectable binding for IgG3 but binds IgG1, 2a and 2b with K_d of $3.03x10^{-7}$ M, $2.38x10^{-6}$ M, $4.55x10^{-7}$ M respectively. FcγR III does not bind IgG3 but has low affinities for IgG1, 2a and 2b with K_d values of $3.23x10^{-6}$M, $1.47x10^{-6}$M, $1.56x10^{-6}$ M respectively (Nimmerjahn *et al.*, 2005). FcγRIV does not bind IgG1 or IgG3 but has strong affinity for IgG2a and 2b with K_d of $3.45x10^{-8}$ M and $5.88x10^{-8}$ M respectively (Nimmerjahn *et al.*, 2005; Nimmerjahn *et al.*, 2010). The binding affinities of murine FcγRs expressed in Ka and Kd are summarized in Table 2. All mouse IgG isotypes bind FcRn with high affinities. However, FcRn is not an Fc gamma receptor but an MHC class I molecule that mediates recycling of internalized IgG (S. Akilesh *et al.*, 2007; T. Suzuki *et al.*, 2010).

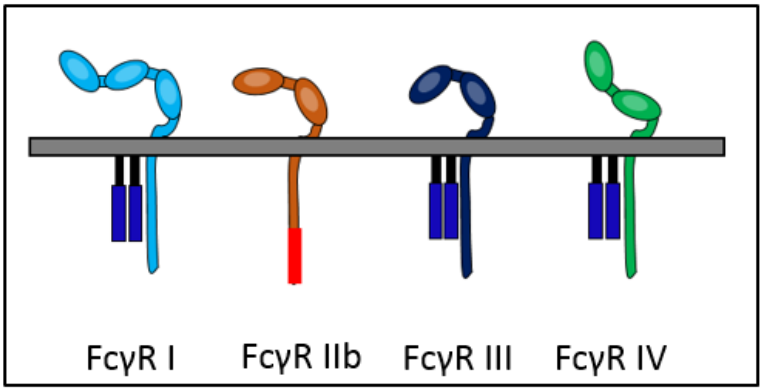

Figure 1. 2 Schematic diagram of murine Fc gamma receptors. FcγR I, III and IV are associated with dimeric FcRγ (blue and black) containing ITAM for recruitment of syk to generate activation signals for endocytosis or phagocytosis of opsonized targets. FcγR IIb is the inhibitory receptor bearing ITIM (red) for the recruitment of SHIP. Signaling generated by FcγR IIb down regulates signaling from all three activating FcγRs.

1.4.3. Mechanism of Fc gamma receptor mediated internalization of opsonized targets

Signaling downstream of the FcR includes multiple kinase and phosphatase activities that coordinate rearrangements of the actin cytoskeleton to facilitate phagosome formation. Syk recruits and activates effector molecules including phospholipase C gamma (PLCγ) and phosphoinositide-3-kinase (PI3K) (Crowley *et al.,* 1997; Law *et al.,* 1996). PI3K converts phosphoinositol-4,5-bisphosphate (PIP_2) to phosphoinositol-3,4,5-trisphosphate (PIP_3) at the plasma membrane. PIP_3 is retained at the plasma membrane by pleckstrin homology (PH) domain containing proteins, where it promotes the activities of guanosine nucleotide exchange factors (GEFs) (Thapa *et al.*, 2015; Vonkova *et al.*, 2015). GEFs such as T-cell lymphoma invasion and metastasis 1 (Tiam), VAV guanine nucleotide exchange factor 1 (Vav1) and ARF nucleotide binding site opener (ARNO) activates small GTPases via binding-induced conformational changes that releases bound GDP in exchange for GTP (Takai *et al.,* 2001). Tiam activates Ras-related C3 botulinum toxin substrate 1 (RAC1) thereby enabling binding and activation of effector proteins that drive cell movement, invasion and actin dynamics during phagocytosis (Bollag *et al.*, 2000). Vav1 activates Rho family GTPases with profound effects on actin dynamics (Heo *et al.*, 2005). ARNO activates members of the ADP ribosylation factor proteins (ARF) which play roles in actin assembly at the site of phagocytosis (Jayaram *et al.*, 2011; Santy & Casanova, 2001).

PLCγ hydrolyzes phosphoinositol-3,4-bisphosphate (PI-3,4-K) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) . IP_3 diffuses from the plasma membrane to the endoplasmic reticulum where it acts as a ligand for IP3 receptor to mediate release of

intracellular calcium (Rhee, 2001). Calcium and PKC are important for actin polymerization and activation of the NADPH oxidase complex. Activation of the complex enables generation of reactive oxygen species to kill internalized microbes (Hempel & Trebak, 2017; Nunes & Demaurex, 2010).

Activation signals must be regulated to prevent tissue damage and pathological conditions related to inflammatory disorders. Downregulation of activation signals is orchestrated by a system of signaling molecules organized by FcγRIIb. Tyrosine phosphorylation of ITIM in FcγIIb enables recruitment of SH2-domain containing inositol 5-phosphatase (SHIP-1) which binds phosphorylated tyrosine within the ITIM with high affinity (Daniele D'Ambrosio, 1996; Sarah J. Famiglietti, 1999). SHIP hydrolyses PIP_3 back to PIP_2 causing its removal from PH domains of Vav1 and Btk at the plasma membrane. This leads to inactivation of GTPases and concomitant inhibition of actin assembly and myosin associated contractile activity required for phagosome formation and closure (Salamon & Backer, 2013). In B cells, FcγR IIb recruits growth factor receptor bound protein 2 (Grb2) and docking protein 3 (Dok-3) (Manno *et al.*, 2016; Pauls & Marshall, 2017). Association of Grb2 with SHIP-1 minimizes its availability to activation effectors such as CD19 and PI3K. This further reduces the concentration of PIP3 available at the plasma membrane. Dok-3 phosphorylation upon recruitment to SHIP-1 drives activation of RasGAP causing downstream inhibition of Erk and intracellular calcium flux (Neumann *et al.*, 2011; Stork *et al.*, 2007). Thus, combined signaling from FcγR IIb and activating FcγRs regulate spatiotemporal signaling to ensure phagocytic cells achieve the necessary threshold required for efficient capture, internalization, destruction of opsonized targets, antigen presentation and generation of inflammatory cytokines.

2. Research Goal and Objectives

The goal of this study was to investigate the role of murine Fc gamma receptors in mediating the clearance of antibody opsonized nanovesicles and the interplay of receptor mediated endocytosis and macropinocytosis, intracellular transport and degradation of antigen. The achievement of this goal revolved around three main objectives as briefly described below.

Objective1: Develop a nanovesicle system for investigating FcγR endocytosis in murine macrophages.

To address the goal of this study, we first developed an appropriate nanovesicle system sensitive for only FcγR endocytosis with minimal uptake by other receptors in macrophages. Current approaches to studying FcγR-mediated endocytosis in phagocytes utilize silica beads or liposomes. Whilst this approach may be beneficial for studies involving scavenger receptors, it is challenging for studies involving FcγRs due to uptake mediated by other receptors and associated nanotoxicity (Fu *et al.*, 2014; Hsu & Juliano, 1982). As a result, it is nearly impossible to distinguish between Fc receptor specific uptake and scavenger receptor mediated internalization in macrophages. For objective one, the following research questions were investigated:

I. Unopsonized liposomes should have minimal uptake.

II. Addition of IgG to their surface should promote significant internalization.

III. IgG opsonized liposomes should enable modulation of macrophage response by changing antigen and antibody concentrations?

Objective 2: Investigate the contributions of FcγRs (I, IIb and III) and FcRγ to endocytosis of IgG2a opsonized nanovesicles, and the influence of FcγR knockout on macropinosome formation in macrophages.

The ideal nanoparticles should be internalized in large quantities by macrophages in the presence of an opsonizing antibody and have minimal to no uptake in the absence of the antibody. The elimination or drastic reduction of non-specific uptake will pave the way for relatively accurate analysis of FcγR endocytosis against a macrophage FcR-receptor-knockout background. Thus, relative contributions of each FcγR and Fcγ chain will be investigated using macrophage cells having one or more of its Fc gamma receptor knockout by a gene disruption method. Furthermore, it will enable investigation of any possible relationship between FcγRs and macropinosome formation in macrophages. The relationship between FcγRs and macropinosome formation in macrophages or phagocytes in general is unknown and there is yet no published work on this study. For objective two, the following research questions will be investigated:

I. How does single and multiple Fc receptor knockouts influence uptake of IgG2a opsonized nanoparticles in macrophages?

II. Which Fc receptors are most important for binding and clustering of antibody opsonized nanoparticles during endocytosis.

III. Does antibody opsonized nanopaticles drive macropinosome formation in macrophages?

IV. Do Fc receptors influence differential macropinosome formation in macrophages?

Objective 3: Investigate trafficking of antibody opsonized nanovesicles in macrophages

The intracellular path of internalized antibody-antigen complexes will be investigated with focus on the interplay of receptor mediated endocytosis and macropinocytosis during the internalization, intracellular transport, segregation and degradation of antibody opsonized nanovesicles. Specifically, the following research questions will be investigated:

I. What is the internalization mechanism of antibody opsonized nanovesicles in murine macrophages; is it a receptor dependent uptake, fluid phase uptake (macropinocytosis) or a combination of both mechanisms?

II. What is the role of macropinosomes in antigen-antibody segregation?

III. Do macrophages recycle internalized IgG and can recycled IgG potentiate uptake of fresh antigen?

3. Research Relevance

1. Elucidate novel mechanisms in antibody-antigen traffic in macrophages

Phagocytosis of large particles has been extensively studied in macrophages. The mechanism of internalization, role of phagocytic receptors and effector proteins have received great attention in the context of phagocytosis more than endocytosis. Understanding the functions of known and novel factors that enable phagocytes to eat or not to eat an opsonized large target are under intense investigation and represent some of the current research efforts to dissect the complexity of phagocytic signaling under various physiological and pathological conditions (Bakalar *et al.*, 2018; Chow & Chen, 2018). Despite the tremendous progress made in studying uptake of large particles, corresponding study of small particle uptake has not received this level of attention; consequently, many of the mechanistic details of FcγR endocytosis in macrophages remain unclear. It is particularly important to investigate FcγR-mediated endocytosis in macrophages due to its central role in innate and adaptive immunity. This effort holds great potential to unravel new pathways of immune complex trafficking, antibody recycling and cargo degradation that will deepen our understanding of macrophage biology. The downstream trafficking steps in macrophages that determine the fate of small soluble immune complexes remain to be investigated. This research is in part, an effort to address the knowledge gap in FcR mediated small particle trafficking in macrophages.

2. Recycling and availability of IgG by improved antibody design

Increasing the affinity of therapeutic antibodies for FcRn causes a corresponding increase in half-life As a result, efforts to increase antibody circulation time and availability have focused on FcRn mediated recycling. FcRn binds IgG with high affinity at low pH and recycles internalized IgG back to the cell surface (Shreeram Akilesh *et al.*, 2007; Datta-Mannan *et al.*, 2012; Kuo & Aveson, 2011). However, this trend is not consistent for all therapeutic antibodies, suggesting that some factors in the trafficking pathway influence the fate of internalized antibody therapeutics. There has been recommendations for a more elaborate examination of antibody trafficking to provide complementary information that will improve the design of therapeutic antibodies (Braster *et al.*, 2017; Gurbaxani *et al.*, 2013). Moreover, most of the models of FcRn mediated recycling are based on studies in epithelial cells whilst information on macrophage antibody traffic and recycling is scanty. Macrophages are primary tissue phagocytes responsible for clearance of soluble immune complexes, regulation of antibody homeostasis and consequently therapeutic antibody half-life (Shreeram Akilesh *et al.*, 2007). Therefore, investigating the trafficking of antibody-antigen complexes in macrophages will offer useful insights to improve the design, application and targeting of therapeutic antibodies.

3. ***Provide a robust and reproducible method for studying FcγR endocytosis in phagocytic cells.***

Studying FcγR-mediated small particle uptake in macrophages is challenging due to the notorious ability of phagocytes, particularly liver macrophages, to quickly internalize particles by non-specific uptake mechanisms (Sadauskas *et al.*, 2007; S. S. Yu *et al.*, 2012). This makes it challenging to interpret experiments involving naked silica beads and liposomes. Successful design and application of robust and reproducible fluorescent nanovesicles system will help to overcome the common challenges encountered in the study of small particle endocytosis. Moreover, the ability to adjust antigen and antibody concentrations will enable studies on different immune complex densities and how it influences macrophage signaling and the immune response.

4. ***Provide nanovesicles system for testing therapeutic antibody uptake in macrophages***

Since the nanoparticle system will be fine-tuned for FcR endocytosis, it can be extended to analyze the uptake, trafficking and degradation of new therapeutic antibodies. Studying clearance of antibody opsonized viral particles in phagocytes is challenging due to competition for the viral particle by scavenger receptors. Our nanoparticle platform could be used for preliminary studies to investigate the uptake and trafficking of new antibodies in phagocytes.

Thus, macrophages have indispensable roles in the immune system. They utilize both macropinocytosis and FcγR mechanisms for antigen uptake thereby ensuring clearance of pathogen and apoptotic cells. However, FcγR mediated trafficking of small particles in macrophages is not as extensively characterized as phagocytosis of large particles. The interplay of macropinocytosis and FcγRs during uptake of small particles remain to be investigated. The following chapters consists data and results aimed at addressing current gaps in our knowledge of small particle trafficking in macrophages.

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CHAPTER 2

Fc gamma receptors mediate uptake of IgG opsonized nanovesicles and promote macropinocytosis in murine macrophages

1. Introduction

Fc gamma receptor (FcγR) mediated endocytosis of antibody opsonized particles is an important immune function performed by macrophages and other cells of the mononuclear phagocytic system (Swanson, 2008). It involves engulfment of antibody coated particles in response to binding of Fc receptors to Fc domains of antibodies on a target (Sobota *et al.*, 2005).

Macropinocytosis is the process for non-specific uptake of extracellular fluid. Macropinosomes form as membrane bound vacuoles when ruffles from cell membranes fold over unto the cell surface capturing and internalizing extracellular milieu. In macrophages, macropinocytosis occurs spontaneously but can be upregulated in response to binding of membrane receptors to ligands. (Buckley & King, 2017; Jieqiong Lou *et al.*, 2014). Macropinosomes are relevant to immune functions of phagocytes because they are routes for nutrient uptake and fluid phase antigen sampling (Liu & Roche, 2015; Swanson, 2008).

Many studies have worked to understand the individual roles of the different FcγRs in physiological and pathological processes. The four FcγR in mice: FcγRs I, IIb, III and IV, have different affinities for different IgG subclasses and downstream signal transduction (Beutier *et al.*, 2017; Bruhns, 2012; Guilliams *et al.*, 2014). Most of these studies focused on phagocytosis of large particles (>3 μm) and to a lesser extent, the endocytosis of

antibodies and soluble immune complexes. Consequently, little is known about their differential roles in mediating endocytosis of antibody bound nanovesicles.

Macropinocytosis contributes to the non-specific uptake of fluid-phase antigens and subsequent antigen presentation by macrophages and dendritic cells (Christopher C. Norbury, 1997; Liu & Roche, 2015). It shares many similarities with phagocytosis including the involvement of common gene products such as PI3K, PLCγ, PIP_3 and actin. Macropinocytosis and phagocytosis differ in that phagocytosis is initiated by receptors engaged at the target surface, whereas macropinocytosis occurs both constitutively and in response to global receptor activation (Araki *et al.*, 1996; Levin *et al.*, 2015; Swanson, 2008; Tse *et al.*, 2003). It is unclear if phagocytic receptors, specifically FcγRs, drive macropinosome formation in macrophages. Recently, constitutive macropinocytosis in macrophages has been shown to be regulated by extracellular calcium and calcium sensing receptors (CasR) (Johnathan Canton *et al.*, 2016). This suggests that some membrane receptors can drive constitutive macropinocytosis, thereby enabling efficient antigen sampling in the absence of an active or strong phagocytic signal.

We investigated the differential role of two murine activating FcγRs (I and III), FcRγ, and the inhibitory FcγIIb in the uptake of IgG2a opsonized nanovesicles and macropinosome formation in macrophages derived from mouse liver (Fetal Liver Macrophages or FLMs). Moreover, since FcγR mediated internalization and macropinocytosis share many regulatory proteins, we investigated whether FcγR knockouts have any impact on the

ability of macrophages to perform macropinocytosis. We utilized CRISPR/Cas9 expressing FLMs and synthetic gRNAs targeting unique sequences to knockout FcγRs and Fcγ. The gRNA is a hairpin-like RNA recruited by the CRISPR/Cas9 enzyme enabling the enzyme to target specific DNA sequences to mediate gene knockout.

2. Results

FcγRs drive differential internalization of IgG2a opsonized liposomes

We tested the hypothesis that FcγRs differentially facilitate internalization of antibody-opsonized nanovesicles. We generated single, double and triple knockouts of FcγR in FLM cells using CRISPR/Cas9 and synthetic gRNAs targeting each receptor and the FcRγ. Liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocoline (POPC, 90 mol %), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-[Methoxy(Polyethylene glycol)-2000] ammonium salt (DSPE-PEG-2000, 9 mol %) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)2000] ammonium salt (DSPE-PEG2000-Biotin, 1 mol %) opsonized with anti-biotin monoclonal IgG2a were added to FLM macrophages bearing FcγRI^{KO}, FcγRIIb^{KO}, FcγRIII^{KO}, FcγRIIb&III^{KO} (DKO), Fcγ^{KO} and FcγR I, IIb & III^{KO} (TKO). for 1 hour. After thorough washing in PBS to remove excess IgG2a opsonized nanoparticles, cells were harvested and analyzed on a BD Accuri flow cytometer to measure the amount of DiD-liposomes internalized across knockout cell lines. Analysis of opsonized liposome uptake showed that FcγRI and FcRγ^{KO} cells were defective for uptake, demonstrating that the high affinity FcγRI and associated FcRγ are

critical for uptake of IgG2a opsonized nanoparticles. Triple knockouts (TKOs or FcγRI/IIb/III^{KO} cells) were indistinguishable from untreated parental cells, demonstrating that all three receptors (I, IIb and III) contributed at varying degrees to endocytosis of IgG2a-nanovesicles (Figure 2.1 A). FcγRIII^{KO} had only a minor deficiency in uptake of IgG2a-liposomes, consistent with a low affinity of FcγRIII for IgG2a (Figure 2.1 A). Double FcγRIIb/III^{KO} showed a stronger defect than either FcγRIII^{KO} or FcγRIIb^{KO}, indicating that both FcRs contributed to uptake of IgG2a-liposomes.

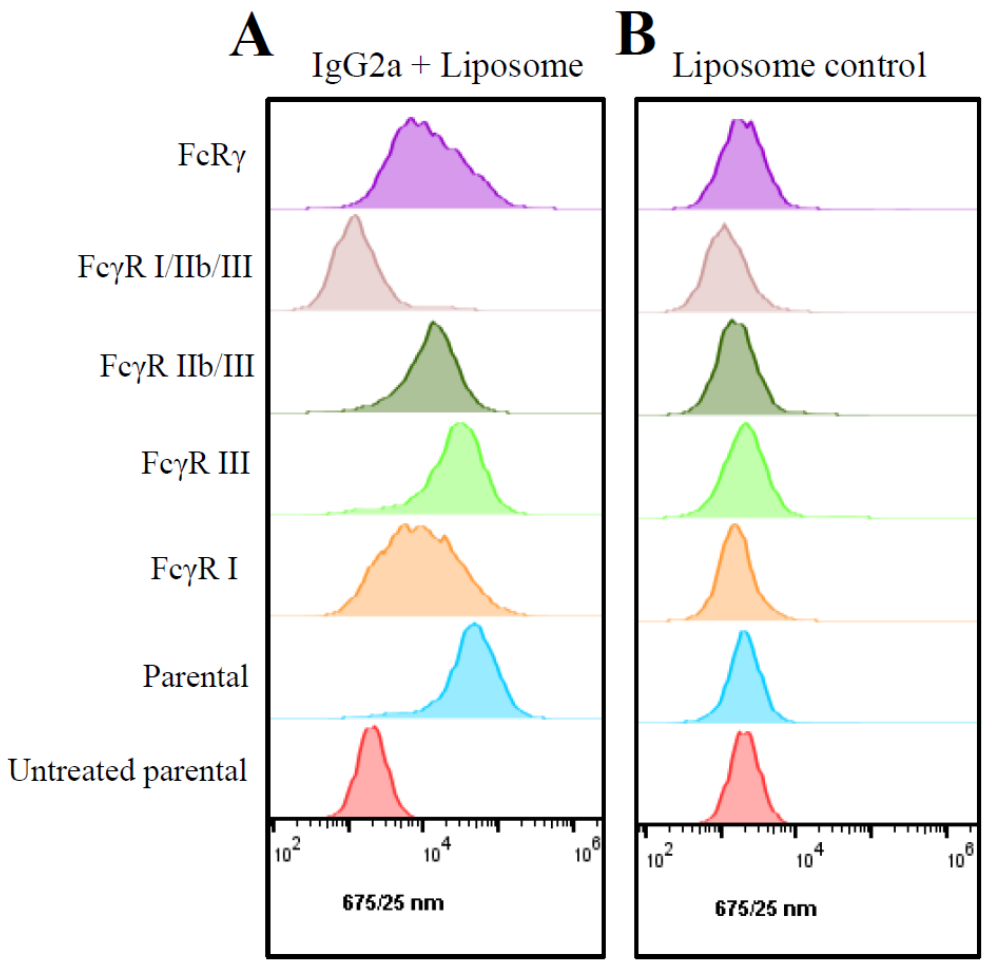

Figure 2.1 FcγRs drive uptake of IgG2a opsonized liposomes in macrophages. Flow cytometry analysis of igG2a-liposome uptake in FcγR^{KO} FLMs after 1-hour exposure. (a) Internalization of IgG2a opsonized liposomes in knockout lines. High affinity receptor FcγR I (orange) and associated Fcγ- (violet) are most critical for uptake, however, triple knockout cells (brown) have highest defect in uptake of liposomes whilst low affinity FcγR III^{KO} has the least defect compared to FcγR IIb/III double knockout (green). (b) Internalization of non-opsonized liposomes in knockout lines. all populations are almost perfectly superimposed demonstrating that the phenotypic differences observed in IgG2a-treated cells are as a result of uptake of antibody bound nanoparticles mediated by fc receptors, and not some non-specific uptake mechanism.

FcγRI and FcRγ mediate binding and clustering of IgG2a-opsonized liposomes

An important step in potentiating phagocytic signal is clustering of FcγRs and aggregation of bound immune complexes (Sobota *et al.*, 2005). To investigate impact of receptor knockout on immune complex binding and cluster size, we exposed cells to IgG2a-liposomes for 5 minutes in the presence of 40 kDa dextran and imaged them on a spinning disk confocal microscope. The area of IgG2a-liposome endosome, integrated intensity, and the number of bound and internalized endosomes were analyzed by applying a Cell Profiler pipeline to the confocal microscopy data. Consistent with our earlier observations in flow cytometry uptake assay (Figure 2.1 A), FcγRI^{KO} and FcRγ^{KO} had the lowest intensity of bound/internalized vesicles, accordingly, they produced smaller endosomes than parental cells (Figure 2.2 A-C and Figure 2.10). FcγRIII^{KO} had decreased binding and reduced endosome sizes compared to parental FLM cells, however, these defects were not as strong as that observed in FcγRI^{KO} and FcRγ^{KO} (Figure 2.2 A-C and Figure 2.11). It is noteworthy that triple knockout cells (TKOs or FcγRI/IIb/III^{KO}) had completely lost the ability to bind and internalize antibody opsonized liposomes, supporting our earlier assertion that removal of the three receptors (I, IIb, III) completely blocks binding and internalization of antibody opsonized nanoparticles (Figure 2.2 A-C and Figure 2.11 C).

FcγRIIb ^{KO} cells have a reduced endosome area of IgG2a-liposome complexes

Analysis of IgG2a-liposome complexes in FcγRIIb^{KO} cells showed a reduced endosome area compared to parental control (Figure 2C). They had more bound/internalized endosome count compared to parental cells (Figure 2B). However, compared to parental

control, FcγRIIb^{KO} cells had similar average intensities for bound IgG2a-liposome complexes (Figure 2.2 A and Figure 2.10).

Similar integrated intensity relative to parental cells suggests that FcγR IIb is not critical for binding compared to FcγRI and FcRγ. However, higher number of bound endosomes suggest that FcγRIIb may be required for the formation of large endosomes as observed in controls. Thus, FcγR IIb^{KO} cells are not defective for binding of IgG2a opsonized liposomes due to the presence of high affinity FcγRI, FcRγ, and low affinity FcγR III but may require FcγR IIb for efficient formation of large endosomes similar to those observed in controls.

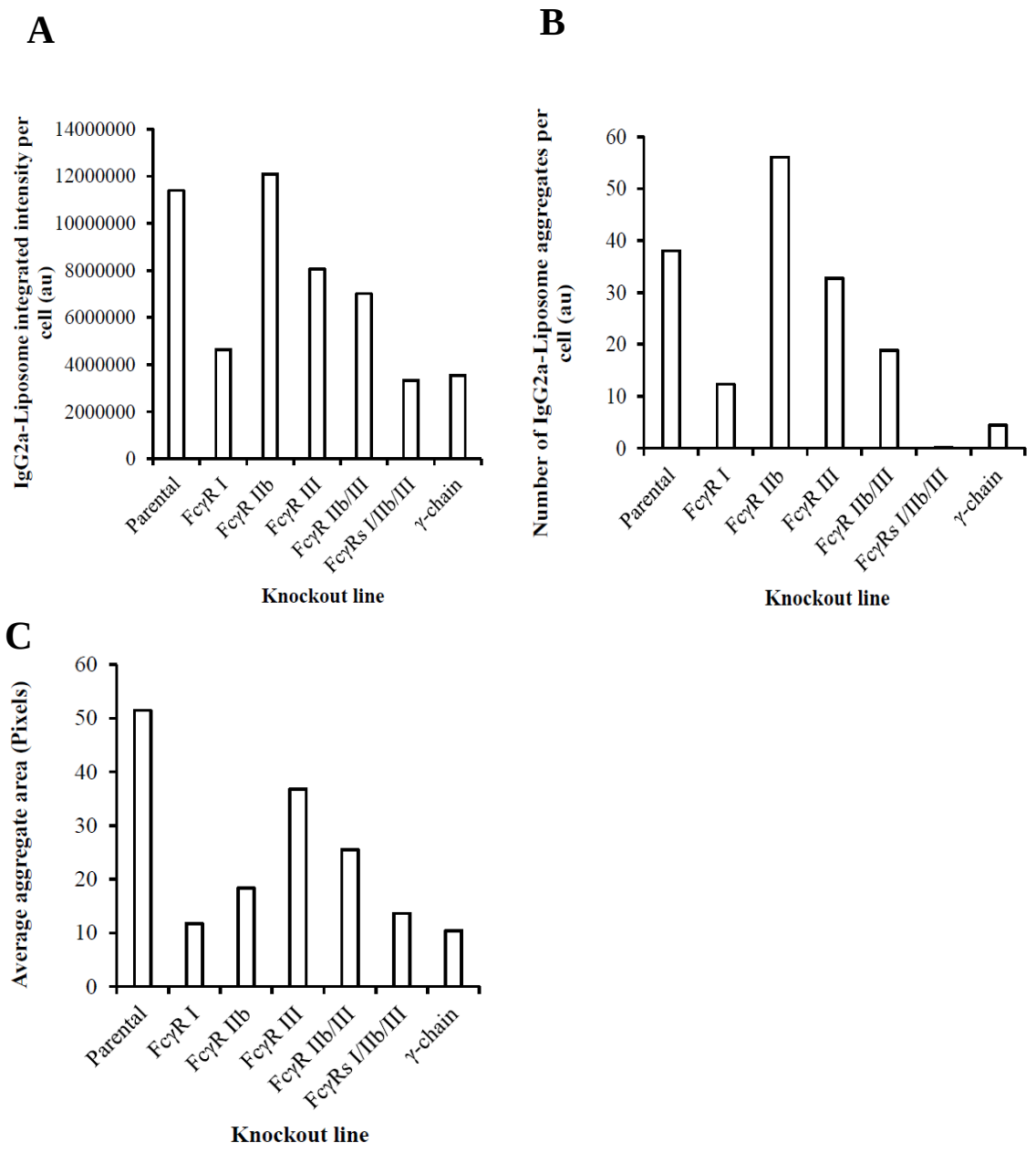

Figure 2.2 FcγRs regulate binding of IgG2a opsonized liposomes to macrophages. (A) Integrated intensity of IgG2a-liposomes in 120 cells sampled across all receptor knockouts. FcγRI, FcγRI/IIb/III and FcRγ knockout cells have the lowest intensity indicative of defective binding or uptake. (B) Number of IgG2a-liposome endosomes counted across $FcγR^{KO}$ cells. $FcγRI^{KO}$ and $FcRγ^{KO}$ cells have the least amount of bound/internalized IgG2a-liposomes whilst TKO is almost completely devoid of them. $FcγRIIb^{KO}$ has the highest number of bound endosomes but are not defective for binding. (C) Average area of bound/internalized endosomes. $FcγRI^{KO}$, $FcγRIIb^{KO}$, TKO and $FcRγ^{KO}$ have the smallest endosomes consistent with defective binding and aggregation associated with high affinity receptor knockout and requirement of all three receptors for uptake of antibody opsonized targets in macrophages. IgG2a-liposome endosomes in $FcγRIIb^{KO}$ has reduced area compared to parental cells demonstrating that FcγRIIb plays an important role in aggregation.

FcγR I, FcγR III and FcRγ drive micropinocytosis attenuated by FcγRIIb

To delineate the contributions of FcγRs to macropinosome formation, we tested the hypothesis that activating FcγRs I and III as well as the FcRγ, drive constitutive macropinocytosis. We used FLM cells consisting single, double and triple knockouts of Fc gamma receptors to analyze uptake of dextran upon stimulation of cells with IgG2a-liposomes. Cells were exposed to IgG2a opsonized DiD labelled liposomes in the presence of dylight-594-conjugated dextran for 5 minutes, washed in PBS and imaged on a spinning disk confocal microscope. Analysis of macropinosome formation in parental FLM cells showed normal levels of macropinosome formation but markedly reduced levels in FcR^{KO} cells (Figure 2.3, Figure 2.10 and Figure 2.11).

We repeated dextran uptake in FcR knockouts on a high content microscope, this time by including a dextran control in which cells were exposed to only dextran, and compared to dextran uptake in the presence of IgG2a opsonized liposomes. Consistent with our earlier findings, dextran uptake in the absence or presence of Fc receptor ligand IgG2a opsonized liposomes did not increase macropinosome formation in FcγR knockout cells (Figure 2.5). Thus, we observed a drop in constitutive and ligand-induced macropinocytosis in activating FcR knockout cells demonstrating that knockout of activating FcγRs causes a drop in macropinosome formation in macrophages (Figure 2.7). However, knockout of inhibitory FcγR IIb resulted in more than twice the number of macropinosomes compared to parental cells (Figure 2.6, Figure 2.7A-B and Figure 2.10C). Thus, knockout of activating Fcγ receptors downregulate macropinosome formation whilst knockout of the inhibitory FcγR IIb upregulates macropinosome formation in macrophages.

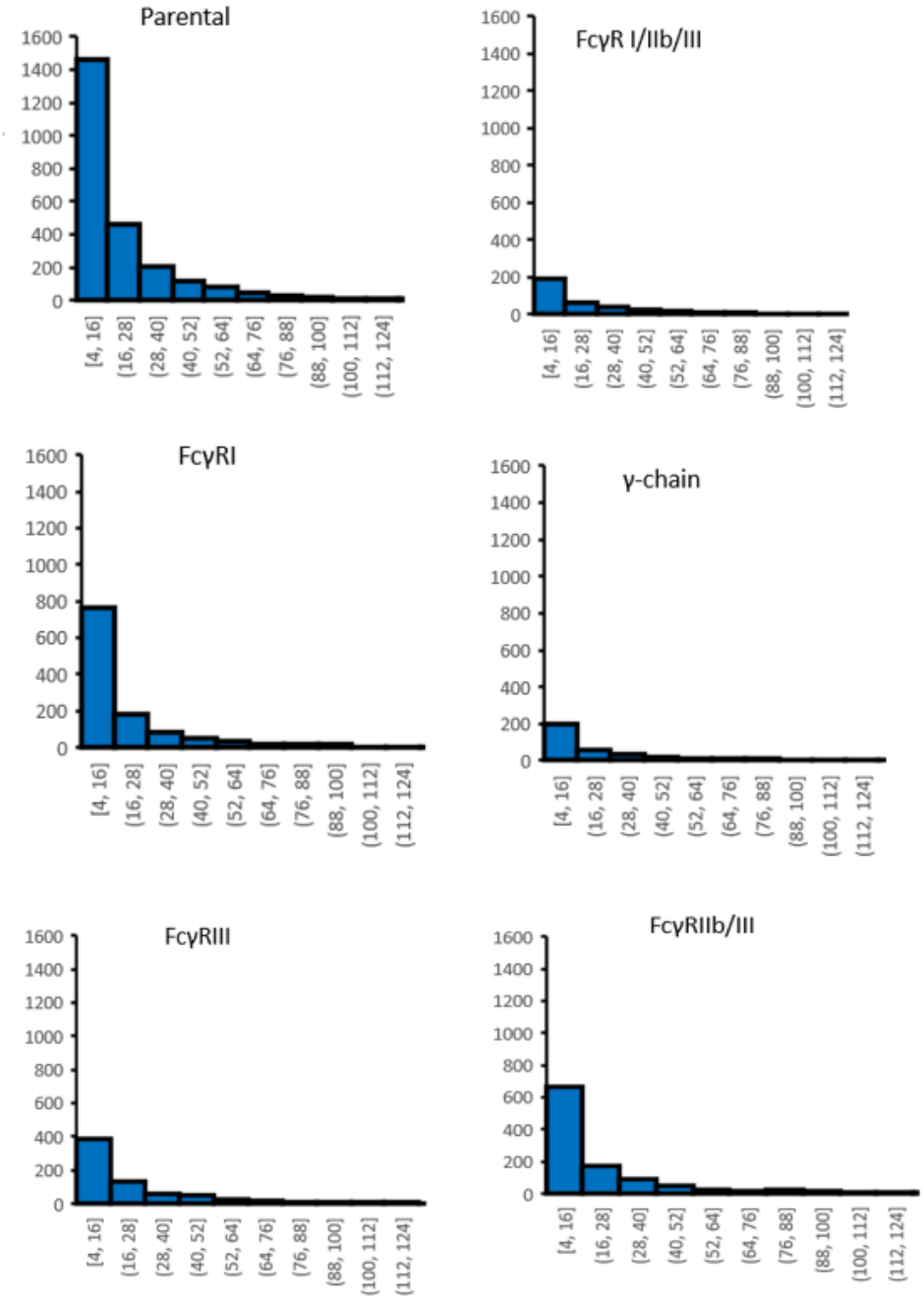

Figure 2. 3 Size distribution of macropinosomes in 120 cells each in FcγR^{KO} FLMs. Comparison of macropinosome areas in pixels in activating FcγR^{KO} cells and parental cells show a reduction in the number of macropinosomes formed in knockout cells compared to the parental.

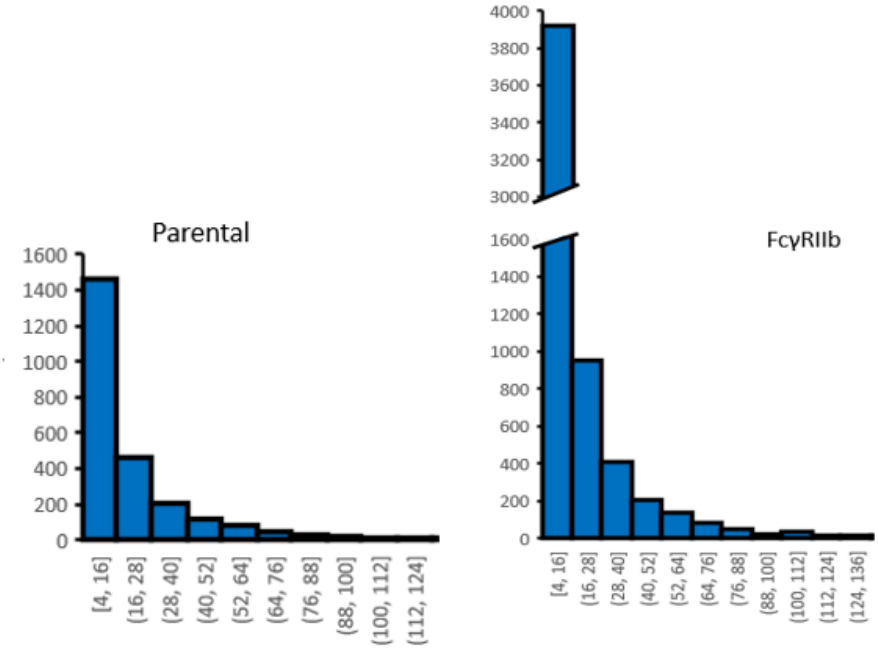

Figure 2.4 Comparison of macropinosome size distribution in FcγRIIb^{KO} FLMs compared to parental cells. FcγRIIb^{KO} cells generate almost three times the number of macropinosomes compared to parental cells demonstrating that FcγRIIb may have a novel role in regulating formation of macropinosomes.

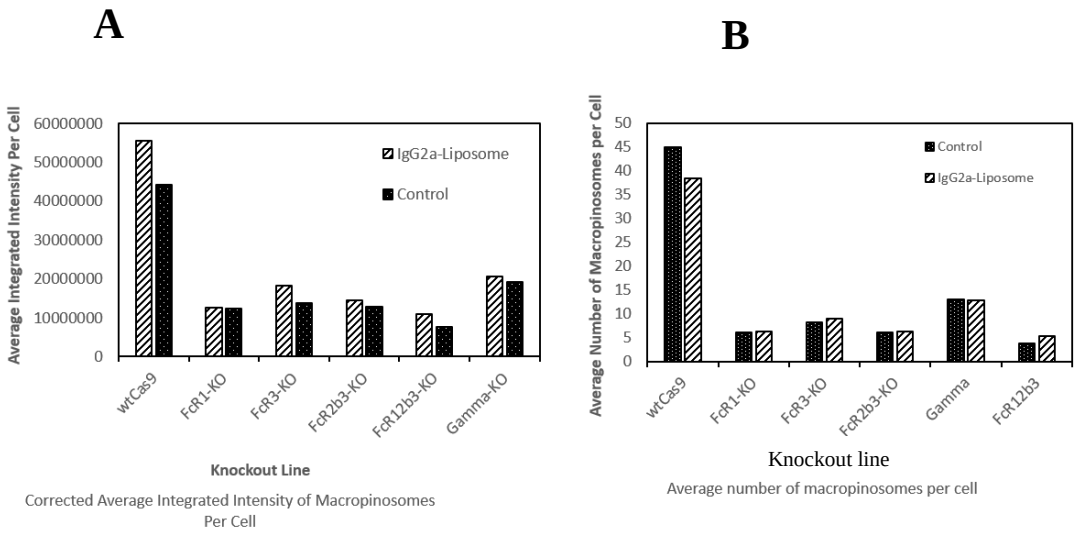

Figure 2.5 Activating FcγRs drive constitutive macropinosome formation. (A) Integrated intensities of dextran measured under two conditions namely IgG2a-liposome exposure in the presence of dextran (striped bar) and dextran only control (solid bar). Knockout of activating FcγRs result in drop in macropinosome formation in conditions (B) Average number of macropinosomes per cell counted across parental and FcγR knockouts. The lower number of macropinosomes supports data in A showing reduced ability of cells to perform macropinocytosis.

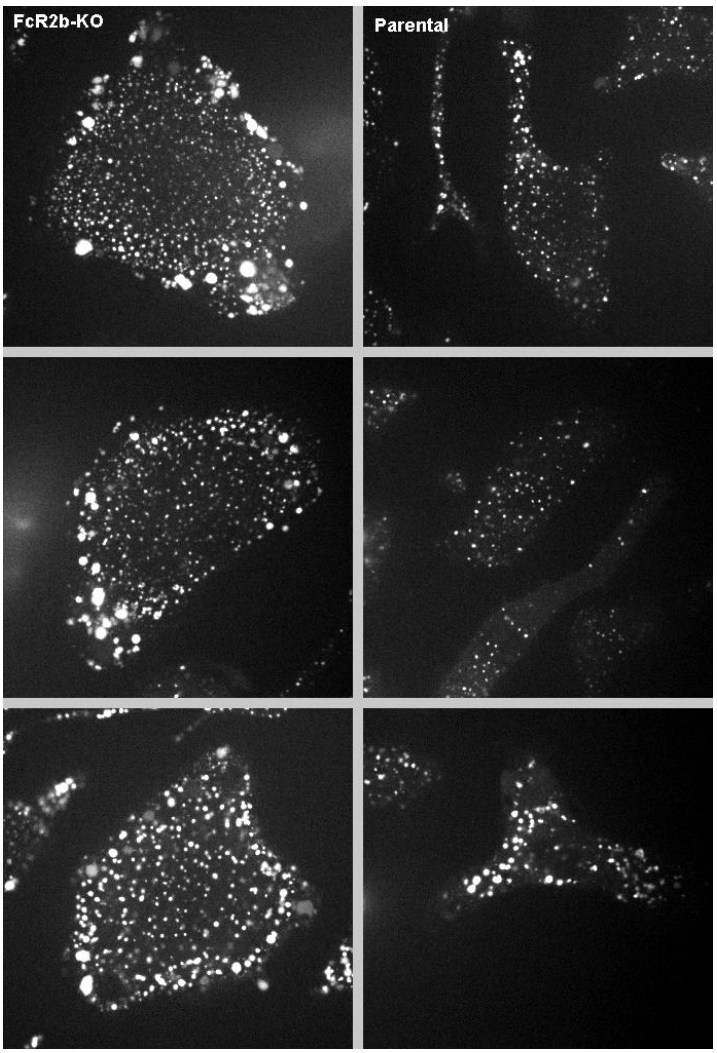

Figure 2.6 Comparison of macropinosome formation in FcγRIIb and parental cells treated with dylight-594 conjugated dextran. Upregulated macropinosome formation in FcγRIIb exists persists in the absence of IgG2a opsonized liposomes indicating that FcγRIIb attenuates constitutive macropinosome formation.

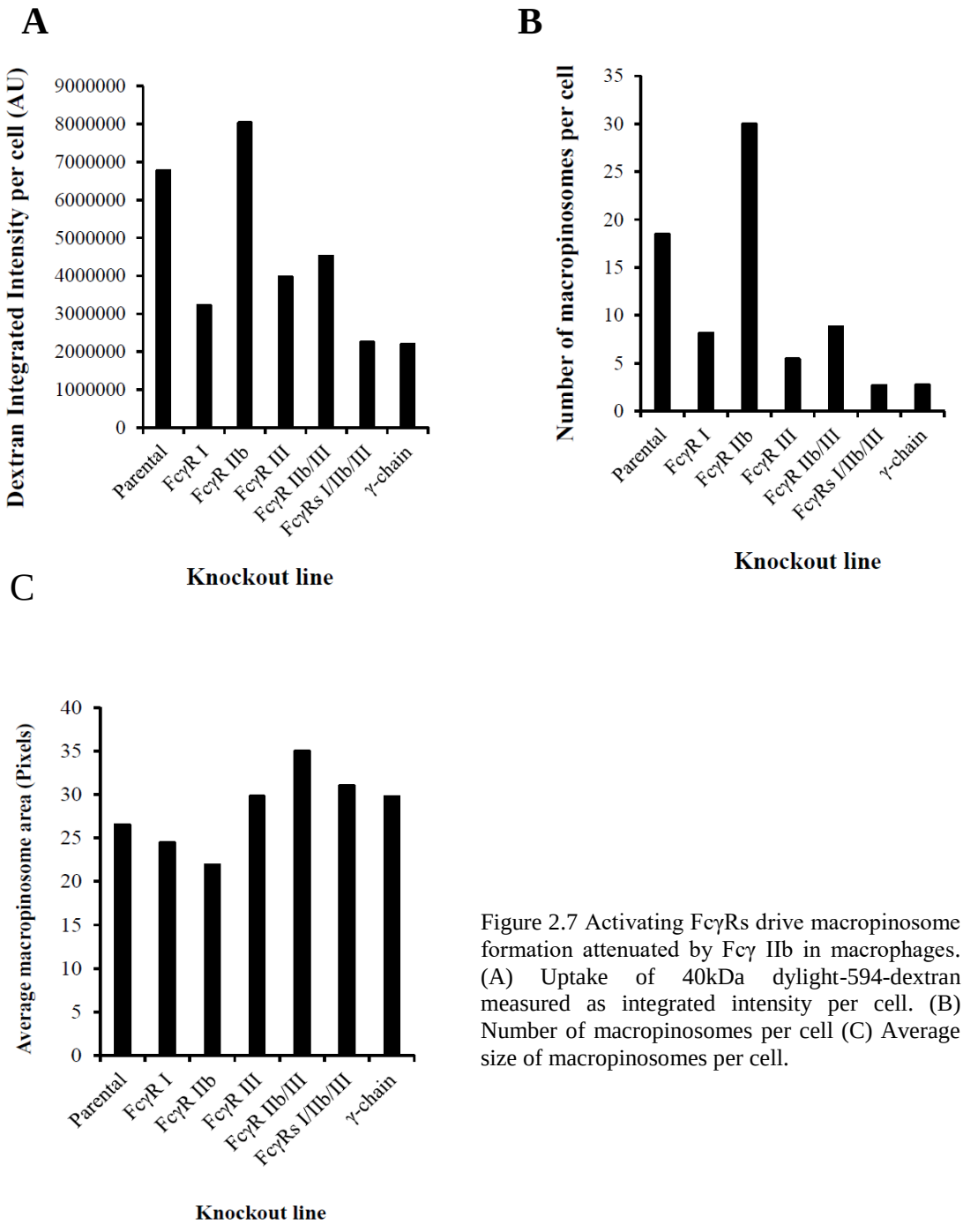

Figure 2.7 Activating FcγRs drive macropinosome formation attenuated by Fcγ IIb in macrophages. (A) Uptake of 40kDa dylight-594-dextran measured as integrated intensity per cell. (B) Number of macropinosomes per cell (C) Average size of macropinosomes per cell.

FcγRs drive macropinosome formation independent of ligand IgG2a-opsonized-liposomes

Since exposure of IgG2a-liposomes to FcγR knockouts did not increase macropinosome formation beyond dextran controls, we sought to investigate the effect of FcγR ligand-induced macropinocytosis in parental cells. First, bone marrow derived macrophages (BMDM) and fetal liver macrophages (FLMs) were cultured in bone marrow media overnight and exposed to IgG2a opsonized DiD labelled liposomes in the presence of 40kDa-dylight-594 dextran for 5 minutes. We observed similar levels of dextran uptake in FLM cells both in IgG2a-liposome test samples, and corresponding dextran controls (Figure 2.8). Next, we compared dextran uptake in CSF-1 starved primary macrophages upon stimulation with IgG2a-liposomes, free IgG2a and CSF-1 growth factor. We observed increased levels of macropinocytosis in cells stimulated with CSF-1 but levels remained low in IgG2a-liposome, free IgG2a and dextran controls (Figure 2.9). Thus, demonstrating that the ligand for Fc gamma receptors, whether bound to liposome cargo or not, does not drive significant increase in macropinosome formation in macrophages. This supports our earlier observations that FcRg receptors control a basal signaling circuit that drives a signaling of macropinosome formation independent of the ligand.

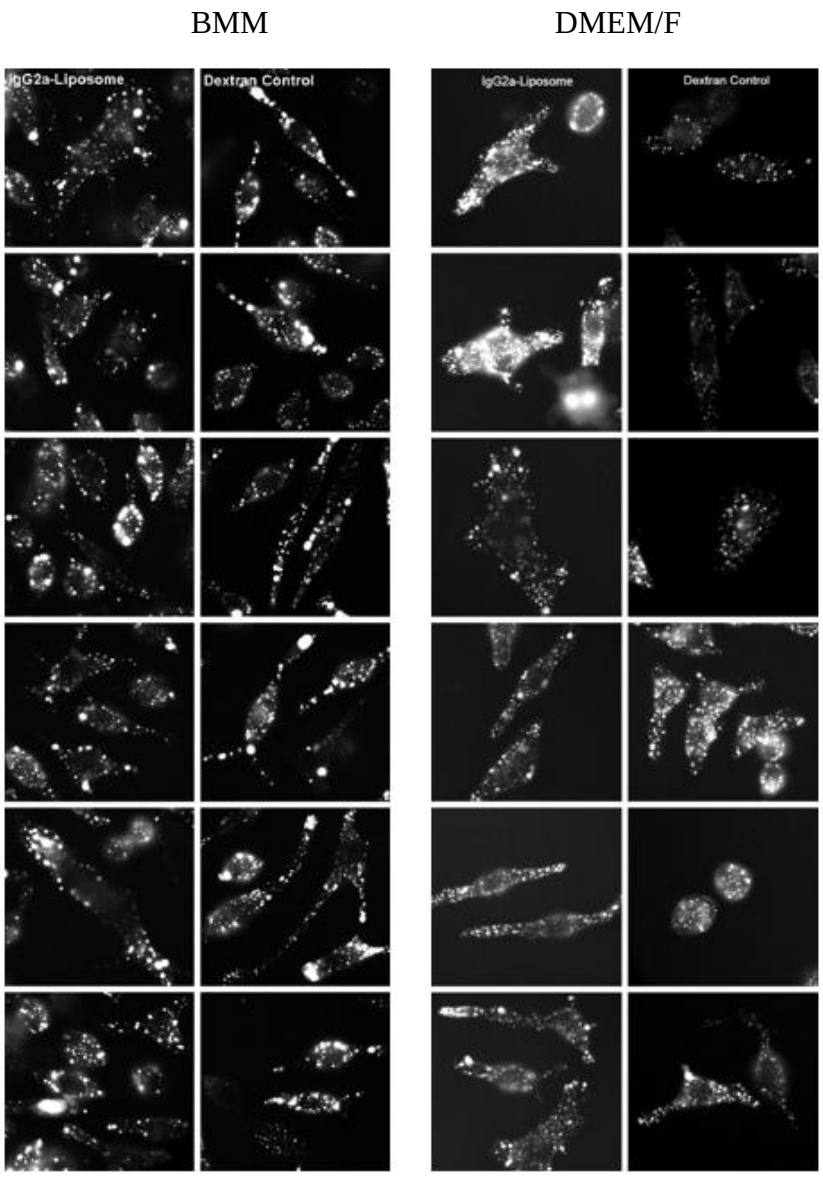

Figure 2.8 Exposure of IgG2a liposomes to FLM cells does not drive increased macropinosome formation in primary macrophages and FLMs. Twelve (12) fields of view each from FLM cells imaged under BMM condition and CSF-1 starved condition in DMEM/FBS.

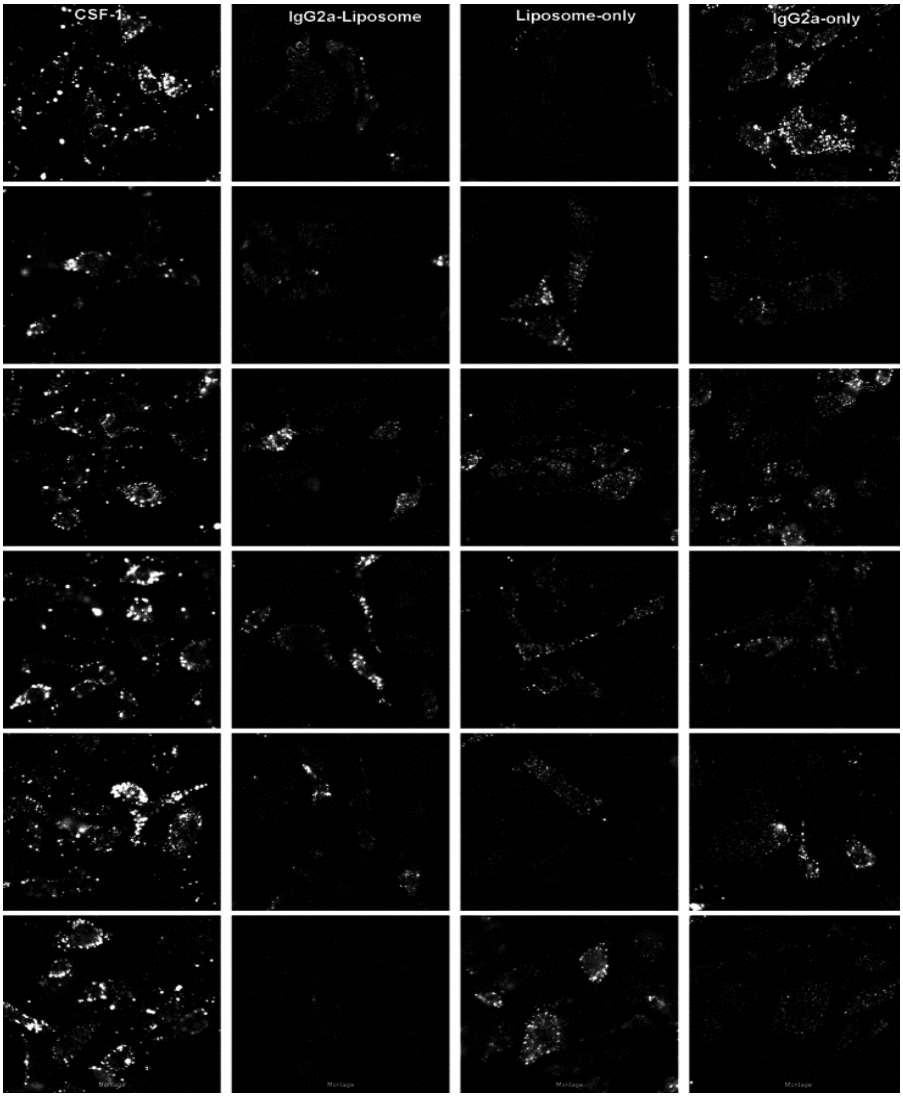

Figure 2.9 IgG2a-Liposome exposure to CSF-1 starved primary macrophages does not increase macropinosome formation. Primary macrophages starved in DMEM/FB and exposed to CSF-1, IgG2a-liposomes, liposome-only and IgG2a-only show marked increase in macropinosome formation in CSF-1 treated cells and reduction IgG2a-liposome, liposome-only and IgG2a-only cells.

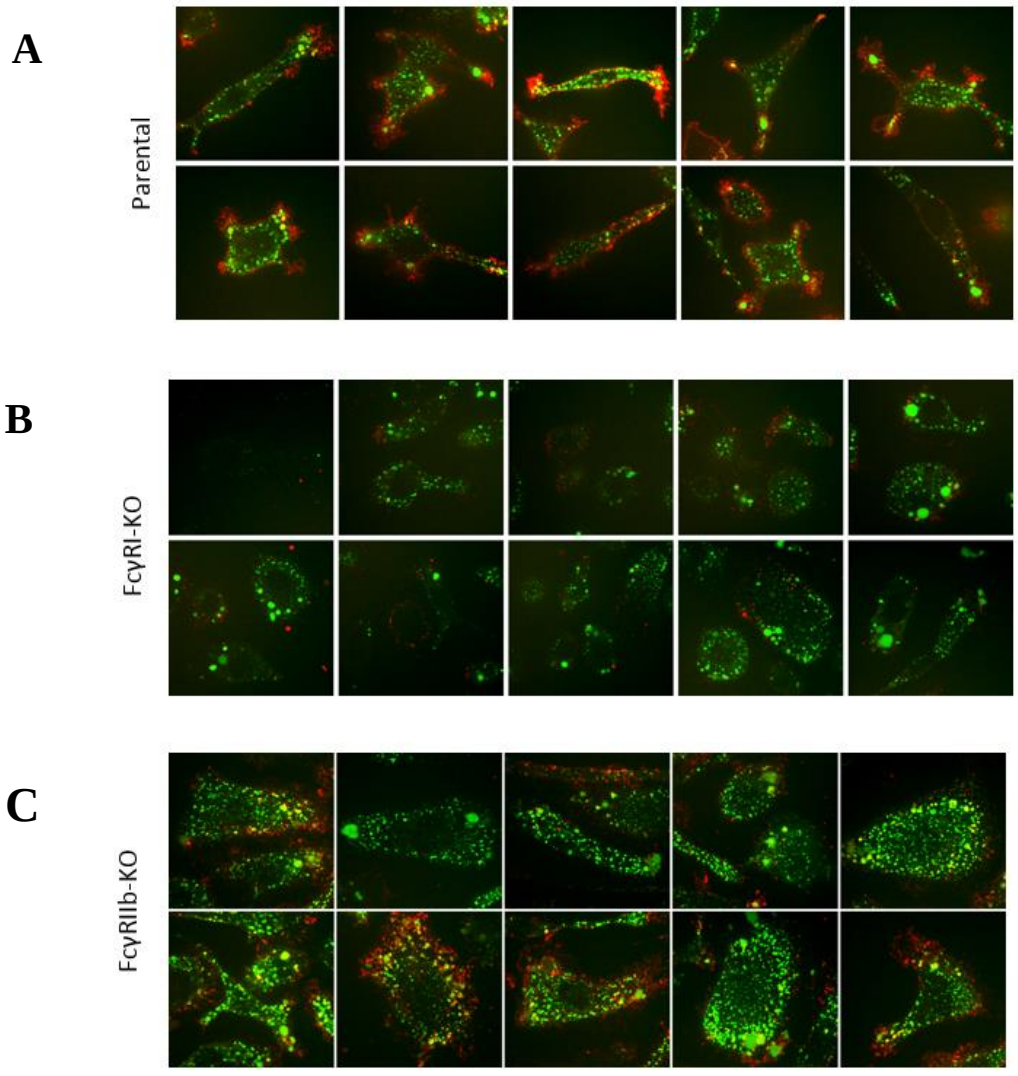

Figure 2.10 Confocal microscopy images (showing 10 fields of view each) of (A) parental, (B) FcγRI^{KO} and (C) FcγRIIb^{KO} FLMs at 5 minutes post exposure to IgG2a opsonized DiD-liposomes (red) in the presence of 40kDa-dylight-594 dextran (green).

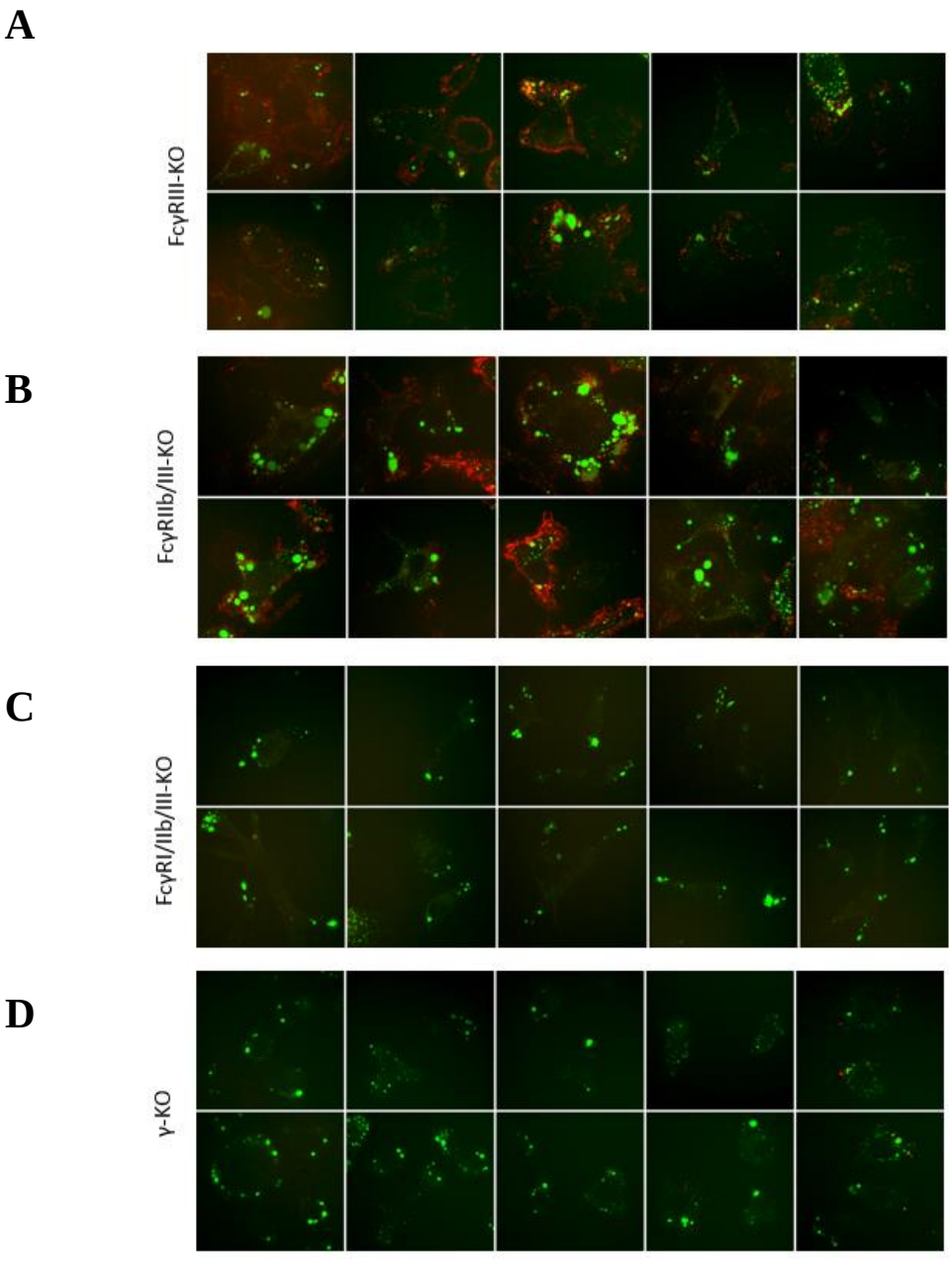

Figure 2.11 Confocal microscopy images (10 fields of view each) of (A) FcγRIII^{KO} (B) FcγRIIb/III^{KO} (C) Triple knockout or FcγRI/IIb/III^{KO} and (D) FcRγ^{KO} cells at 5 minute post exposure to IgG2a opsonized DiD-liposomes (red) and 40kDa dextran (green).

IgG2a opsonized liposomes are internalized by CME

Since uptake of antibody opsonized liposomes was receptor dependent and fluid phase independent. We investigated the hypothesis that internalization of IgG2a opsonized liposome nanovesicles occurs by clathrin mediated endocytosis (CME). FLMs expressing yellow fluorescent protein (YFP) fusion of clathrin light chain (clc-YFP) were made by retroviral transduction of PIB2-clc-YFP plasmid. Enriched population of clathrin expressing cells were obtained via flow sorting on a BD FacsJazz, and cultured in bone marrow media for ~1 week. Exposure of IgG2a-liposomes to these cells resulted in enhanced recruitment of clathrin to the membrane of the cells where it co-localized with IgG2a-liposomes on the membrane. Live cell movies also showed recruitment of IgG2a-liposomes to clathrin positive endosomes and their subsequent traffic to the macropinosome. Thus, we demonstrate that IgG2a-liposome endocytosis in macrophages involve clathrin mediated endocytosis, an observation consistent with other studies on uptake of immune complexes in phagocytic and non-phagocytic cells (Tse *et al.*, 2003).

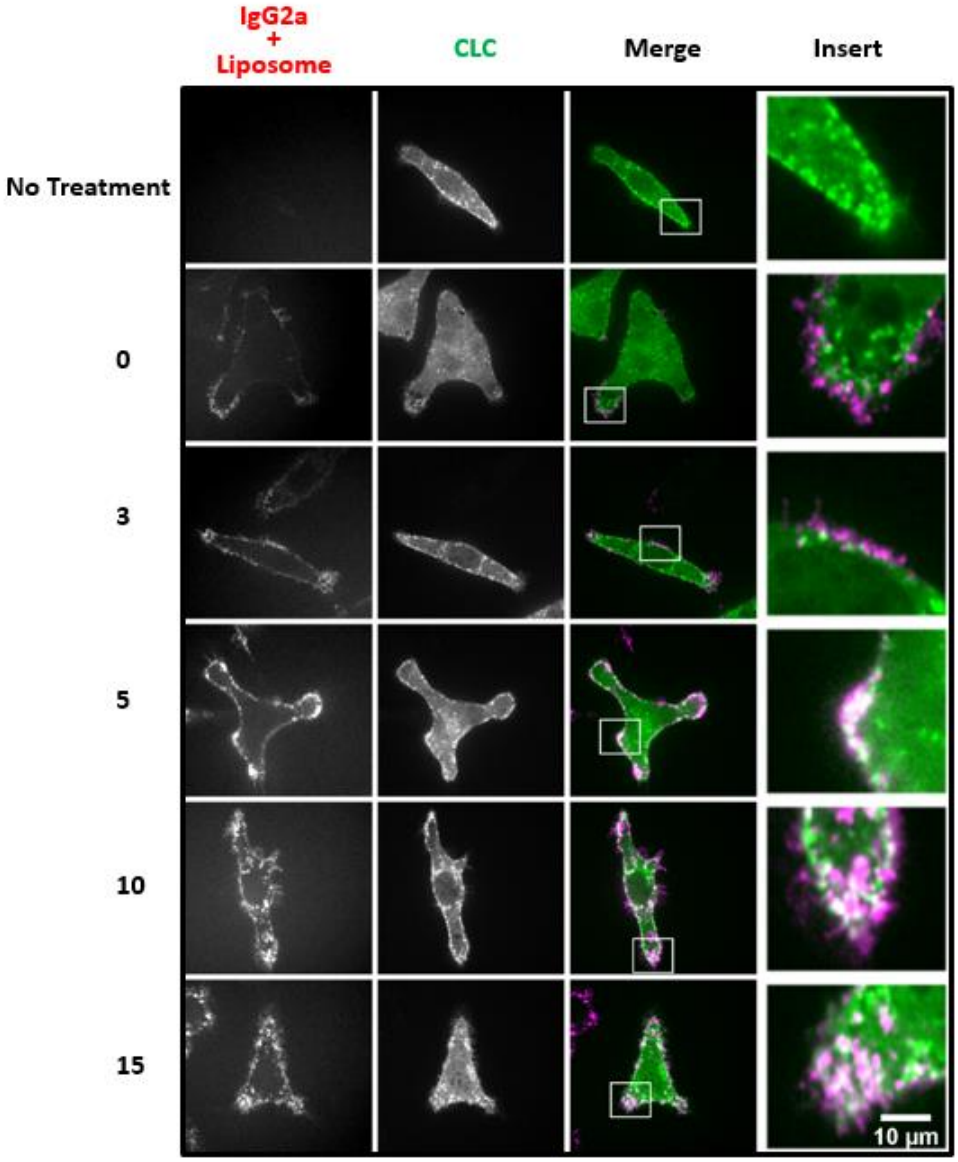

Figure 2.12 IgG2a-liposome endosomes are internalized by CME. FLM cells precooled on ice and exposed to IgG2a opsonized DiD-liposomes for 10 minutes on ice were washed in PBS and incubated in warm media for specific time from 3 through 15 minutes post exposure. Cells were fixed with PFA and imaged on confocal microscope. Co-localization of IgG2a-liposomes (red) with clc-YFP (green) is observed from 3 minutes through 15 minutes chase. Moreover, uptake of liposomes is blocked at zero minutes upon cooling on ice demonstrating that endocytosis is sensitive to CME and requires a receptor mediated uptake mechanism.

3. Discussion

We found that FcγRs I, IIb, III and FcRγ are all important in mediating uptake of IgG2a bound nanoparticles. This differential contribution of FcγRs underscores the significance of all three receptors in the spatiotemporal regulation of phagocytic signaling. This is supported by the observation that knockout of all three receptors completely abrogated binding. High affinity receptor FcγRI and associated FcRγ are the most significant factors required for binding, aggregation and internalization of opsonized nanoparticles. Moreover, our study highlights a new role for FcγRIIb as important for aggregation of bound nanoparticles. FcγRIIb^{KO} cells did not have a defect for binding due to expression of FcγRI but were defective for aggregation of liposomes similar to FcγRI^{KO} cells suggesting that binding is highly dependent on FcγRI but clustering requires FcγRI, and FcγRIIb and FcγR. Thus, both activating and inhibitory signals are required for aggregation of bound nanoparticles, which may have a direct impact on receptor clustering and downstream phagocytic signaling. This is consistent with the findings of Nimmerjahn and colleagues who showed that in vivo differences of IgG subclass activity were as a result of formation of different ratios of activating to inhibitory receptor complexes (Falk Nimmerjahn & Jeffrey V. Ravetch, 2005). Our findings on deficient aggregation of immune complexes in FcγR IIb^{KO} cells show a potentially important role in generation of phagocytic signals. Gallo and colleagues showed that the density of immune complexes had a direct effect on signal amplitude via increased phosphorylation of ERK, production of IL-10 and inhibition of IL-12 (Gallo *et al.*, 2010). Moreover, a critical step to generation of phagocytic signal is receptor binding and clustering (Sobota *et al.*, 2005); our data

identify FcR IIb as playing an important role in aggregation of IgG2a opsonized liposomes to form FcR-ligand complexes with possible implications for signals downstream of FcγRs.

Furthermore, our studies show that FcγRs drive constitutive macropinocytosis in macrophages independent of antibody opsonized ligand. CSF-1 starvation of primary macrophages before exposure to IgG2a-liposomes did not increase macropinosome formation above levels in untreated control cells but a drastic increase in macropinosome number upon treatment with CSF-1 was observed, showing that the ligand IgG2a-liposome did not drive increase macropinocytosis. This is a previously undescribed relationship between FcγRs and fluid phase uptake. Furthermore, analysis of dextran uptake in $Fc\gamma R^{KO}$ cells show that knockout of activating receptors and FcRγ reduce fluid phase uptake levels below that observed in control cells. This implies the existence of an uncharacterized basal signaling network orchestrated by FcγRs that drive constitutive macropinosome formation in macrophages.

Src family kinases phosphorylate tyrosine residues within the ITAM of FcRγ leading to recruitment of syk on phosphorylated tyrosines for downstream signaling (J C Cambier, 1995; Takeshi Suzuki *et al.*, 2000). In dendritic cells, Lyn and Fyn are constitutively activated and associated with Toll-like receptors (TLRs) (Dallari *et al.*, 2017). A link between FcγR signaling and the actin cytoskeleton has been found to involve the formation of SLP-76 complex consisting Nck, VASP and WASP, enabling transmission of signals from Fc receptors to the actin cytoskeleton. Whilst this mechanism mediates phagocytosis, it is possible that the formation of this complex also regulates constitutive macropinocytosis, a process highly dependent on actin remodeling. Moreover, the role of src family kinases in constitutive macropinocytosis in myeloid cells has been described

(Kasahara *et al.*, 2007), however, no connection has been made as yet to signaling from FcγRs in macrophages. FcγR signaling is coupled to actin dynamics, and utilizes effector proteins like Ras, PIP_3, PLCγ and Arp2/3 complex to drive actin mediated internalization of large particles (Swanson & Hoppe, 2004). Many of these proteins are also involved in macropinocytosis and therefore suggests the possible existence of a macropinosome signaling axis regulated by FcγRs.

4. Materials and Methods

Cell lines

Fetal liver macrophages were differentiated from fetus liver of Cas9 transgenic mice by culturing in bone marrow media enriched with CSF-1 growth factor for 6 weeks. Bone marrow derived macrophages (BMDMs), were differentiated from the femur of mice and cultured in bone marrow media for 10 days before experiments. Details of bone marrow macrophage differentiation has been previously described (Jieqiong Lou *et al.*, 2014).

Preparation of PEGylated liposome nanovesicles

The following lipids were obtained from Avanti Polar Lipids Incorporated: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocoline (POPC, #850457), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-[Methoxy(Polyethylene glycol)-2000] ammonium salt (DSPE-PEG-2000, #880120) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-

[biotinyl(polyethylene glycol)2000] ammonium salt (DSPE-PEG2000-Biotin, #880129). Stock solutions of lipids were prepared by dissolving solid samples in chloroform to make 25mg/mL POPC, 10mg/mL DSPE-PEG2000 and 1mg/mL DSPE-PEG2000-Biotin. Solubilization of solid fluorescent dyes were performed in DMSO to make 1mg/mL solution of 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate (DiD; Life Technologies) or 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-5,5'-disulfonic acid (DiI; molecular probes). The liposome formulation was made by mixing POPC, DSPE-PEG-2000, DSPE-PEG-2000-Biotin and DiD or DiI in the molar ratio 89:9:1:1 respectively. Liposomes were either air-dried overnight or dried in a speedvac and reconstituted in 1mL DPBS (Hyclone, GE Healthcare Life Sciences). The solution was sonicated in JSP ultrasonic bath sonicator for 10 minutes and extruded 45 times through a 100nm Whatman nuclepore track-etch membrane (Sigma-Aldrich) using manual extruder (Avanti Polar Lipids) to make small unilamellar vesicles (SUVs). Vesicle size was confirmed to be ~119 nm using a DLS particle analyzer (__). Vesicle number was estimated by quantifying absorbance of DiI on Synergy H1 microplate reader (BioTek).

Opsonization of liposomes

To make antibody opsonized vesicles, 5uL of IgG2a (clone ID4-C5, Biolegend) or fluorescently conjugated version (containing dylight 594) was added to an Eppendorf tube containing 85uL of PBS, the solution was mixed thoroughly using a pipette and 10uL of stock liposomes was added, mixed thoroughly and incubated at room temperature for 1hour.

Flow cytometry uptake of IgG2a-liposomes in FcγR^{KO} cells

Parental and FcγR^{KO} cells FLMs were plated at a density of 32 000 cells per knockout line in a 24 well plate and incubated in bone marrow media (BMM) overnight. For each well 3uL of opsonized liposome mix was added to 300uL of DMEM (pre-equilibrated in CO2 incubator overnight) and added to the cells after removal of bone marrow media (BMM). Cells were incubated at 37°C in a CO2 incubator for 1 hour, washed three times in cold PBS, re-suspended in cold PBS and kept kept on ice at 4°C before analysis. Data on approximately 5000 cells was acquisition for each cell line on C6 BD Accuri flow cytometer (BD Biosciences) and data analysis was carried out in FlowJo (version10). See figure 2.13 for schematic representation of this procedure.

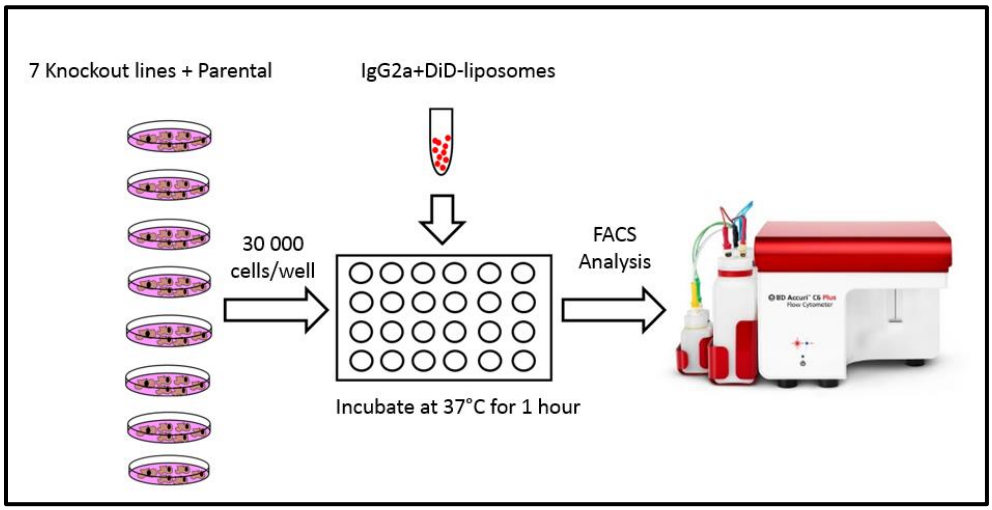

Figure 2.13 Schematic diagram of flow cytometry analysis of IgG2a-liposome uptake in FcγR knockouts (image of BD Accuri was adapted from www.bdbiosciences.com).

Dual color imaging on confocal microscope

All cell lines were seeded on a 25mm cover glass at a density of 300, 000 cells per well and incubated in bone marrow media (BMM) overnight. For each time point, cells were pulsed for 5 minutes with a mixture of IgG2a opsonized DiD-liposomes and 0.1mg/mL dylight-594 conjugated dextran (40kDa). The solution used consisted 700uL of BMM, 25uL of IgG2a-liposomes and 0.1mg/mL dextran. Cells were quickly washed with PBS and incubated for a fixed time, fixed with ~2% PFA , mounted in leiden chambers and imaged on a spinning disk confocal microscope. See schematic diagram of experimental procedure in figure 2.14.

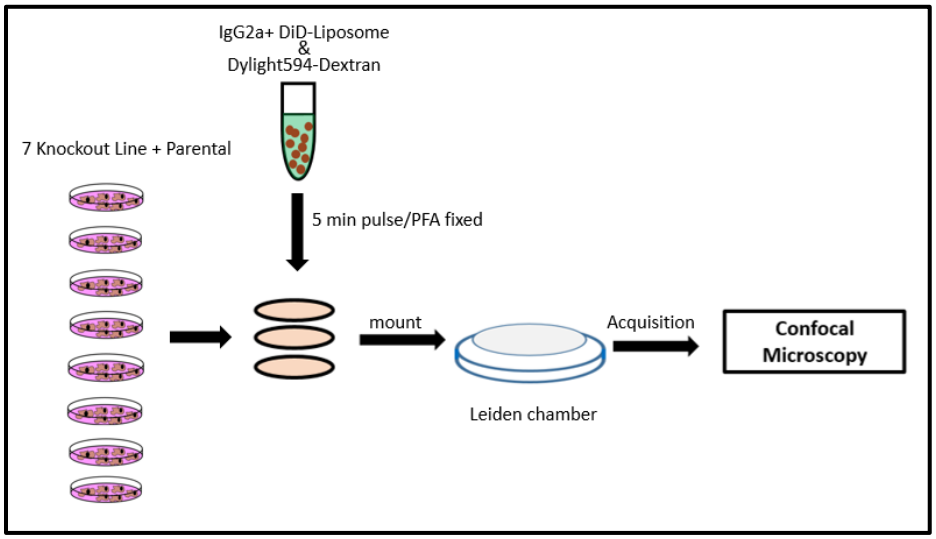

Figure 2.14 Schematic diagram of confocal microscopy image acquisition on simultaneous uptake of IgG2a opsonized DiD-liposomes and dylight-594 conjugated dextran in FcγR^{KO} FLMs

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CHAPTER 3

The macropinosome organizes sorting and recycling of IgG opsonized nanoparticles in macrophages

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1. Abstract

IgG transport and antigen processing is an important step in the clearance of targets such as viruses and tumor cells. Macrophages are the principal tissue-resident cells that mediate clearance of antibody-antigen complexes. Therefore, understanding the fate of antibodies in macrophages is important for improving antibody and vaccine design. Here, we show that macrophages are able to recycle IgG2a from internalized liposomes and present them at the cell surface on Fc receptors allowing them to phagocytose new targets including erythrocytes. Initial internalization of IgG opsonized liposomes occurs by Fc receptor mediated uptake, with little contribution from macropinocytosis. Following endocytosis, IgG-liposomes are trafficked to the limiting membrane of macropinosomes where low pH triggers sorting of IgG off of the liposomes. Subsequently, a portion of the IgG2a is sorted back to the cell surface whereas liposomes are targeted to the lysosome for degradation. These results indicate that a sorting compartment associated with the macropinosome, serves as an antibody-antigen sorting nexus. This idea was reinforced by experiments that demonstrated that traffic of liposomes is not predicted by fluid phase movement. Together, these show that the macropinosome may have a novel function in controlling antibody antigen sorting.

2. Introduction

Antibodies of immunoglobulin class G (IgG) activate Fc gamma receptors (FcγRs) and are attractive therapeutic agents because of their ability to promote phagocytosis and killing of tumor cells, capture and presentation of associated antigens and cytokine responses (Dahan *et al.*, 2016; DiLillo & Ravetch, 2015). FcγRs bind the Fc domain of antibodies which, in macrophages initiates a signaling cascade that coordinates the uptake of opsonized targets. Murine Fc gamma receptors consist of activating FcγRI, FcγRIII and FcγRIV, and the inhibitory FcγRIIb; similar receptors exist in human macrophages with the addition of the activating receptor FcγRIIa and lack of FcγRIV (Bruhns, 2012). Murine FcγRI has the highest affinity for the Fc domain of immunoglobulin 2a (IgG2a) and FcγRIIb and III have moderate to low affinities for IgG2a. This is similar to humans with the exception that IgG2a's functional homologue is human IgG1. FcγRI and III are transmembrane proteins associated with dimeric FcRγ required for signal transduction (Gavin *et al.*, 1998a; Nimmerjahn *et al.*, 2005). Binding to the Fc domain of particle-associated IgG results in receptor clustering and phosphorylation of tyrosine residues in a conserved immunoreceptor tyrosine activation motif (ITAM) by Src family kinases (J C Cambier, 1995; Sobota *et al.*, 2005). Phosphorylated ITAMs serve as docking sites for spleen tyrosine kinase (Syk) to propagate downstream signals that drive actin remodeling, particle internalization and secondary inflammatory responses such as generation of reactive oxygen species and cytokine secretion (Gallo *et al.*, 2010; Park *et al.*, 1999).

A prominent feature of macrophages is their ability for macropinocytosis. Macropinocytosis is a nonspecific endocytic mechanism that involves actin-dependent

uptake of bulk extracellular fluid (Johnathan Canton *et al.*, 2016). In macrophages and dendritic cells, macropinocytosis is an important route for antigen uptake and antigen presentation on major histocompatibility complex class II (MHC II) (Johnathan Canton *et al.*, 2016; Federica Sallusto, 1995b; Lim *et al.*, 2012). In macrophages, macropinosomes mediate mTORC1 dependent uptake of amino acids as nutrients for growth and differentiation (Yoshida, Pacitto, *et al.*, 2015). We previously showed that macropinosomes are important for signaling and down-modulation of CSF-1R in macrophages. Here, ligand-bound CSF-1R is internalized by micropinocytosis independent mechanisms and trafficked to the lumen of macropinosomes where its signals are amplified prior to its degradation in the macropinosome lumen (Huynh *et al.*, 2012; Jieqiong Lou *et al.*, 2014).

Recent efforts in the field of antibody therapeutics have focused heavily on the design of antibodies with pH dependent antibody-antigen dissociation properties with high affinity for neonatal Fc receptor (FcRn) (Borrok *et al.*, 2015; Datta-Mannan *et al.*, 2012; Devanaboyina *et al.*, 2013; Kuo & Aveson, 2011). FcRn is an MHC class I molecule that binds IgG with high affinity at acidic pH (~6.5) and low affinity at neutral pH (~7.4). Acidification of endosomes leads to dissociation of IgG from FcγRs and favors binding of IgG to FcRn, which mediates recycling of IgG back to the cell surface (Lencer & Blumberg, 2005; Simister & Mostov, 1989; Tzaban *et al.*, 2009). However, increasing binding affinity of IgG for FcRn does not always correlate with prolonged serum half-life and improved efficacy of therapeutic antibodies, suggesting that other factors influencing endosomal trafficking may be critical to understanding the fate of internalized IgG (Braster *et al.*, 2017; Gurbaxani *et al.*, 2013).

Macrophages are the principal tissue resident phagocytes that mediate clearance of antibody-antigen complexes. Consequently, they are critical for IgG recycling and maintenance of IgG homeostasis. Despite this importance, trafficking of IgG in macrophages is poorly understood (Shreeram Akilesh *et al.*, 2007; Zhu *et al.*, 2001). Rather, current understanding of IgG trafficking and recycling is predominantly based on studies in epithelial cells due to their presence in tissue vasculature. Epithelial cells lining tissue vasculature are in direct contact with serum IgG and regulate its recycling (Lencer & Blumberg, 2005; Pyzik *et al.*, 2015). Notwithstanding, there is a need to investigate IgG recycling in macrophages because it is important for improving therapeutic antibody design to prime macrophages to sustain phagocytic response. This is necessary to ensure efficient clearance of tumor and pathogen infected cells during a pathological condition or pathogen invasion (DiLillo & Ravetch, 2015; Gül & van Egmond, 2015).

Antigen can be taken up by phagocytes via both receptor-dependent uptake mechanisms and macropinocytosis (Liu & Roche, 2015; Mercanti *et al.*, 2006; Swanson, 2008). However, trafficking of antibody opsonized nanovesicles once internalized by IgG-FcγR mediated endocytosis is unclear. Furthermore, the interplay of macropinocytosis and FcγR endocytosis during the uptake, intracellular traffic, antibody segregation and degradation of antigen remains to be investigated. Here, we utilized IgG2a opsonized fluorescent nanovesicles to delineate the roles of FcγR endocytosis and macropinocytosis during traffic of antibody opsonized nanovesicles in fetal liver macrophages (FLMs).

3. Results

Antibody recycling primes macrophages for phagocytosis of new antigen

In addition to their role in mediating IgG-dependent functions, macrophages contribute to maintaining soluble IgG homeostasis by FcRn mediated recycling of IgG (Shreeram Akilesh *et al.*, 2007). We tested the hypothesis that IgG2a could be recycled from soluble antigen bearing nanoparticles. To address this hypothesis, FLMs were exposed to ~120nm PEGylated liposomes opsonized with monoclonal anti-biotin IgG2a (mIgG2a) for 5 minutes. Cells were washed with PBS to remove unbound IgG2a-liposomes. After chasing for 15, 60 and 120 minutes post wash, they were exposed to biotinylated sheep red blood cells (SRBCs) labeled with pHrodoRed, and followed by another 30-minute chase. High content microscopy images of the pHrodoRed (bright at low pH) revealed that the FLMs gained phagocytic ability as a function of time after initial liposome uptake, demonstrating that recycled IgG2a mediated phagocytosis of biotinylated SRBCs (Figure1.A and C). To determine if this recycling was pH dependent as would be expected for an FcRn mechanism, a 30-minute pre-treatment with bafilomycin, the vATPase inhibitor, before exposure to IgG2a-liposomes abolished phagocytic activity (Figure 3.1.B-C). Thus, low endosomal pH was critical for release of antibody from antigen/nanoparticles and recycling back to the cell surface where it can enable phagocytosis of new antigen.

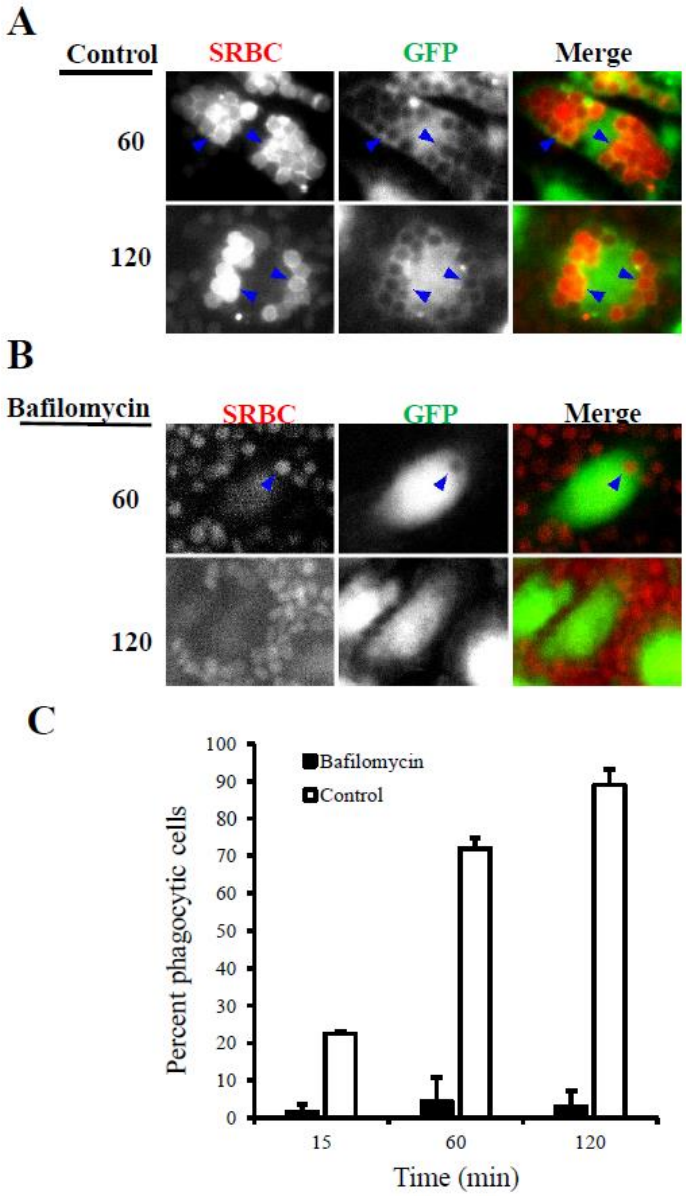

Figure 3. 1 IgG2a recycling can potentiate phagocytosis of SRBCs. (A) FLM cells pretreated with IgG2a opsonized liposomes phagocytose biotinylated sheep red blood cells (SRBCs) at 60 and 120 minutes chase following a 5-minute pulse and wash with the opsonized nanoparticles. Internalized SRBCs displace GFP in the cytoplasm of FLMs and can be observed as 'dark holes' in GFP channel as shown by blue arrow heads. (B) Bafilomycin treatment impairs recycled IgG2a dependent phagocytosis. Unlike control cells in (A), FLM cells pretreated with bafilomycin for 30 minutes followed by exposure to IgG2a-liposomes are unable to internalize biotinylated. (C) Quantification of phagocytic cells under control and bafilomycin treatment conditions. Hundred cells were counted in each condition and the number of phagocytosing cells expressed as a percentage. Error bar is standard deviation for two independent experiments.

Internalized antibody opsonized nanovesicles traffic to the macropinosome membrane

To track the fate of IgG-liposomes after FcγR mediated endocytosis, we produced liposomes that were selectively internalized by FcγRs. Liposomes can be internalized by non-specific interactions with the macrophage cell surface, however, ~10 mol percent polyethylene glycol (PEG) prevents this uptake route (Johnstone *et al.*, 2001). Liposomes containing 1 mol % PE-PEG-biotin and 10 mol % PE-PEG were selectively internalized by IgG-FcγR dependent uptake (Figures S1.1, S1.2 and S1.3). To determine endocytic uptake relative to fluid-phase uptake, fetal liver macrophages (FLMs) were exposed for 5 minutes to dylight-594 conjugated 70kDa dextran and DiD-labelled PEGylated liposomes (DiD-liposomes) opsonized with mIgG2a. The DiD signal was distinct from the fluorescent dextran containing macropinosomes (Figure 3.2A). We observed that IgG2a opsonized DiD-liposomes were not internalized by macropinosomes. Rather, DiD-liposomes were observed to traffic to the limiting membranes of macropinosomes observed as a ring-like appearance (Figure 3.2B). Line profile analysis of macropinosomes showed that mIgG2a opsonized liposomes were predominantly associated with the limiting membrane of macropinosomes and not in the intraluminal milieu (Figure 3.2.B-C). Accumulation of DiD-liposomes on macropinosome membranes increased rapidly reaching a peak at 10 minutes chase (Figure 3.3.B-C). Thus, we show that in macrophages, uptake of mIgG2a-opsonized nanovesicles is predominantly receptor dependent but occurs concurrently with macropinocytosis, and involves trafficking of internalized complexes to the macropinosome membrane.

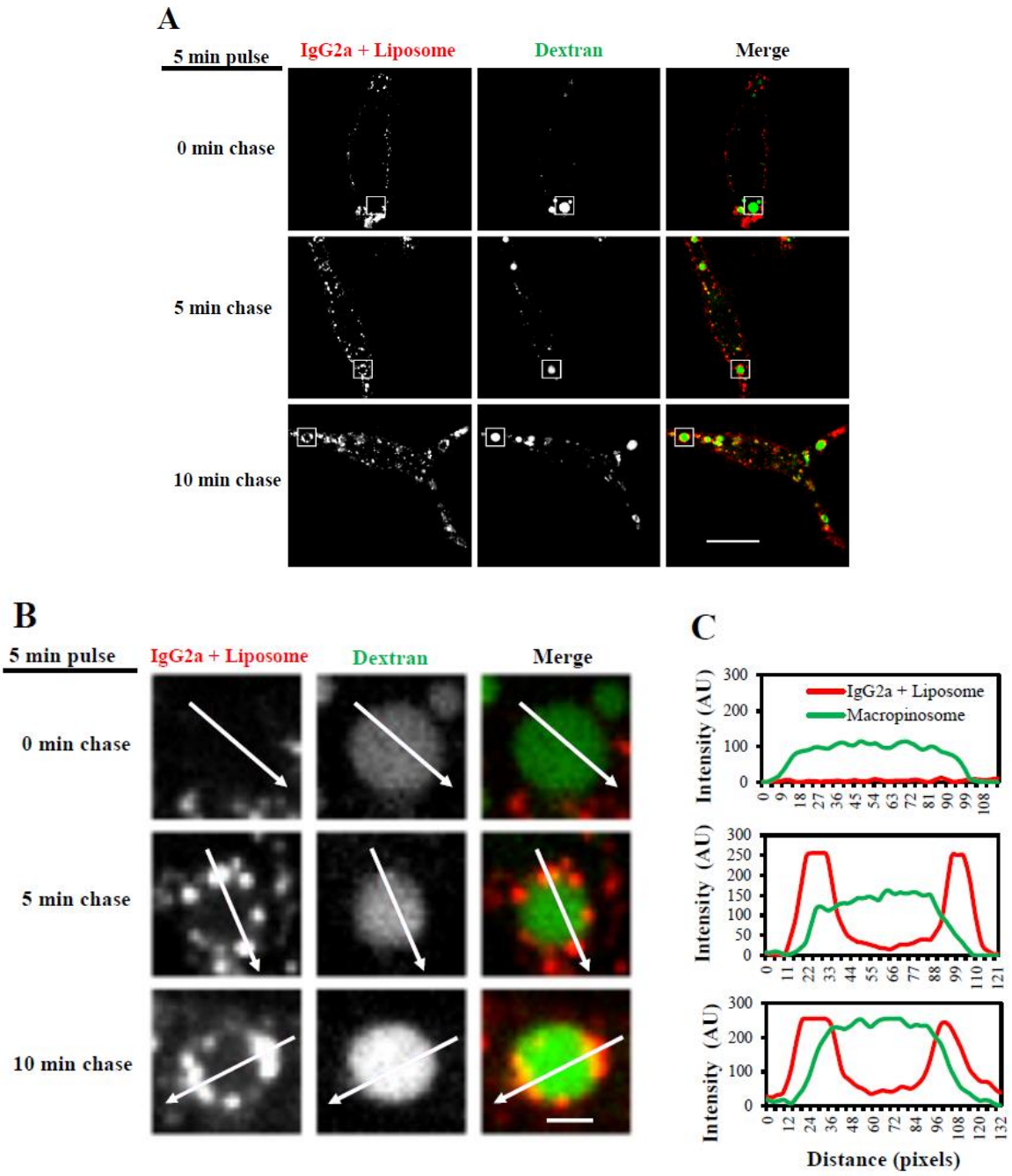

Figure 3. 2 Antibody opsonized nanovesicles traffic to the macropinosome membrane. (A) Simultaneous uptake of 70kDa dextran and IgG2a opsonized liposomes in FLM cells show that antibody opsonized liposomes are internalized predominantly by a receptor mediated or non-fluid phase uptake pathway. Scale bar is 10µm. (B) Inserts from panel A; opsonized liposomes traffic to the macropinosome membrane at 5 through 10 minutes chase with limited intraluminal budding. Scale bar is 5µm. (C) Line profiles along white arrows across macropinosomes in B show no accumulation of antibody-opsonized liposomes at 0 minutes chase and increasing accumulation on macropinosome membranes from 5 through 10 minutes. Note the exclusion of opsonized liposome signal from the lumen of macropinosomes at 5 and 10 minutes chase.

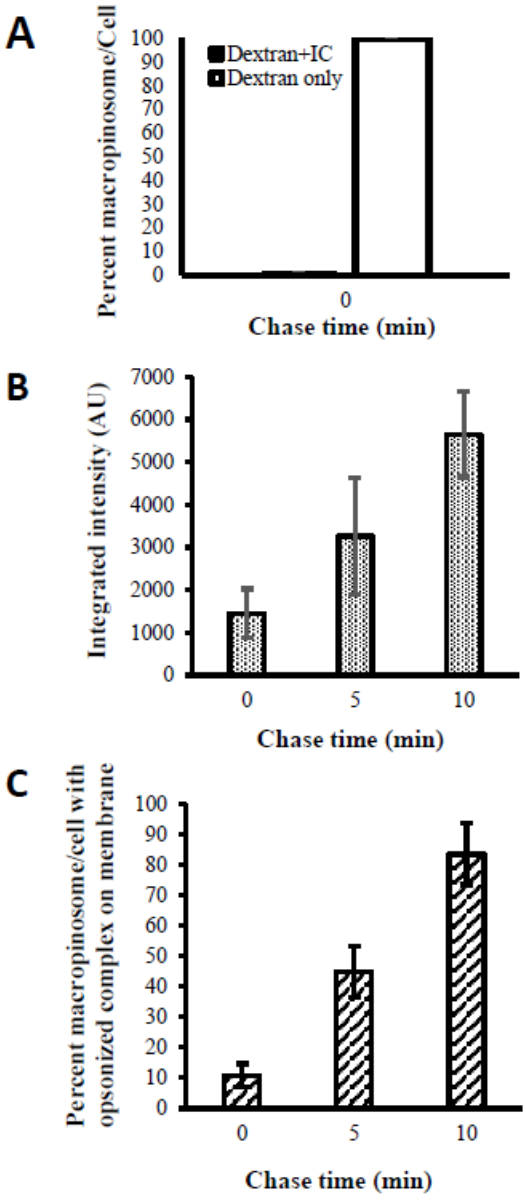

Figure 3. 3 Antibody opsonized nanovesicles accumulate on the macropinosome membrane. (A) Cells were pulsed with solution containing immune complex (IC or IgG2a-liposome) and 40kDa dextran for 5 minutes and fixed with PFA. The number of macropinosomes in 20 cells having their lumen filled with IC or without IC were counted manually and scored as a percentage. Over 99% of the macropinosomes counted at 0 minutes chase did not have IgG2a-liposomes in their lumen, demonstrating that macropinosomes formed during FcγR endocytosis are not used for direct uptake of antigen. (B) Integrated intensity of IgG2a opsonized liposomes on macropinosome membranes increases from 0 through 10 minutes chase, showing accumulation of ICs on macropinosome membranes. Error bar is SEM for two independent experiments consisting 60 macropinosomes at each time point. (C) Percentage macropinosomes per cell with opsonized liposomes on their membranes were observed to increase steadily from 0 through 10 minutes chase. Twenty (20) cells at each time point were counted and scored as a percentage. Error bar is SEM for two independent experiments.

The macropinosome membrane is a nexus for accumulation and segregation of IgG2a from opsonized nanovesicles

The unique interaction of opsonized nanovesicles with macropinosome membranes raised a fundamental question about the fate of the antibody: does IgG2a remain bound to nanovesicles as they traffic to macropinosomes or it segregates from the cargo before reaching the macropinosome membrane? To understand the traffic of IgG relative to antigen DiD labelled liposomes were opsonized with dylight594-IgG2a. FLMs were incubated for 5 minutes, washed and imaged live movies were recorded on a spinning disc confocal microscope. IgG2a-liposomes trafficked to the macropinosome membrane as an intact complex appearing as punctate structures on macropinosome membranes at 5 minutes chase (Figure 3.4.A-B). However, upon reaching the macropinosome membrane, segregation of IgG2a from the cargo occurred, leading to the formation of IgG2a containing compartments that were separate from the liposome containing compartments at ~12 minutes chase; segregated IgG2a only and liposome containing vesicles were organized around the limiting membrane of the macropinosome (Figure 3.4.A-B). Unlike our previous observations of the rapid delivery of the CSF-1R into the macropinosome (Jieqiong Lou *et al.*, 2014), we did not observe directed transport of IgG2a into the macropinosome lumen, indicating that the macropinosome does not mediate the immediate destruction of the IgG in the same fashion as the CSF-1R. As accumulation of liposomes on macropinosome membranes increased (Figure 3.3. B-C), limited intraluminal delivery liposomes was observed in some cases resulting in macropinosomes with a hazy lumen containing liposomes (Figure S1.4.mov1).

To determine if the observed IgG2a sorting event was pH dependent, FLM cells were pretreated with bafilomycin for 30 minutes prior to a 5-minute pulse with dylight-594-IgG2a opsonized DiD-liposomes. Blocking of endosome acidification prevented the dissociation of IgG2a from the antigen. Analysis of dylight-594-IgG2a and DiD-liposomes showed persistent antibody bound liposome complexes within the cell even after complete clearance of macropinosomes at 25 minutes chase, demonstrating that antibody-antigen separation from the surface of nanoparticles is pH dependent (Figure 3.5A-C). Quantification of antibody-liposome co-localization showed that at 25 minutes chase, mIgG2a remained associated with liposome endosomes in control cells with co-localization coefficient of 0.41 whilst the antibody remained associated with liposome endosomes in bafilomycin treated cells with a co-localization coefficient of 0.87 (Figure 3.5.C). Thus, in macrophages, segregation of antibodies from antigen can occur in a pH dependent fashion at a sorting compartment organized by the macropinosome .

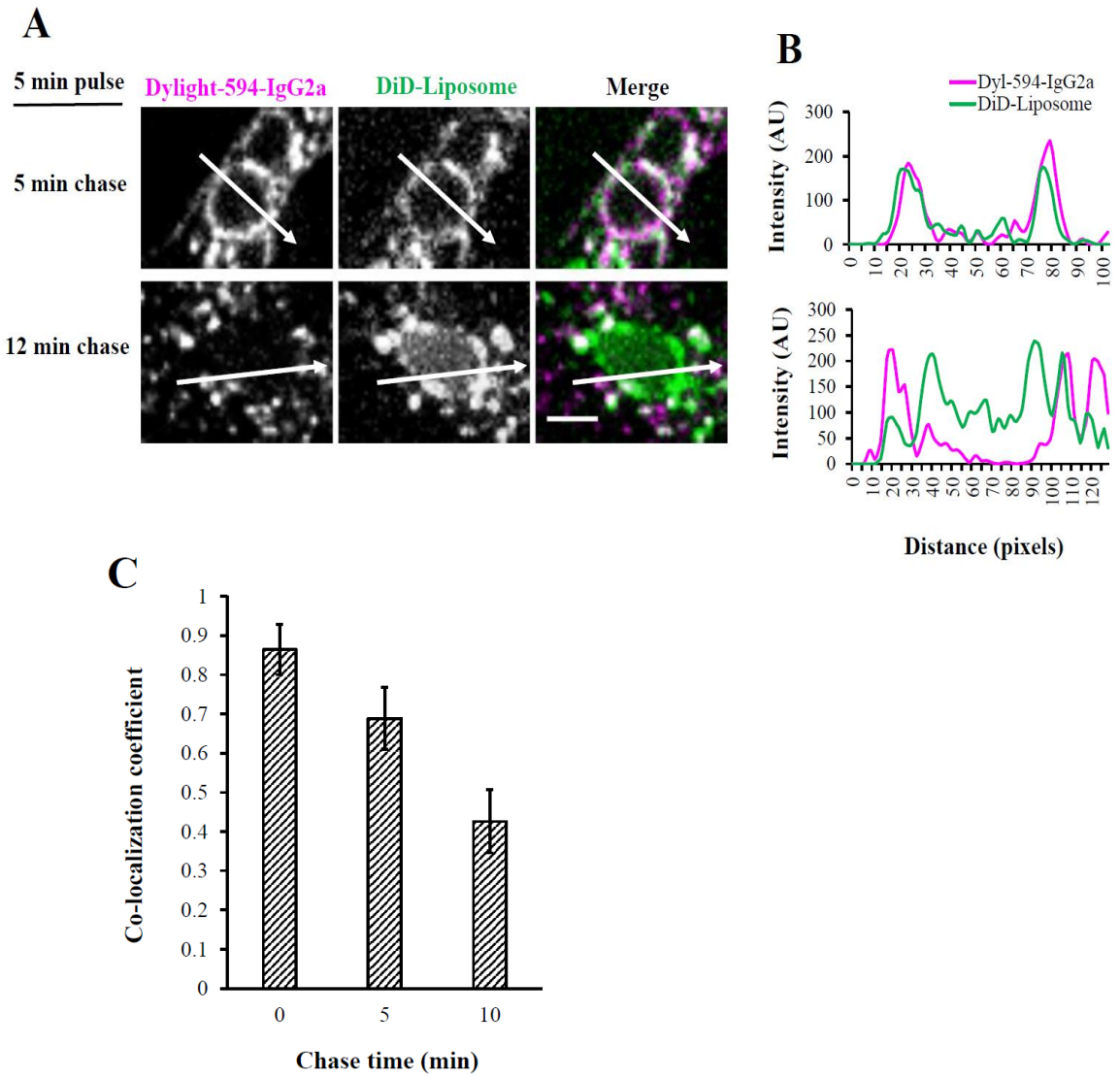

Figure 3. 4 Antibody-Antigen complexes traffic to the macropinosome membrane before segregation. (A) FLMs pulsed with dylight-594-IgG2a opsonized DiD labelled liposomes for 5 minutes show that antibody-bound-liposomes traffic to macropinosome membranes as intact complexes (white endosomes) at 5 minutes chase, afterwards, antibody-antigen complexes become segregated at the macropinosome membrane into IgG2a-only (magenta) and liposome-only (green) endosomes as observed at 12 minutes chase. Scale bar is 5µm. (B) Line profiles along white arrows shown in A: at 5 minutes chase, DiD-liposomes remain associated with Dylight-594-IgG2a on the macropinosome membrane but become separated at 12 minutes chase. Data is representative of three independent experiments. (C) Manders' coefficient of co-localization measuring association of DiD-Liposomes with Dylight-594-IgG2a show increasing segregation of antibody and liposomes from 0 through 10 minutes chase. Error bar is standard deviation for five macropinosomes measured across five cells at each time point. Data is representative of three independent experiments.

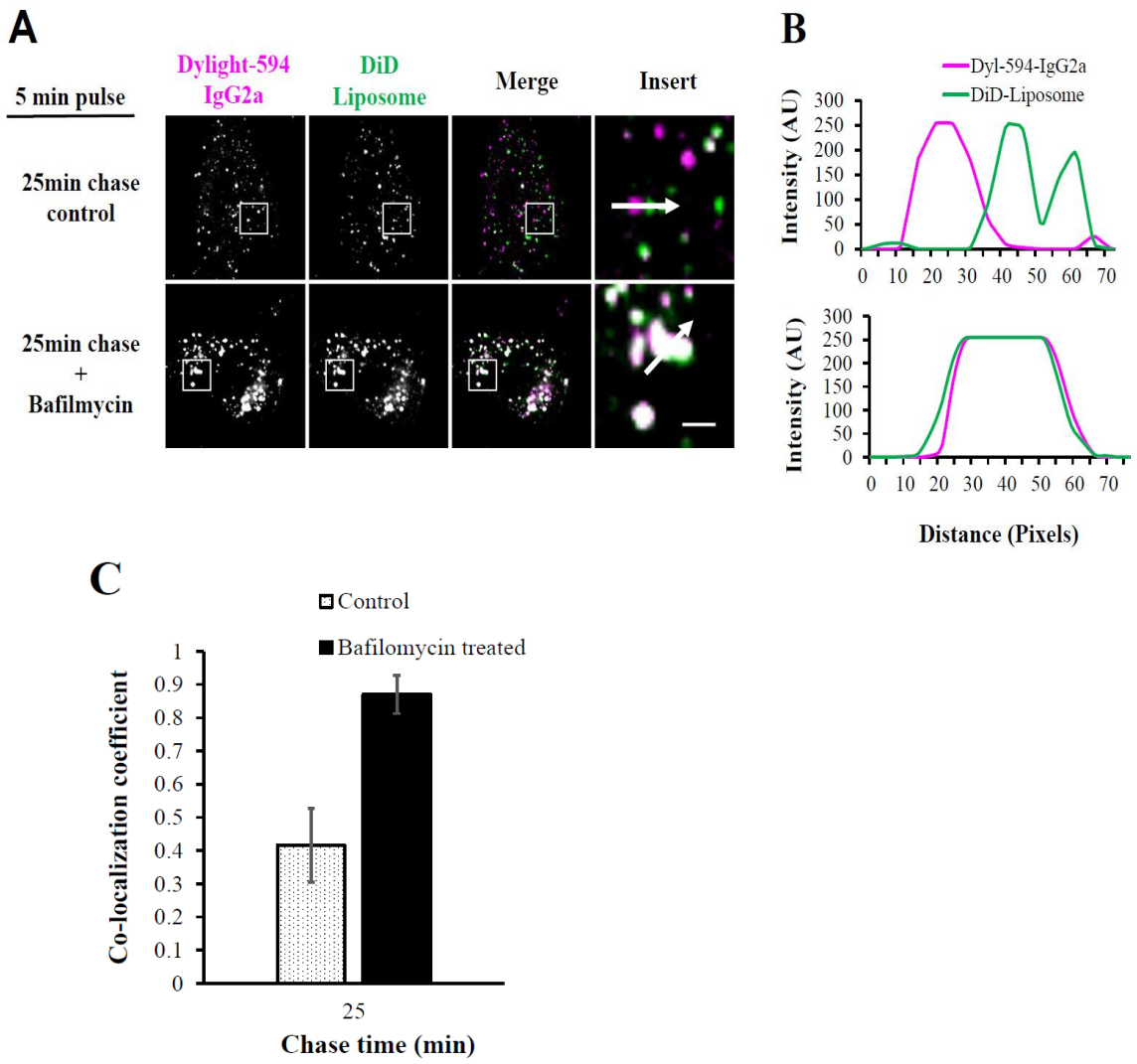

Figure 3. 5 Antibody-antigen segregration occurs on macropinosome membranes in a pH dependent manner. (A) Treatment of FLMs with bafilomycin to block acidification of endosomes and macropinosomes abrogates segregation of Dylight-594-IgG2a (magenta) from DiD-liposomes (green) which persists even after clearance of macropinosomes at 25 minutes chase. Scale bar is 5µm (B) Line profiles of Dylight-594-IgG2a and DiD-liposomes along white arrows shown in D. (C) Manders' co-localization coefficient of Dylight594-IgG2a and DiD-Liposomes at 25 minutes chase show a low co-localization indicative of segregation in control cells and a high co-localization indicative of abrogated segregation in bafilomycin treated cells. Error bar is standard deviation for 4 cells at each condition.

Trafficking of nanovesicles to lysosomal compartment is delayed relative to contents of macropinosome lumen

Multicolor imaging of liposome endosomes and fluid phase marker (40kDa dextran) in FLM and primary bone marrow macrophages showed that clearance of macropinosomes resulted in transition of the fluid phase marker into tubulovesicular compartments reminiscent of lysosomes. However, transition of liposome endosomes to these compartments was delayed until ~40 minutes chase. Although, opsonized nanovesicles trafficked to the macropinosome membrane to form a multivesicular structure, the clearance of fluid phase marker in macropinosomes appeared to precede clearance of liposomes.

Macropinosomes have been shown to interact with the lysosome to mediate delivery of their contents to the lysosome via a process known as squidlysis or piranhalysis (Yoshida, Pacitto, *et al.*, 2015). Given that recruitment of the antigen onto the macropinosome membrane did not appear to drive delivery into the macropinosome lumen, we used high resolution microscopy to interrogate the interaction between the lysosome and the macropinosome proximal sorting compartment (see schematic diagram in Figure S1.6). Consistent with the work of Swanson and collogues, we observed rapid interactions between the tubular lysosome and the macropinosome. FLM cells were preloaded with dylight594-40kDa dextran overnight, washed in PBS and incubated in bone marrow media for ~1 hour at 37°C. This provided enough time for loading of dextran into lysosomal compartments as performed in other studies. After 5 minute-pulse with IgG2a opsonized DiD-liposomes in the presence of Lucifer yellow, cells were washed and imaged in HBSS

media on a spinning disk confocal microscope. We observed two modes of interaction of lysosomes with nascent macropinosomes; first, small lysosomal vesicles budded from tubulovesicular compartments of lysosomes and fused with nascent macropinosomes leading to quick acquisition of lysosomal contents by macropinosomes. Secondly, momentary fusion of tubular extensions of lysosomes with macropinosomes led to 'drinking' of fluid phase marker from macropinosome lumen by lysosomes. Direct and immediate 'drinking' of Lucifer yellow by tubular lysosomes supports earlier observations that the fluid phase marker transitioned to tubulovesicular compartments much earlier than liposome endosomes.

Three-color imaging of nanovesicles (DiD-liposomes), macropinosomes (Lucifer yellow) and lysosomes (dylight-594 40kDa dextran) showed that lysosomes did not directly internalize opsonized nanovesicles on the surfaces of macropinosomes during piranhalysis, rather, lysosomes contacted liposome endosomes through 'kissing' events leading to acquisition of lysosomal contents by liposome endosomes. Line profile and co-localization analyses showed that liposome endosomes, macropinosomes and lysosomes interacted with each other but remained predominantly separate compartments through 10 minutes chase. Co-localization of Lucifer yellow in macropinosomes and dylight594-dextran in lysosomal compartments increased from 0.25 at 7 minutes chase to 0.69 and 0.89 at 15 minutes and 55 minutes chase respectively. However, co-localization of DiD in liposome endosomes and dylight-594-dextran in lysosomes remained low at 0.1 and 0.2 at 7 minutes and 15 minutes chase respectively. At 55 minutes chase, co-localization of liposome endosomes and lysosomes had increased to a high of 0.79. These observations demonstrate

that, during FcγR trafficking of antibody-opsonized nanovesicles, lysosomes interact simultaneously with macropinosomes and cargo nanovesicles on macropinosome membranes by piranhalysis and ‘kiss-run’ events respectively leading to early transition of macropinosome contents into tubular lysosomes and a delay in the clearance of liposome endosomes.

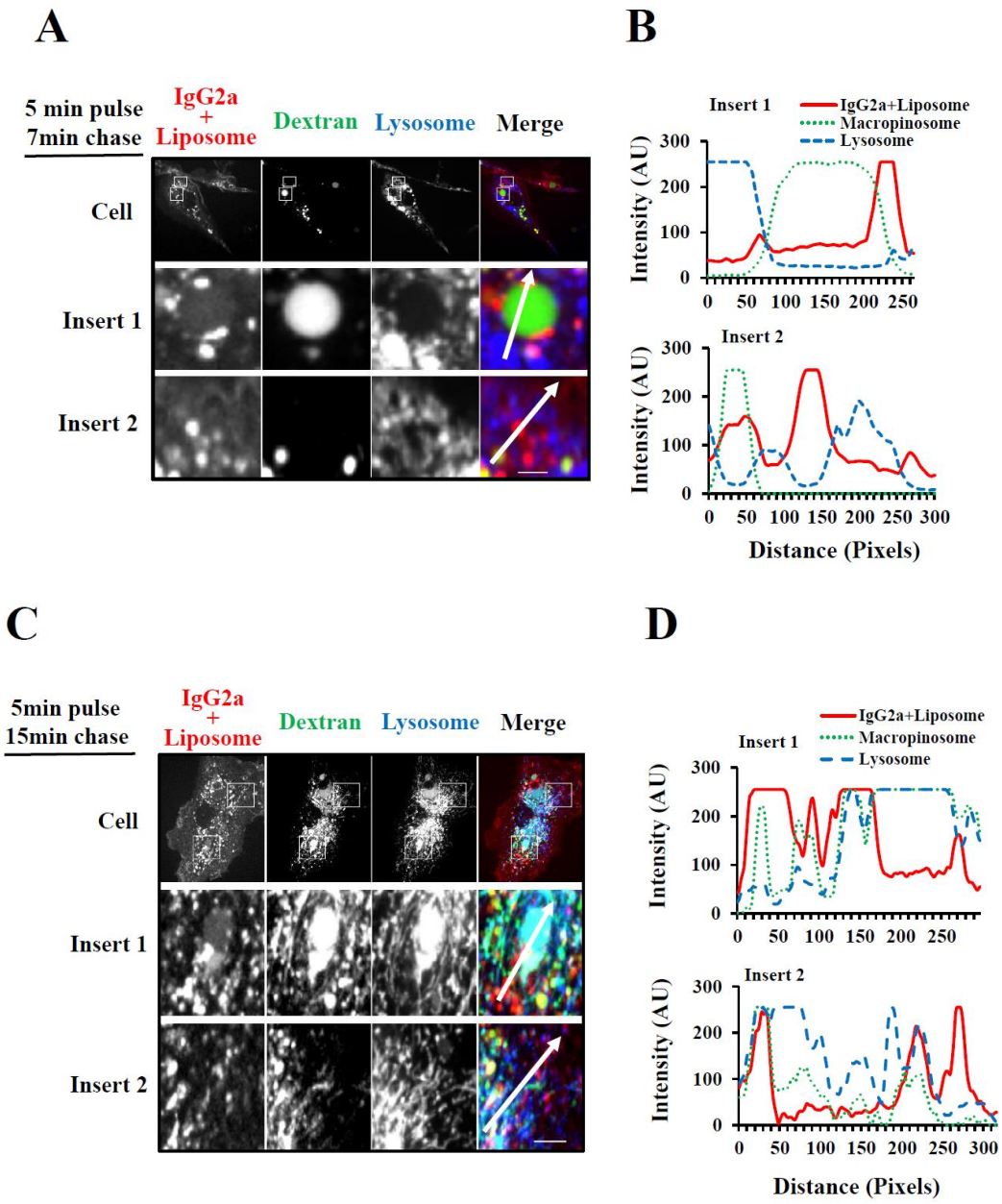

Figure 3. 6 Trafficking of liposome endosomes to the lysosome is delayed relative to fluid phase marker in macropinosomes. (A) FLMs preloaded with dylight-594-dextran to label lysosomes were pulsed with IgG2a opsonized with DiD labelled liposomes in the presence of Lucifer yellow. At early time points (~7 minutes chase), the lysosomal compartment interacts with both IgG2a opsonized liposomes and macropinosomes but all three components remain in distinct compartments. (B) Line profiles from inserts 1 and 2 in A showing distinct localization of liposomes (DiD signal), macropinosomes (Lucifer yellow signal) and lysosomes (dylight-594 signal). (C) Increased co-localization of Lucifer yellow (from macropinosomes) and dylight-594-dextran (from lysosomes) is observed at 15 minutes chase. However, co-localization of DiD labelled liposome endosomes (red) with lysosomes (blue) remain low and comparable to 7 minutes chase. (D) Line profiles of inserts 1 and 2 in D showing enhanced association of Lucifer yellow in macropinosomes (green) with dylight-594-dextran in lysosomes (red); however, association of lysosomes with liposomes have only increased slightly by this time..

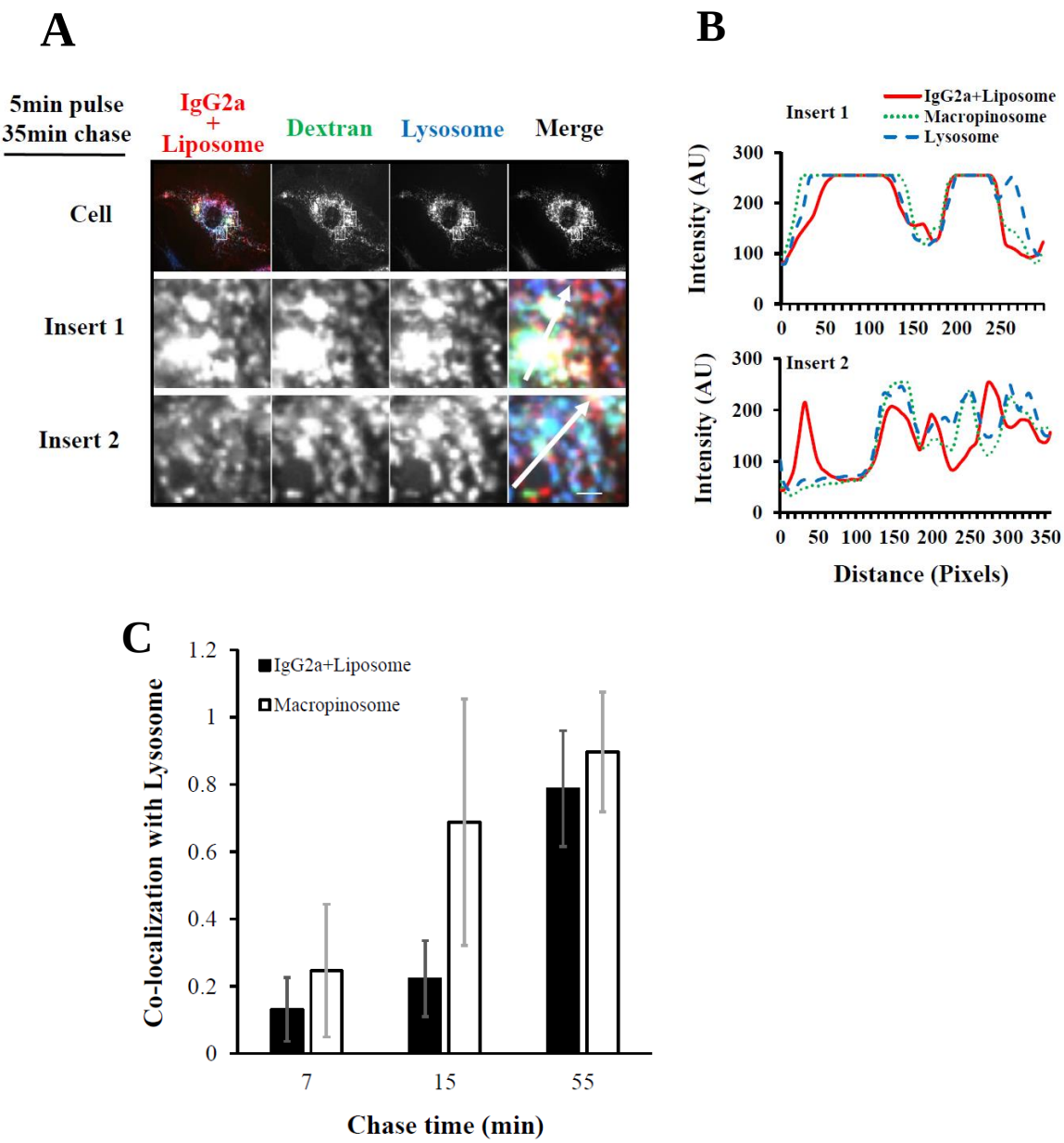

Figure 3. 7 Transition of liposome endosomes to lysosomes occur at late time point following a delay. (A) After mid time point as described in Figure 3.6 C and D, both fluid phase marker (Lucifer yellow) and DiD labelled liposomes transition to the lysosomes at ~55 minutes chase. (B) Line profile analyses from both inserts 1 and 2 show increased association of lysosomes (blue), fluid phase marker Lucifer yellow (green) and DiD-liposomes (red). (C) Quantification of Manders' coefficient at three time points show increasing co-localization of Lucifer yellow and dylight-594-dextran (in lysosomes) at 15 and 55 minutes chase, whilst there is a delayed increase in co-localization of DiD-liposomes with lysosomes at 15 minutes, followed by an increased association of liposomes with the lysosome at 55 minutes chase. Error bar is SD for measurements on four cells.

4. Discussion

FcγR/IgG-mediated internalization of small particles shares some similarities with uptake of large particles as many gene products ranging from phagocytic receptors, non-receptor tyrosine kinases and modulators of actin cytoskeleton dynamics have been found to be involved in both processes (Araki *et al.*, 1996; Kuhn *et al.*, 2014; Levin *et al.*, 2015; Swanson, 2008; Tse *et al.*, 2003).

However, the mechanism of uptake and downstream processing of internalized cargo have some unique differences; whilst uptake of large particles is regarded as largely dependent on actin, small particle uptake is more clathrin dependent and less sensitive to disruption of the actin cytoskeleton (Tse *et al.*, 2003). Consistent with these findings, we found that antibody opsonized nanovesicles were internalized predominantly by a receptor dependent pathway instead of macropinosomes in FLM cells (Figure 3.2). Moreover, antibody bound complexes co-localized with clathrin light chain fused to yellow fluorescent protein in co-localization experiments (Figure S1.5), demonstrating that the endosomes are most likely internalized by clathrin mediated endocytosis (CME) or a process involving clathrin (Banerjee *et al.*, 2013; Tse *et al.*, 2003).

In macrophages and dendritic cells, macropinocytosis is a route for non-specific uptake of antigen (Christopher C. Norbury, 1997; Federica Sallusto, 1995b). The prevailing idea is that the antigen or pathogen is captured in the lumen of the macropinosome during its

formation. Here, we present a new insight into how macrophages respond to nanoparticles internalized exclusively by FcγR mediated endocytosis. Instead of direct uptake of antibody bound nanoparticles by macropinosomes, opsonized complexes are rather almost exclusively, internalized by a receptor dependent pathway and trafficked to the macropinosome membrane without intraluminal budding (Figure 3.2 and Figure S1.5). Moreover, restriction of antibody-antigen complexes from budding into the lumen of macropinosomes is in contrast to the behavior of receptor-ligand complexes on the macropinosome during internalization of CSF-1 receptor-ligand complexes where intraluminal budding occurs soon after complexes reach the macropinosome surface (Jieqiong Lou *et al.*, 2014). Thus, the macropinosome surface can mediate different sorting mechanisms depending on the nature of the cargo, mode of internalization and type of membrane receptors involved.

Antibody-antigen punctae on macropinosome membranes were similar to the distribution of WASH complexes on macropinosome membranes in Dictyostelium. In Dictyostelium, WASH and its associated proteins mediate sorting and recycling of plasma membrane proteins from macropinosomes back to the cell surface (Buckley *et al.*, 2016). We anticipate that in macrophages and other phagocytic cells, localization of internalized opsonized vesicles to the macropinosome membrane will coincide with accumulation of WASH complex and sorting nexins on the macropinosome outer membrane and not within the macropinosome lumen as segregated antibody complexes appear to be concentrated outside the macropinosome. This will potentially enhance antibody-antigen sorting, Fc receptor and antibody recycling, cargo degradation and resolution of the macropinosome

(Freeman & Grinstein; Lim *et al.*, 2012). Furthermore, we demonstrate visually, that antibody-antigen segregation occurs in a pH dependent fashion on the macropinosome surface and this is consistent with the interaction of lysosomes with the macropinosome surface during trafficking of internalized complexes (Figures 6 and 7).

Our observations underscore the central importance of macropinosomes in multiple trafficking and signaling processes in the cell. Multiple studies show that the macropinosome is required for nutrient uptake, growth factor receptor signal attenuation, pathogen entry, antigen internalization and presentation. Our studies provide additional insight into how the macropinosome surface functions as a platform for accumulation, aggregation, sorting and clearance of antibody-opsonized vesicles during FcγR endocytosis. We report a novel interaction of internalized antibody opsonized vesicles with macrophages and show that whether IgG is located in the lumen of the macropinosome or docked on its surface will have implications for its fate for recycling or degradation.

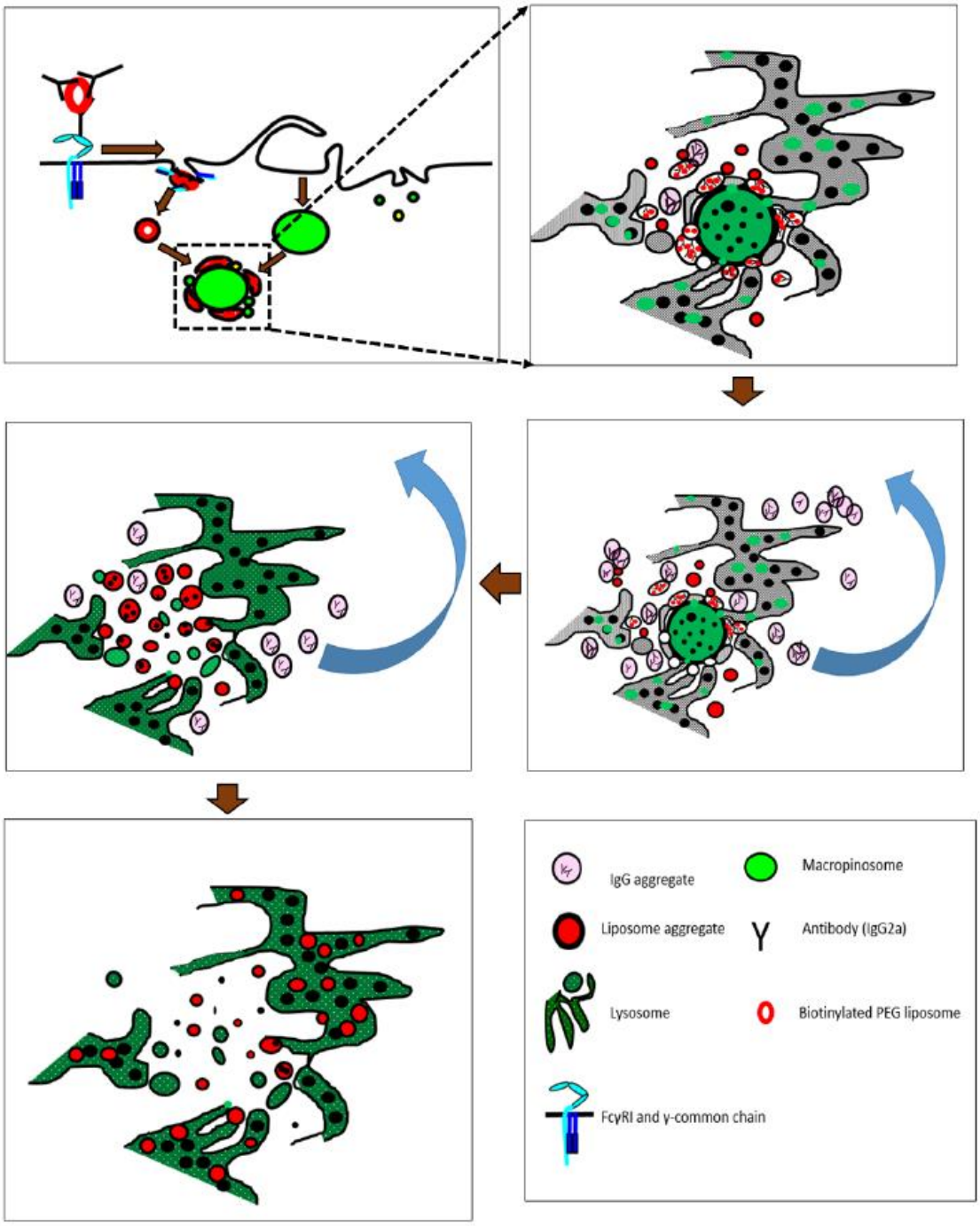

Figure 3. 8 Model for FcγR mediated endocytosis of antibody opsonized nanoparticles

5. Materials and Methods

Cell lines

Fetal liver macrophages were differentiated from liver of BALB/c mice fetus by culturing in bone marrow media enriched with CSF-1 growth factor for 6 weeks. Bone marrow macrophages were differentiated from the femur of BALB/c mice as previously described (Jieqiong Lou *et al.*, 2014). Cells were cultured in bone marrow media for 10 days before being used for experiments.

Preparation of PEGylated liposome nanovesicles

The following lipids were obtained from Avanti Polar Lipids Incorporated: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocoline (POPC, #850457), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-[Methoxy(Polyethylene glycol)-2000] ammonium salt (DSPE-PEG-2000, #880120) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)2000] ammonium salt (DSPE-PEG2000-Biotin, #880129). Stock solutions of lipids were prepared by dissolving solid samples in chloroform to make 25mg/mL POPC, 10mg/mL DSPE-PEG2000 and 1mg/mL DSPE-PEG2000-Biotin. Solubilization of solid fluorescent dyes were performed in DMSO to make 1mg/mL solution of 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate (DiD; Life Technologies) or 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-5,5'-disulfonic acid (DiI; molecular probes). The liposome formulation was made by mixing POPC, DSPE-PEG-2000, DSPE-PEG-2000-Biotin and DiD or DiI in the molar ratio 89:9:1:1 respectively. Liposomes were either air-dried

overnight or dried in a speedvac and reconstituted in 1mL DPBS (Hyclone, GE Healthcare Life Sciences). The solution was sonicated in JSP ultrasonic bath sonicator for 10 minutes and extruded 45 times through a 100nm Whatman nuclepore track-etch membrane (Sigma-Aldrich) using manual extruder (Avanti Polar Lipids) to make small unilamellar vesicles (SUVs). Vesicle size was confirmed to be ~119 nm using a DLS particle analyzer (Malvern Panalytical). Vesicle number was estimated by quantifying absorbance of DiI on Synergy H1 microplate reader (BioTek).

Opsonization of liposomes

To make antibody opsonized vesicles, 5uL of IgG2a (clone ID4-C5, Biolegend) or fluorescently conjugated version (containing dylight 594) was added to an Eppendorf tube containing 85uL of PBS, the solution was mixed thoroughly using a pipette and 10uL of stock liposomes was added, mixed thoroughly and incubated at room temperature for 1hour.

Uptake of opsonized nanovesicles in murine macrophages

Fetal liver macrophages were plated on a 25mm cover glass at density of ~300 000 cells per well and cultured in 2mL of bone marrow media overnight or for approximately 4 hours. 700uL of bone marrow media from the cultured cells was pipetted into an Eppendorf tube and 25uL of the antibody-opsonized vesicles was added and mixed thoroughly. The rest of the media on the cells was harvested from the dish, transferred to an Eppendorf tube and incubated at 37°C. Opsonized solution in bone marrow media was added to the cells

and incubated at 37°C for 5 minutes. This solution was washed off and bone marrow media was added to cells and incubated at 37°C for a set time as shown in the time point experiments. Cells were fixed with 1% PFA for ~7 to 10 minutes. For simultaneous simultaneous uptake of opsonized vesicles with dextran, 0.1mg/mL 40kDa dextran was added to

Microscopy and Flow cytometry

Images were acquired using a spinning disk confocal microscope (Till Photonics, Germany) and analyzed in FIJI. For flow cytometry, approximately 5000 cells were analyzed on BD Accuri and flow histograms were made in FlowJo (version 10). Cells were either kept on ice in preparation for the analysis or were fixed with 1% PFA.

Supplemental Data

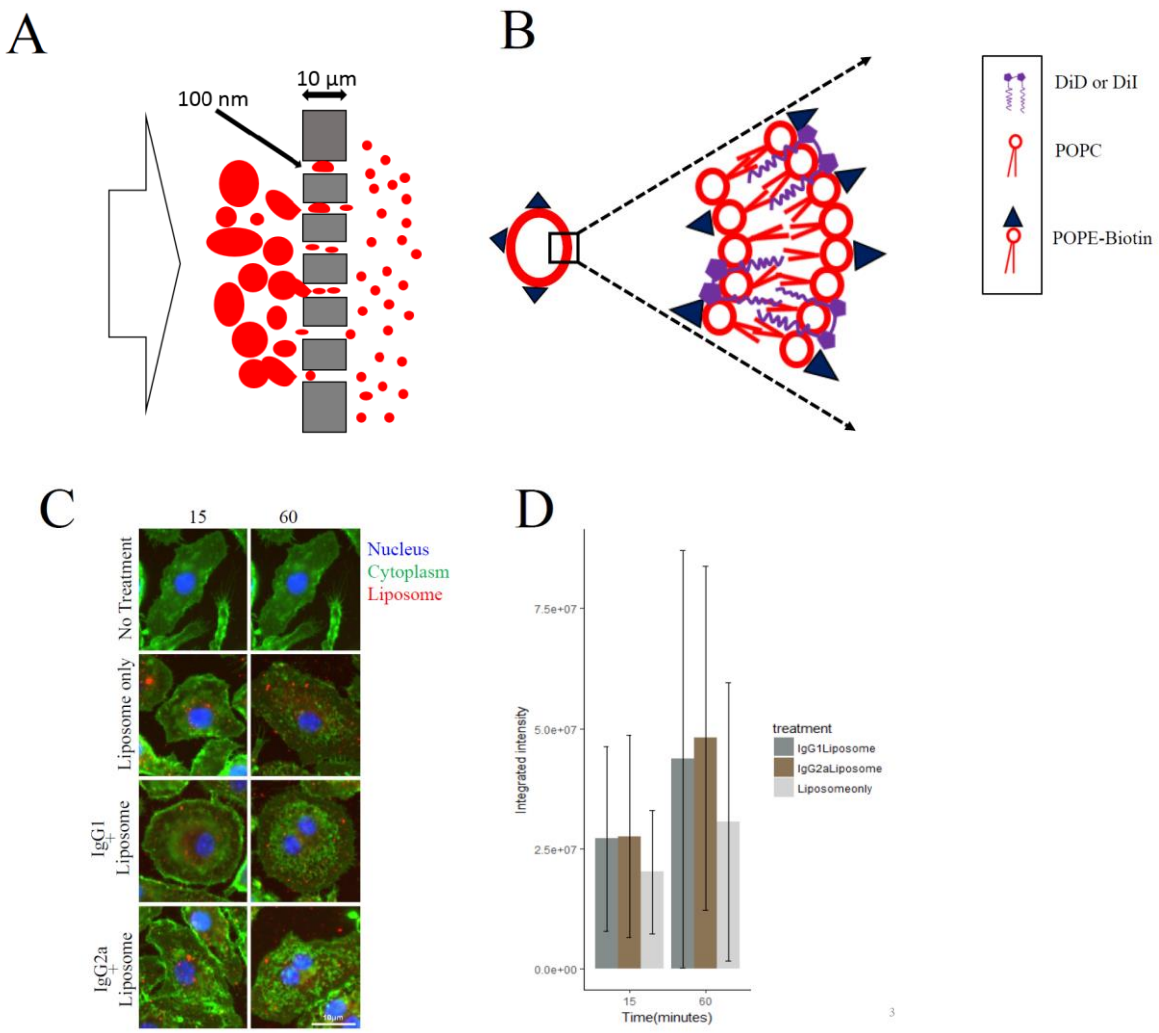

Figure S1. 1 Nonspecific uptake of non-PEGylated liposomes in macrophages (A) Schematic of liposome extrusion through ~100nm pores of polycarbonate membrane. (B) Schematic of non-PEGylated liposome (C) Uptake of IgG1 and IgG2a opsonized liposomes in FLM cells. Non-opsonized liposomes are internalized by non-specific endocytosis. (D) Quantification of liposome uptake in C.

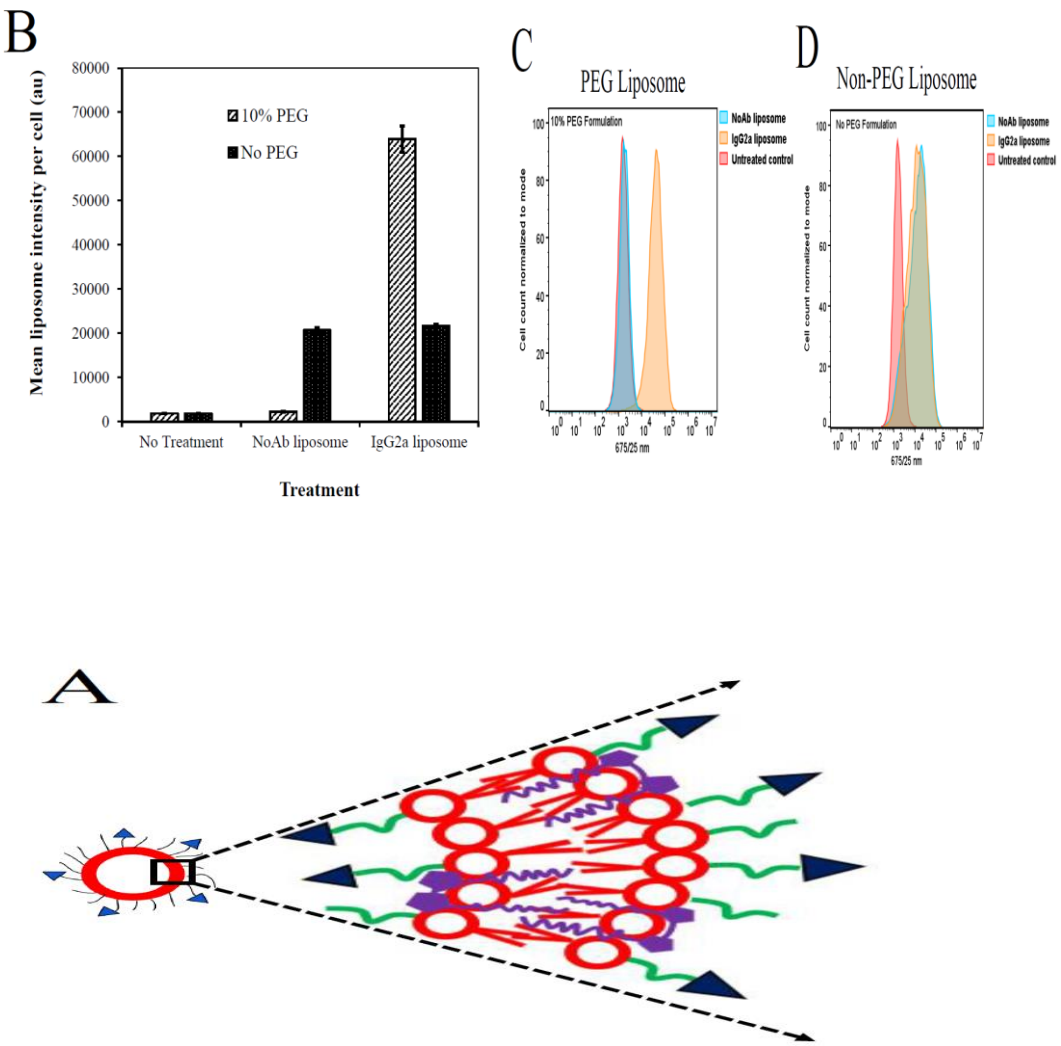

Figure S1. 2 Uptake of PEGylated liposomes in macrophages. (A) Schematic of PEGylated liposome PEG-2000 is shown in green (B) Mean liposome uptake per cell obtained from flow cytometry data in C and D. (C) Uptake of IgG2a PEGylated liposomes is well resolved from uptake of non-opsonized PEG-liposomes enabling relatively accurate measurement of antibody opsonized endocytosis in macrophages. (D) Uptake of free non-PEGylated liposomes (without IgG2a) overlaps with uptake of IgG2a opsonized non-PEG liposome demonstrating strong non-specific uptake.

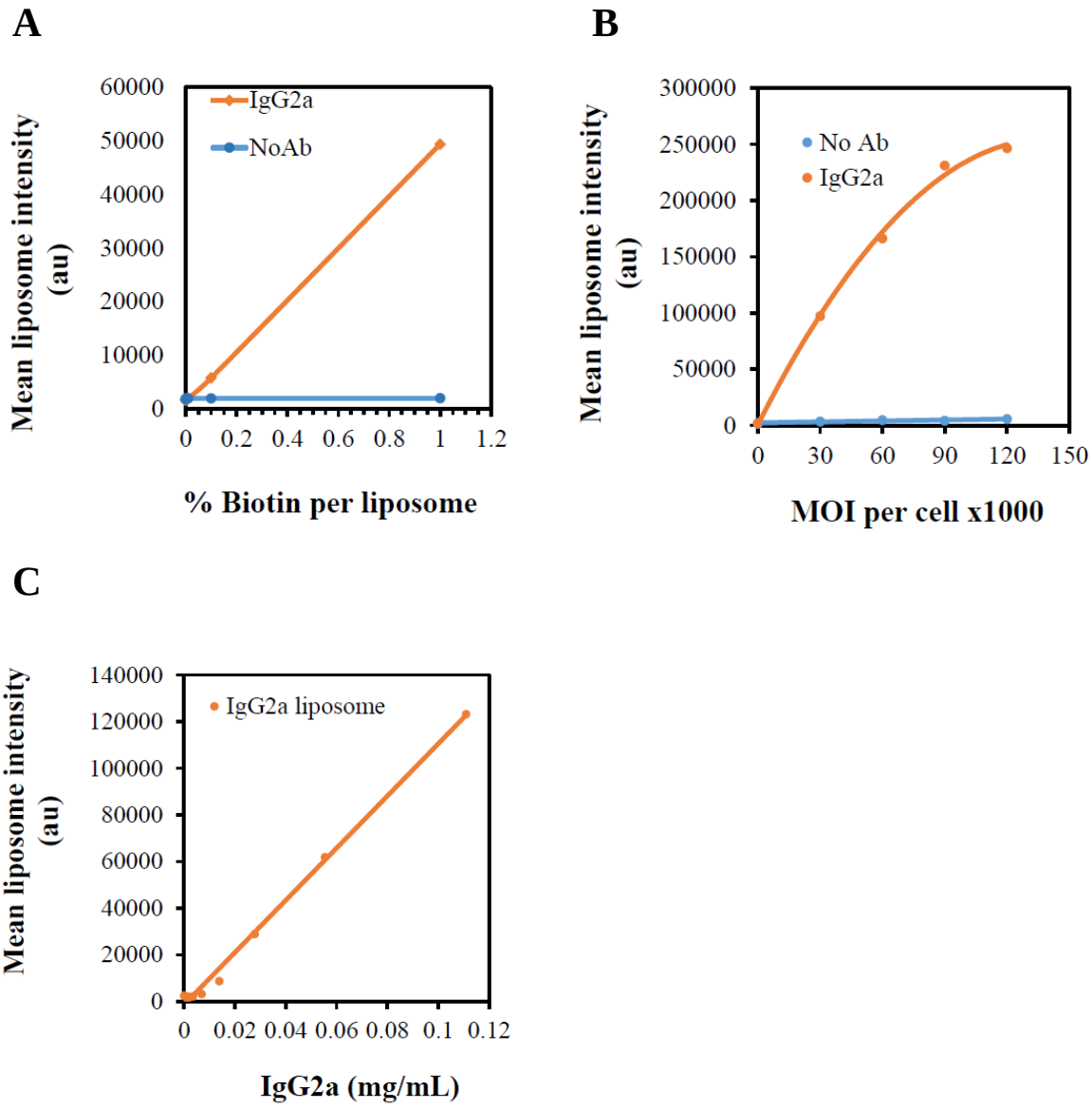

Figure S1. 3 PEGylated liposomes enable modulation of macrophage response. (A) Increasing biotin and corresponding antibody concentration on PEG liposomes increases uptake by macrophages but does not permit uptake in the absence of IgG. (B) Increasing number multiplicity of infection (MOI per cell) of IgG2a opsonized liposomes results in near saturation of receptors at ~120 000 particles per cell whilst non-opsonized PEG-liposomes does not record any significant uptake. (C) At 30 000 MOI, increasing antibody concentration through 0.12mg/mL causes a corresponding increase in cellular uptake of nanoparticles in a linear fashion. Thus, within the limits of the concentrations used for our experiments, biotin antigen was not saturated demonstrating that there were no excess IgG in the solution.

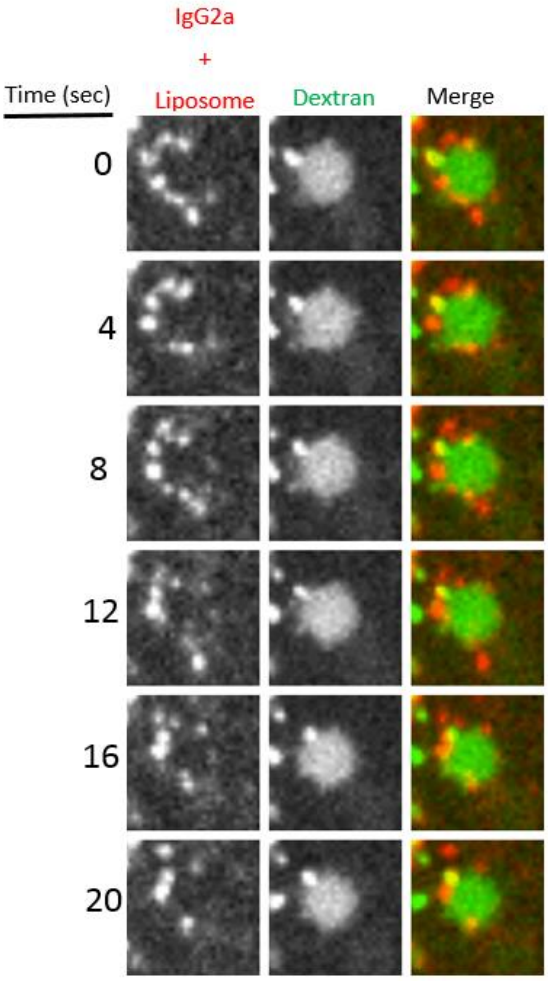

Figure S1. 4 Trafficking of IgG2a opsonized liposomes to the membrane of a macropinosome in bone marrow derived macrophage cell.

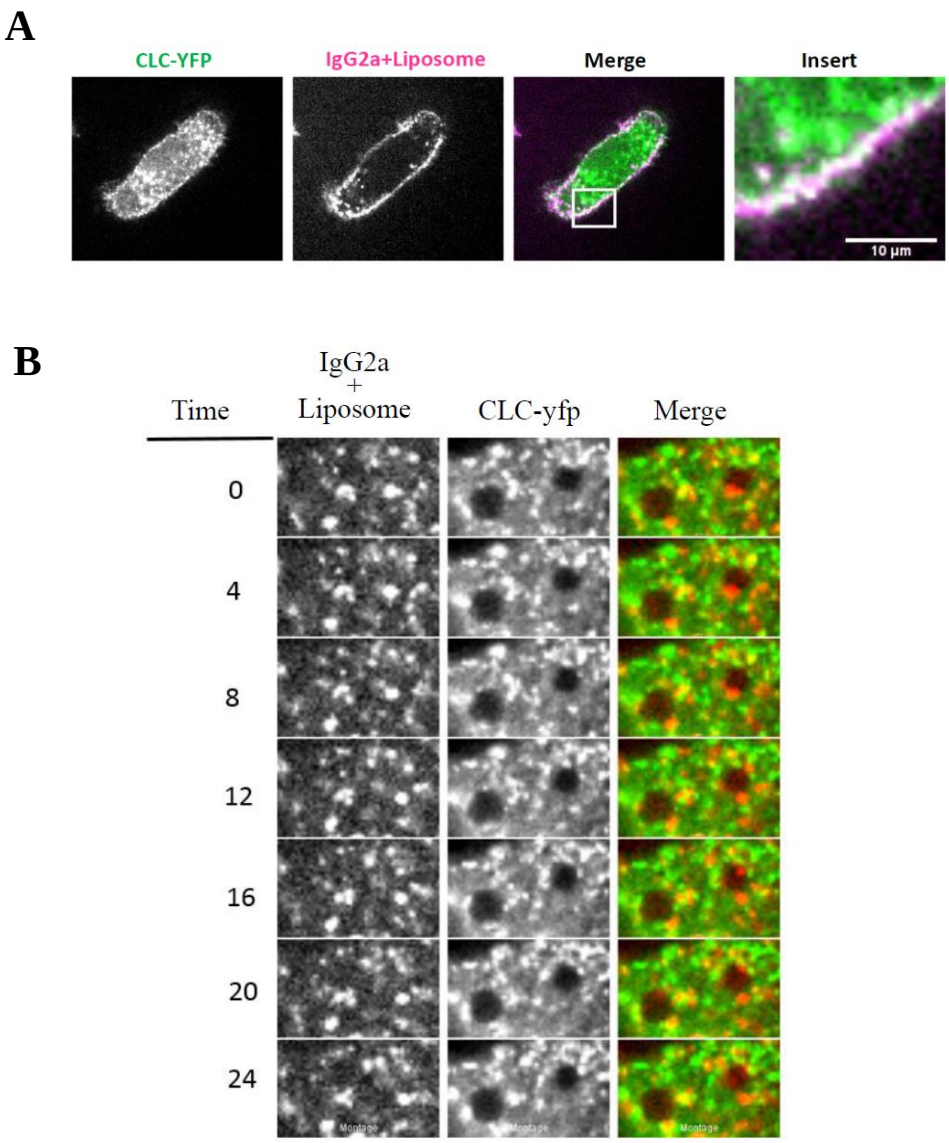

Figure S1. 5 Clathrin co-localizes with IgG2a opsonized liposomes at onset of nanoparticle internalization and on macropinosomes. (A) An FLM cell expressing clathrin light chain-yfp fusion protein was incubated with IgG2a opsonized liposome on ice, fixed in PFA and imaged on a confocal microscope.Patches of clathrin (green) were seen to associate with patches of IgG2a-liposomes (red). (B) Clathrin and IgG2a-liposomes traffic to the membrane of the macropinosome. Images taken from a time lapse movie shows patches of clathrin only endosomes (green), IgG2a-liposome only endosomes (red) and both clathrin+IgG2a-liposome endosomes (yellow) on the membranes of two macropinosomes. The macropinosomes appear in the clathrin channel (green) and the merge channel as two dark holes.

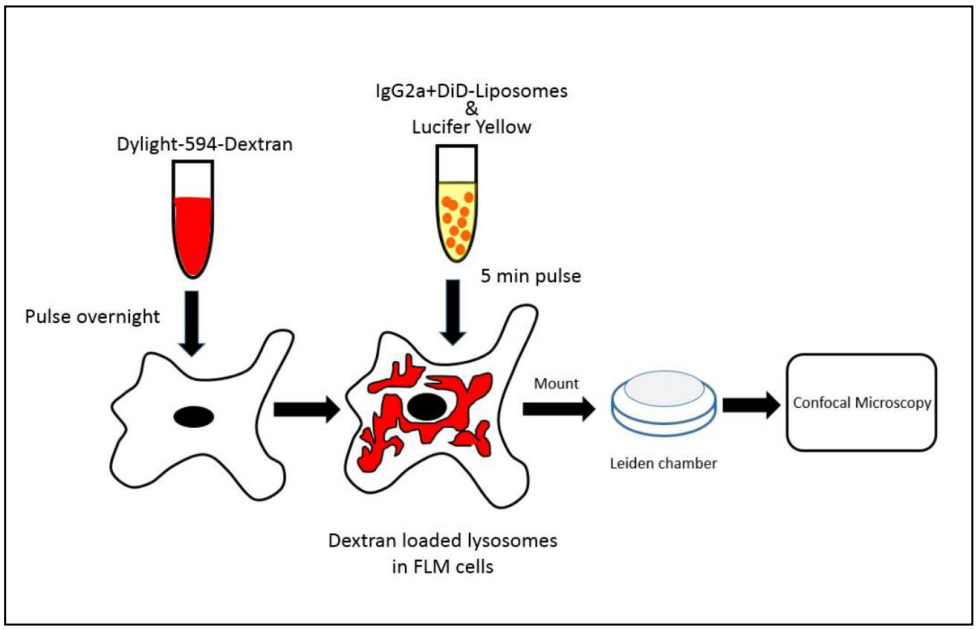

Figure S1. 6 Schematic of 3-color experiment involving live imaging of lysosomes, macropinosomes and IgG2a-liposomes. Lysosomal were preloaded with dylight-594-dextran, washed in PBS and incubated in bone marrow media before exposure to a solution containing IgG2a labelled liposome and Lucifer yellow.

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CHAPTER 4

New insights into trafficking of antibody opsonized nanoparticles in macrophages

Trafficking of antibody opsonized nanoparticles following uptake by FcγRs follows a novel trafficking path in macrophages. Here, I discuss new insights in small particle trafficking gained through this research effort.

Efficient investigation of antibody opsonized nanoparticle uptake by FcγRs require that particles do not adsorb to the cell surface non-specifically or be internalized by scavenger receptors. Addition of PEG to liposome formulations enable nanoparticles to evade non-specific uptake and promote antibody dependent internalization. Following uptake by high affinity FcγRI, immune complexes are trafficked to the macropinosome membrane where IgG segregates from liposomes in a pH dependent manner, and recycles back to the cell surface. Interestingly, fluid-phase-marker degradation precedes liposome degradation as lysosomes interact with macropinosomes and immune complexes differently. Together, these lines of evidence support a novel role for macropinosomes in antibody-antigen segregation.

PEG blocks non-specific uptake and enhances antibody dependent internalization of IgG2a opsonized liposomes

Investigation of small particle uptake in macrophages has been challenging due to non-specific internalization associated with use of naked silica beads and liposomes (Dos Santos *et al.*, 2007; Lee *et al.*, 2015). Other studies on small particle uptake have utilized heat aggregated IgG (Ptak *et al.*, 1998; St. Clair *et al.*, 2017). While this approach is ideal for understanding trafficking of antibodies, it does not give any insight on the influence of antigen on trafficking of intact antibody-antigen complexes in phagocytic cells.

Consequently, an understanding of antigen-antibody trafficking of small particles in macrophages and phagocytes has remained unclear.

Here, we successfully used PEGylated liposomes to investigate the trafficking of immune complexes enabling analysis of FcR-IgG endocytosis independent of other modulating factors such as non-specific adsorption to the cell surface and uptake by scavenger receptors. Addition of DiI and DiD in the formulation process enables quantitative and visual analysis of subcellular trafficking of immune complexes on high resolution microscopes and flow cytometers. Thus, the development of this method has paved the way for investigation of a previously uncharacterized trafficking path in macrophages that is important in immune response.

Binding of antibiotin mIgG2a to FcγRs enables rapid internalization of liposomes in a receptor dependent manner. Based on estimation of nanoparticle number, uptake of IgG2a opsonized nanoparticles nears saturation at approximately ~120,000 vesicles per cell. However, maintaining particle MOI at 30,000 and adjusting the concentration of IgG does not result in saturation of uptake but rather produces a linear relationship within the limits of IgG concentration tested. Thus, endocytosis of antibody opsonized nanoparticles demonstrates saturation kinetics, supporting uptake by a receptor dependent mechanism rather than a fluid phase non-specific uptake pathway.

Differential FcR internalization of antibody opsonized nanovesicles is dependent on high affinity FcγRI and associated Fcγ.

Objective 2 sought to investigate the differential role of three FcγRs (I, IIb and III) as well as FcRγ in mediating uptake of IgG2a opsonized liposomes in fetal liver macrophages. The results demonstrate that high affinity FcγRI and associated FcRγ drive binding and clustering of IgG2a opsonized nanovesicles.

Furthermore, we show that FcγRIIb may aid clustering of immune complexes as knockout of this inhibitory receptor does not affect binding and internalization, but results in formation of smaller endosomes similar to FcγRI and Fcγ knockouts. This underscores the importance of both activating and inhibitory receptors in generating phagocytic signals. The role of FcγRI in driving clustering and internalization of soluble complexes has been characterized (Gillis *et al.*, 2017; Kiyoshi *et al.*, 2015; Maresco *et al.*, 1999). Nimmerjahn and colleagues showed that binding of IgG drives clustering of activating and inhibitory receptors in a ratio that is IgG subclass dependent (Falk Nimmerjahn & Jeffrey V. Ravetch, 2005). They show that this ratio successfully predicts in vivo functions of IgG subclasses. Moreover, Gallo and colleagues show that the density of immune complex has a direct effect on phagocytic signal and downstream activation of ERK (Gallo *et al.*, 2010).

Our findings show that in macrophages, inhibitory FcγRIIb may aid FcγRI in forming large endosomes of soluble antibody-antigen complexes in a way that may have consequences for downstream regulation of phagocytic signals. However, internalization of clustered immune complexes requires FcγRI and the associated Fcγ.

In macrophages, internalized antibody-antigen complexes traffic to the macropinosome membrane.

The trafficking of antibody opsonized vesicles was investigated based on research questions outlined in objective 3. We show that clusters of IgG2a opsonized liposomes are most likely internalized by clathrin mediated endocytosis as strong association of immune complexes and clathrin light chain occur on the plasma membrane and within the cell through 15 minutes post exposure to immune complexes. Moreover, we observed trafficking of clathrin-liposome complexes to the membranes of macropinosomes in live cell experiments. This results is corroborated by other studies demonstrating that FcγR endocytosis of small particles is dependent on clathrin whilst uptake of large particles is more dependent on actin (Banerjee *et al.*, 2013; Fratini *et al.*, 2018; Tse *et al.*, 2003).

Following internalization, IgG2a opsonized nanovesicles are trafficked to the membranes of macropinosomes that form simultaneously. Accumulation of immune complexes on the macropinosome membrane occurs through 15 minutes post exposure without budding of antibodies into the macropinosome lumen. This novel interaction suggests that accumulation of sorting complexes may be enriched on the outer membrane of macropinosomes compared to the luminal membrane, since antibody-antigen segregation occurs on the outer membrane. In fact, a study on plasma membrane protein recycling from macropinosomes in *Dictyostelium discoideum* showed that a recycling complex organized by WASH (Wiscott Aldrich protein and scar homolog) accumulated on the macropinosome membrane in a fashion similar to the distribution of immune complexes on macropinosomes observed in our experiments (Buckley *et al.*, 2016). Since WASH complex is involved in macropinosome and endosome recycling, we speculate that a

sorting complex organized by WASH complex will drive segregation and recycling of antibodies from the surfaces of macropinosomes in macrophages (Marques *et al.*, 2017). The mechanism by which sorting complexes drive segregation of antibody-antigen complexes whilst restricting intraluminal budding of segregated IgG remains to be investigated.

Fc gamma receptors may drive ligand independent macropinocytosis in macrophages

The exclusive internalization of IgG2a opsonized liposomes by receptor dependent pathway and subsequent trafficking of opsonized complexes to the macropinosome membrane, prompted an investigation into the role of FcγRs in mediating macropinosome formation. We observed that macropinosome formation was independent of the ligand IgG2a-opsonized-liposomes irrespective of CSF-1 growth factor starvation. However, knockout of activating FcRs caused a drop in macropinosome formation in a manner that appeared to match corresponding defects in phagocytic activities. Accordingly, knockout of inhibitory FcγR IIb resulted in more than two fold increase in the number of macropinosomes formed in macrophages. Therefore, we propose for the first time, that FcγRs orchestrate a basal signaling network that drive constitutive macropinosome formation in macrophages and possibly other members of the mononuclear phagocytic systems.

Differential interaction of lysosomes with immune complexes and macropinosomes drive early transition of fluid phase molecules to the lysosomal compartment whilst delaying lysosomal uptake of immune complexes

Investigation of lysosome mediated degradation of liposomes showed that delivery of fluid phase molecules to the lysosomal compartment precedes delivery of liposomes. As previously described by Swanson and co, we identify the mechanism of squidlysis or piranhalysis as the means by which lysosomes rapidly internalize luminal content from macropinosomes (Willingham & Yamada, 1978; Yoshida, Pacitto, *et al.*, 2015). Although IgG2a-liposomes accumulate on the macropinosome membrane, lysosomes appear not to directly internalize these complexes but rather engage in a kiss-run interaction resulting in acquisition of lysosomal contents by liposome containing endosomes. This interaction most likely promotes pH drop in liposome endosomes, which is important for IgG segregation from its cargo. Consequently, we found that inhibiting vATPase mediated acidification via pretreatment of cells with bafilomycin blocks segregation of IgG from liposomes. In control cells, segregated IgG is recycled back to the cell surface possibly mediated by high affinity FcγRI or FcRn. The recycled IgG is capable of potentiating phagocytosis of fresh antigen demonstrating that it was competent and not immediately digested by lysosomal enzymes.

Conclusion and Recommendations

In conclusion, we show that uptake of IgG2a opsonized liposomes is heavily dependent on high affinity FcγRI and Fcγ leading to trafficking of antibody-antigen complexes via a novel pathway in macrophages. We demonstrate that the macropinosome plays a role in mediating segregation of antibody from antigen, by organizing the accumulation and sorting of antibody-antigen complexes on a macropinosome membrane limited proximal compartment. Moreover, we describe a new phenomenon in which fluid phase molecules

within the lumen of macropinosomes reach lysosomal compartments by the mechanism of piranhalysis or squidlysis, whilst transition of antibody bound complexes to the lysosomes are delayed, until segregation of antibodies from cargo liposomes. Liposome cargo is eventually transported to the lysosome, leaving a portion of antibody complexes within the cytoplasm where they are recycled back to the cell surface to potentiate endocytosis or phagocytosis of new antigen. Thus, in addition to the known functions of macropinosomes, we identify a novel function that related to antibody-antigen segregation and IgG recycling. This implies that future modulation of the macropinosome or its associated sorting components could have consequences for therapeutic antibody half-life.

Characterization of macropinosome formation in FcR knockouts reveal a potential novel role for activating and inhibitory FcγRs in mediating differential formation or attenuation of macropinosomes in macrophages. The mechanism of this phenomenon should be investigated to identify the molecular players involved. Furthermore, investigation of the effect of FcγRs on trafficking of immune complexes to the macropinosome surface will provide insight on the roles of various FcRs in mediating intracellular trafficking. Sorting of antibody-antigen complexes on the macropinosome surface will most likely be organized by the WASH complex and this requires investigation to elucidate the mechanism of vesiculation on the macropinosome surface during sorting of immune complexes. The method development aspect of this study paves the way for an investigation of the impact of macrophage polarization on uptake, intracellular traffic and recycling of antibody-antigen complexes as well as detailed analysis of the mechanism of uptake by CME.

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