FCγ RECEPTOR SIGNALING
IN RESPONSE TO
SOLUBLE VERSUS IMMOBILIZED IgG

By

SANDEEP GUPTA

A thesis submitted in conformity with the requirements for
degree of Master of Science
Graduate Department of Immunology
University of Toronto

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ABSTRACT

Fcγ Receptor Signaling in response to Soluble versus Immobilized IgG

Sandeep Gupta

Master of Science

Graduate Department of Immunology

University of Toronto

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Fcγ receptors present on monocytes and macrophages clear pathogens engaged by antibodies through the process of endocytosis. Upon being clustered by immune complexes, Fcγ receptors initiate signaling that lead to cellular activation and cytokine secretion, processes that are likely terminated upon receptor internalization and subsequent degradation. In many disease states, however, Fcγ receptors encounter their ligands in the tissue deposited form that cannot be readily internalized. In this project, we examined kinetics of Erk 1/2 phosphorylation and TNF-α secretion in enriched human primary monocytes in response to stimulation of Fcγ receptors by soluble heat aggregated IgG or plastic surface-bound IgG, to mimic immune complexes and tissue-deposited IgG, respectively. Soluble aggregated IgG induced transient and robust signaling, leading to peak levels of phospho-Erk 1/2 by 15 minutes and TNF-α secretion by 1 hour, in contrast to surface-bound IgG, which caused sustained signaling lasting for hours, supporting the hypothesis that signal termination is dependent upon receptor internalization.
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LIST OF ABBREVIATIONS

AA  Arachidonic Acid
ADCC  Antibody Dependent Cell-mediated Cytotoxicity
AgIgG  Aggregated IgG
BCR  B Cell Receptor
CD  Cluster of Differentiation
CRD  Complimentarity Determining Region
DAG  Diacylglycerol
DNA  Deoxyribose Nucleic Acid
EGFR  Epidermal Growth Factor Receptor
ERK1/2  Extracellular signal-regulated Kinase1/2
FcγR  Fcγ Receptor
FcRn  Neonatal Fc Receptor
GM-CSF  Granulocyte-Macrophage Colony Stimulating Factor
GPI  Glycosyl Phosphatidyl Inositol
GTP  Guanosine Triphosphate
IC  Immune Complex
IFN-γ  Interferon gamma
Ig  Immunoglobulin
IKK  IkB Kinase
IL  Interleukin
IP₃  Inositol triphosphate
ITAM  Immunoreceptor Tyrosine Activation Motif
ITIM  Immunoreceptor Tyrosine Inhibition Motif
IVIG  Intravenous Immunoglobulin
JNK  c-Jun N-terminal Kinase
K_d  Dissociation coefficient
MAPK  Mitogen Activated Protein Kinase
M-CSF  Macrophage Colony Stimulating Factor
NF-κB  Nuclear Factor κB
NK Cells  Natural Killer Cells
PBMC  Peripheral Blood Mononuclear Cells
PDGFR  Platelet-derived Growth Factor Receptor
PDK  Phosphoinositide-dependent Kinase
PI3-K  Phosphatidylinositol 3-Kinase
PIP_2  Phosphatidylinositol bisphosphate
PKC  Protein Kinase C
PLA_2  Phospholipase A_2
PLC_γ  Phospholipase C_γ
RANK  Receptor Activator of Nuclear factor κB
ROS  Reactive Oxygen Species
SDS-PAGE  Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SH  Src Homology
SHIP  Src homology 2-containing Inositol Phosphatase
SLE  Systemic Lupus Erythematosus
TACE  TNF-α Converting Enzyme
TCR  T Cell Receptor
TGFβR  Transforming Growth Factor-β Receptor
TLR  Toll-like Receptor
TNF-α  Tumor Necrosis Factor α
I. INTRODUCTION

Circulating immunoglobulin molecules are extremely important for the body’s immune system to carry out immune surveillance and provide protection against invading pathogens. These immunoglobulin molecules form immune complexes upon recognition of foreign antigens [1]. The immune complexes are in turn cleared by different cells via binding, internalization and degradation [2]. During this process, these cells also get activated and respond via a variety of effector mechanisms, including production and secretion of inflammatory cytokines that further promote defense against any potential invasive pathogens [3]. Disease symptoms are a consequence of not only the direct invasive and virulent activity of the pathogens, but also because of the immune system’s reaction to contain subsequent progression and propagation of disease-causing invaders.

Fc Receptors present on a large variety of immune cells play the key role of recognizing these immune complexes, mediating uptake, and initiating signaling that leads to cellular responses such as production of inflammatory cytokines and mediators [4]. Once Fc Receptors and the immune complexes are internalized and degraded and the instigating antigen is cleared, the cellular responses subside, due to signal termination. The immune system reverses its activity and helps the body return to a healthy and normal state.

There exist many instances, however, where the antigens that the immunoglobulin molecules encounter are insoluble and bound to tissue surfaces that cannot be internalized readily [5]. Although immunoglobulin molecules recognize their antigenic ligands, they
remain tethered to the tissue surface and the immune cells fail to internalize them, potentially leading to a persistence of immune complex-Fc receptor engagement. We believe that the inability to internalize and degrade the antigen-antibody complex in such circumstances potentially leads to a more sustained and prolonged cellular response, due to continuous Fc Receptor engagement and stimulation, and that such a prolonged response may ultimately be to the body’s own detriment, contributing to the persistent pathology in these conditions.

1.1. Innate Immunity

In humans, innate and adaptive immunity work in tandem to defend the body against intrusion and invasion by any pathogen or foreign organism, employing a myriad of cells and protein molecules that have acquired specialized functions over the course of evolution. With the ability to recognize something as foreign, the ability to mount a response against the foreign agent, and the capacity to generate memory of previous encounters, the immune system is remarkable in its complexity and efficiency.

Defense against pathogens begins at the level of the integumentary system and the mucosal barrier that limits entry into the body [6]. However, once the surface barrier is breached and inner tissue is invaded, the evolutionarily ancient system of innate responses acts promptly to contain the growth and dissemination of any pathogens. Key cells involved in the process are neutrophils, eosinophils, mast cells, macrophages, natural killer cells as well as epithelial cells [7] [8]. These cells have the ability to distinguish foreign from self through their expression of germ-line encoded cell-surface
and intra-cellular molecules known as pattern recognition receptors that recognize unique and conserved pathogenic molecular patterns absent in self [9]. This recognition results in a non-specific non-clonal effector response, involving factors such as cytokines and peptides that have an immediate effect to limit invasion. Beyond reacting to the initial onslaught of infection, innate immunity has the vital role of activating the adaptive and more specific branch of immunity that helps to render the body much more competent and efficient to counter future invasions by similar pathogens.

1.2. Immunoglobulin and Immunoglobulin Receptors

1.2.1. Functions of Immunoglobulin

Immunoglobulin molecules drive the humoral immune responses. They are gamma globulin proteins that are present in blood and other body fluids that carry out immune surveillance [10]. Some of the direct effects of antibodies include opsonization [11], neutralizing antigens [12], as well as mediating complement fixation by the classical pathway [13].

Immunoglobulins contain highly variable Fab regions in their structure, the total repertoire of which can recognize almost any antigenic pattern with high specificity, achieved by the processes of V (D) J recombination, somatic hypermutation and affinity maturation. While the variable Fab regions of immunoglobulins are important for recognition of diverse antigens, the constant region, also known as the Fc portion (Fragment crystallizable) allows cells of the innate immune system to act as effectors of adaptive humoral response [14] [15] [16] by binding to Fc Receptors. A large number of
immune cells including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, Natural Killer cells, mast cells and B cells express receptors for the distinct classes of Ig molecules [17] [18] [19] [20]. Upon receptor engagement and clustering, depending on the class of Ig molecule and the type of cell, a wide variety of responses are generated. These include phagocytosis, endocytosis, respiratory burst by neutrophils, degranulation by mast cells, antibody-dependent cellular cytotoxicity (ADCC) and cytokine production [21] [22].

1.2.2. Fc Receptor classification and functions

Fc Receptors comprise a set of molecules that are expressed on the surface of various immune cells and are named based on the specific class of Ig molecules they bind, determined by the Fc portion of the molecule [23]. Based on the antibody class (IgA, IgG, IgE and secreted IgM) recognized, they are classified as FcαR, FcγR, FcεR and Fcα/μR. There exists a structurally distinct receptor for IgG called FcRn, found in neonates, which also binds to IgG.

IgA molecules are critical in the context of mucosal immunity and are present in secretions that bathe the mucosal surfaces such as tears, saliva, colostrum, intestinal juices, vaginal fluid and in the secretions of the prostate and respiratory epithelium. FcαRI are expressed by monocytes, macrophages, neutrophils and eosinophils and have a low affinity for IgA [24]. Fcα receptor cross-linking induces microbial killing via phagocytosis [25] [26].
Fcε/μR present on B cells, mesangial cells and macrophages has a medium affinity for IgA and high affinity for IgM and can induce endocytosis in these cells [27] [28].

FcεRs are of two types, FcεRI and FcεRII that bind to IgE molecules with high and low affinities, respectively and are involved in allergy and defense against parasitic infection. FcεRI is a tetrameric complex that consists of an α-chain, a β-chain and two γ-chains. Expressed predominantly on mast cells and basophils, due to their high affinity these receptors remain bound to monomeric IgE molecules on the cell surface [29] [18]. When an allergen or parasitic pathogen cross-links two or more bound IgE at the cell surface, these receptors initiate signaling that results in degranulation of inflammatory and allergic mediators in mast cells such as histamines, interleukins, prostaglandins and cytokines including IL-1, IL-3, IL-4, IL-5, IL-6, IL-13 and TNF-α, and major basic protein and peroxidase in eosinophils. FcεRII are C-type lectin molecules and have a low affinity for IgE. They are found on B cells, activated macrophages, eosinophils, follicular dendritic cells and platelets [19]. Both FcεRI and FcεRII expression are known to be upregulated by IgE stimulation [30].

FcRns are transiently expressed during the early few days of life and have an important role in conferring passive immunity from the mother to the neonate [31]. The neonatal monocytes, macrophages, dendritic cells, epithelial cells, endothelial cells and hepatocytes express these receptors that not only bind the maternal IgG transferred through the placenta during fetal life and milk after birth, but also help protect the maternal IgG from degradation [32] [17] [33].
1.3. IgG and Fcy Receptors

1.3.1 IgG: structure and function

IgG are the most abundant immunoglobulin molecules in circulation and in tissues and are predominant humoral effectors of the systemic adaptive immunity. IgG is also found in lymph and other secreted body fluids. It accounts for about 75% of all the circulating antibodies in the body. IgG antibodies are secreted by plasma cells upon successful B cell stimulation and co-stimulation by T cells. Each plasma cell secretes a monoclonal variant of IgG molecules that is soluble and monomeric that bears the complimentarity determining regions (CRD) specific to a particular antigen [34] [35]. Serum contains about 3-20 mg/ml of the various IgG isotypes. The serum half-life of IgG is about 2-4 days. There exist four sub-classes of IgG in humans and the average proportions are: IgG1 67%, IgG2 22%, IgG3 7% and IgG4 4%, although variations exist between individuals [36]. Soluble IgG molecules (sIgG) are monomeric, have two heavy chains and two light chains (H2L2 form) and have a molecular mass of 146 kD on average. However, the small differences in the amino acid sequence in the heavy chains (CH2 domain) and presence of disulfide bonds in some subclasses confer differences in physical properties between the isotypes [37]. A ribbon depiction of the crystal structure of a mouse IgG2a antibody is illustrated in Figure 1.1 [38, 39].

IgG carries out important immune functions. IgG antibodies, released by plasma cells are predominately involved in the secondary antibody response and are a major component of the humoral adaptive immunity, which occurs following antigen recognition. Some of the direct effects of IgG are recognition of bacterial, viral and
**Figure 1.1. Immunoglobulin molecule.** Ribbon depiction of the crystal structure of a mouse IgG2a antibody with the N-linked carbohydrate structure attached at the Fc in ball-and-stick form. Heavy and light chains with their termini are shown in dark and light grey, respectively, and the inter-chain disulphide bridges are indicated. The asymmetric arrangement of the Fc and Fab fragments with respect to each other reflects the flexibility of the molecule. (Reproduced from P. Sondermann and V. Oosthuizen, Biochemical Society Transactions (2002) 30, (481–486), X-ray crystallographic studies of IgG–Fcγ receptor interactions.)
fungal pathogens, their agglutination, neutralization and immobilization and complement fixation by the classical pathway. IgG also exert indirect effects through Fcγ Receptor engagement on FcγR-bearing cells causing endocytosis, phagocytosis, cellular activation, cytokine and inflammatory mediator secretion, degranulation, respiratory burst and antibody-dependent cell-mediated cytotoxicity (ADCC) [40] [41]. Due to their small and monovalent structure, IgGs can diffuse well between the serum and extracellular fluid, and are the only IgGs that can cross the mammalian placenta, conferring passive immunity from the mother to the newborn in the early months of life [42].

1.3.2. Fcγ Receptors

Although the phenomenon of serum elements augmenting bacterial particle phagocytosis was appreciated as early as in 1903 [43], the specific mediators involved, antibodies, were not identified until the mid twentieth century. The subsequent cloning of cell surface Fcγ Receptors expressed on hematopoietic cells facilitated further understanding of this complex group of membrane-bound glycoprotein molecules [44, 45] [46].

Three distinct classes of FcγRs have been defined that are expressed on the cell surface of various human leukocytes: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) each possessing extracellular immunoglobulin-related domains [47], a single transmembrane domain and an intra-cytoplasmic domain of variable length, all products of alternative splicing and gene duplication on chromosome 1q23 [48]. The different types of the Fcγ receptors are illustrated in Figure 1.2 [49].
Figure 1.2. Fcγ Receptor classification. Schematic representation of different Fcγ Receptors expressed by human immune cells with their alleles and affinity. (Reproduced from Falk Nimmerjahn & Jeffrey V. Ravetch, Nature Reviews Immunology 8, 34-47, January 2008, Fcγ Receptors as regulators of immune responses.)
1.3.3. Properties of Fcγ Receptor types

FcγRI or CD64, constitutively expressed by macrophages and monocytes and inducible by IFN-γ in neutrophils [50] [51], binds to monomeric soluble IgG molecules with high affinity with a $K_d$ of $10^{-9}$ M. Because of this strong association, FcγRIIs are thought to remain occupied by monomeric IgG in the circulation due to the high concentration of the IgG in serum. The extracellular portion of FcγRI is composed of three Ig-like domains, one more than FcγRII and FcγRIII classes, and has preferential binding for IgG1 and IgG3 subclasses [52] [33]. The cytoplasmic domain does not have a signaling motif of its own, rather FcγRI associates with the FcR γ-chain that bears an ITAM motif required for signaling and cellular activation [53].

Upon clustering on the cell surface by polyvalent ligands, FcγRI is thought to be a key mediator of ADCC, especially after its upregulation by IFN-γ, derived from T cells [54] [55] [56]. However, the role of FcγRI in immune complex clearance is not well understood.

FcγRIII or CD16 exists in two distinct forms: FcγRIIIA is expressed on macrophages, neutrophils and NK cells [57]. Roughly 10% of CD14+ monocytes are also CD16+. FcγRIIIA has two Ig-like domains in the extracellular portion, a transmembrane part, and a small distinct intracellular domain. FcγRIIIA associates with FcR γ-chains or TCR ζ-chains, both of which contain ITAM motifs required for signaling [58] [59] [60]. FcγRIIIB on the other hand is expressed only on neutrophils and is anchored to the outer leaflet of the plasma membrane by a glycosyl-phosphatidylinositol (GPI) moiety [61]. FcγRIIIIs are low affinity receptors with a $K_d$ in the range of $10^{-6}$ M and are thus unable to
bind circulating soluble monomeric IgG, but play a role in phagocytosis and induction of ADCC in NK Cells and macrophages [62] [63] [64]. While FcγRIIIA and FcγRIIIB both bind to immune complexes (IC), only FcγRIIIA is able to internalize the bound ligand, whereas, FcγRIIIB is postulated to sequester ICs from the circulation. Furthermore, FcγRIIIA can act synergistically with FcγRIIA in the presence of functional γ-chain or ζ-chain to initiate signaling [65] [66].

FcγRIIs are expressed by a wide variety of cells and have a significant role in IC clearance by endocytosis and in immune regulation. They are found on virtually all immune cells including monocytes, macrophages, neutrophils, eosinophils, Langerhans Cells, B cells and on platelets. The expression level varies widely based on the type of cell, being low on platelets [67] and much higher on macrophages and monocytes [68]. FcγRIIIs have low affinities for monomeric IgG, with a K_d of 10^{-7} M, comparable to that of FcγRIII; like FcγRIII, they can only bind to multivalent ICs with strong avidity. FcγRII in humans has a complex genetic locus, encoded by a minimum of three genes [69], which results in six distinct transcripts [48]. Three of these six transcripts are derived from the FcγIIB gene by alternative splicing of cytoplasmic exons with an additional form derived from alternative splicing of the signal sequence [48] [69]. In addition, two other transcripts, FcγIIA and FcγIIC, are derived from two distinct genes [70]. There exists high homology between the FcγRII molecules in the extracellular and transmembrane domains. However, the unique FcγRIIIB that bears an ITIM motif in the cytoplasmic portion differs considerably from FcγRIIA and FcγRIIC, which contain ITAMs in their cytoplasmic domains. The FcγIIB1 and FcγIIB2 variants are distinct in their cell expression and function as well. While both inhibit the activity of other FcγRs
when co-clustered and block cellular activity, FcγIIB2, expressed on macrophages, neutrophils and eosinophils can mediate endocytosis, whereas FcγIIB1 expressed on B cell and mast cells cannot. Neither FcγIIB1 nor FcγIIB2 can mediate phagocytosis of large opsonized particles.

1.4. Signaling by FcγRII

FcγRs can be classified functionally into activating and inhibitory receptors: FcγRI, FcγRIIA and FcγRIIIA are considered activating receptors by virtue of their ability to induce signaling. The activatory roles of the FcγRs I, IIA and IIIA have been determined by their capacity to initiate tyrosine phosphorylation, alter [Ca2+], effect phagocytosis of opsonized particles, influence the synthesis of cytokines such as interleukins and TNF-α, effect ADCC and respiratory burst in multiple transfected, knockout and primary cell systems [71] [72] [73] [74] [75].

FcγRI associates with the FcR γ-chain that bears ITAM motifs to initiate signaling and cellular activation [76] [77]. Similarly, FcγRIIIA associates with either a homodimer of the common γ-chain, homodimer of the ζ-chain or a heterodimer of the γ-ζ chains causing signal induction [78]. FcγRIIA is unique among the group in having a longer cytoplasmic tail that has considerable homology to the common gamma chain and possesses an ITAM motif. The FcR γ-chain is expressed by a large variety of cells such as macrophages, monocytes, NK cells, platelets, eosinophils, mast cells, and some T cells and forms part of various receptor complexes, including antigen receptors for T and B lymphocytes [79] and activating NK cell receptors. Studies with human FcγRI have
shown that the FcR γ-chain is also necessary for IgA mediated activation of leukocytes, stimulating cells for respiratory burst, phagocytosis, cytokine production and antigen presentation [80] [81]. Similar studies on human FceRI have revealed the significance of the common γ-chain in basophil activation and mast cell degranulation [82] [83]. The γ-chain contains pairs of tyrosines and leucines within the consensus ITAM sequence D/E-X7-D/E-X2-(Y-X-X-L)-X7-(Y-X-X-L), where X represents any amino acid [84]. The tyrosines become phosphorylated upon receptor clustering and then act as docking sites for Syk kinase to initiate further signaling. The ζ-chain that associates with FcγRIIIA however has one more Y-X-X-L motif than the γ-chain. The cytoplasmic tail of the low-affinity FcγRIIA in humans is distinct from the FceRIγ, FcεRIβ, CD3γ, and the common FcγRγ chains [85], containing an atypical ITAM sequence (Y-X-X-L)-X12-(Y-X-X-L), required for tyrosine phosphorylation [86]. Signaling from the FcγRIIA α-chain is also Syk dependent [87] [76].

FcγRIIB, a low affinity receptor (Kd > 10^{-7} M), is the only known inhibitory Fc receptor and has an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain [22] [88]. The ITIM sequence is (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val), where X denotes any amino acid. FcγRIIB was initially described on B cells [89] by its ability to attenuate B cell activation, antibody production, lymphokine release, proliferation and survival when co-clustered with the BCR. These single chain inhibitory receptors share considerable homology in their extracellular domains to their activatory counterpart, FcγRIIA, and are expressed on nearly all hematopoietic cells, including macrophages, monocytes, mast cells, neutrophils and B cells. When co-clustered with activating receptors, FcγRIIB can inhibit calcium mobilization, PKC activation and
activation of GTPases and cytoskeleton rearrangement [90], phagocytosis [91] and cytokine production [92] [93]. Studies in autoimmune mouse models have identified FcγRIIB as a key regulator of the immune system and its polymorphisms have been implicated in diseases like systemic lupus erythematosus (SLE) [94], autoimmune thrombocytopenia [95] [96], and Goodpasture syndrome-like glomerular disease [97], in regulation of mast cell function in allergy [98]; it also may play a role in the efficacy of IVIG therapy for autoimmune diseases [99] [100] [101].

1.5. Mediators of FcγR signaling

Signaling from Fcγ receptors has been studied in multiple cell systems and many signaling pathways and molecules have been implicated. These include Src family kinases [102], Syk kinase [87], diacylglycerol and Ca^{2+}, protein kinase C (PKC) [103] [104], phospholipase A2 (PLA2), phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI-3K) [105], mitogen activated protein kinases (MAPK) such as extracellular signal-regulated kinase (ERK) [106] [107] and GTPases of the Rho family [108].

1.5.1. Early phosphorylation events

The earliest event that is known to occur as a result of clustering of activatory receptors is phosphorylation of the tyrosine residues within the ITAM motifs by Src family kinases, which include Src, Fyn, Fgr, Hck and Lyn [84] [86]. These kinases have a myristoylated amino terminal that anchors them to the cell membrane in proximity to the receptors [109]. The kinases also carry SH3, SH2 and catalytic domains [110]. The requirement of Src family kinase activity has been shown to be essential in phagocytic
activity of various immune cells [102]. Phosphorylated tyrosine residues serve as docking sites for the protein tyrosine kinase Syk, which is in turn activated at these sites [111]. Syk is a 72 kD non-myristoylated protein kinase that is present in all hematopoietic cells. Cross-linking of FcγRI and FcγRIIA [112] has been shown to result in recruitment of Syk to the phosphorylated ITAM motifs on the common γ-chain in macrophages [87]. Although FcγRIIA signaling requires Syk activity as do FcγRI/γ and FcγRIIIA/γ, there are a few differences that are present attributable to the difference in structure of the ITAM-containing α-chain of the FcγRIIA, which exhibits higher affinity for Syk [113]. It has been shown in transfected systems that FcγRIIA is able to carry out more efficient phagocytosis than FcγRIIIA/γ chain complex, both of which have similar affinities for IgG [76]. Syk is important for phagocytosis, superoxide production, antibody-dependent cell-mediated cytotoxicity (ADCC), cellular maturation [114] [112] and secretion of cytokines in mouse macrophages and neutrophils [115]. Syk thus acts as a divergence point for many of the subsequent downstream signaling pathways such as activation of PI3-K and PLCγ, as discussed below. Syk activation is initially dependent upon the Src kinase activity for recruitment to the phosphorylated ITAM, but Syk trans-phosphorylation is the main mechanism by which the Syk activation loop is tyrosine phosphorylated, which is required for its enzymatic activity [116].

As mentioned before, macrophages, monocytes, mast cells among others express FcγRIIB, which, when co-clustered with activating receptors, can inhibit signaling. Studies on B cells, mast cells, human peritoneal macrophages and mouse macrophages have illustrated the importance of this mechanism [117] [118]. Tyrosine phosphorylation of the ITIM motifs by Src kinases initiates the inhibitory process, creating docking sites
for protein tyrosine phosphatases SHP-1 and SHP-2, which trans-dephosphorylate the phospho-tyrosine residue on the ITAM of FcγRIIA or the γ-chain of others, thus opposing the activity of the Src and Syk kinases [119].

1.5.2. Phospholipase Cγ

The enzymes PLCγ1 and PLCγ2 are responsible for the induction of secondary messengers inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG), which are critical for Ca²⁺ regulation and activation of protein kinase C (PKC), respectively, in platelets [120] and macrophages [121]. Particularly in macrophages, it has been reported that FcγRI and FcγRIIA stimulation induces IP₃-dependent PLCγ1-mediated Ca²⁺ increase [122] [123]. PLCγ2 is abundant in hematopoietic cells. Studies in platelets, which express only FcγRIIA, have shown that Syk activates PLCγ2 directly or via PIP₃, which is downstream of Phospho-Inositol 3-Kinase (PI3-K) activity [124]. DAG acts on PKC, leading to induction of Ras activity [125] as discussed in subsequent sections.

1.5.3. PI-3K system

Syk activates PI3-K in response to FcγR signaling in macrophages and neutrophils [126] [127]. PI3-Ks are a family of three different classes of enzymes capable of phosphorylating the 3-position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns). Type I PI3-K converts phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol (4,5)-bisphosphate, PI (4,5)P₂, into PI (3,4)P₂ and PI (3,4,5)-P₃, respectively. PI (3,4,5)P₃ can allow membrane recruitment of PH domain-bearing molecules, which is necessary for PLC activation and Ca²⁺ homeostasis [128] [129]. Type I PI3-K is a heterodimer composed of an 85 kD regulatory component and a
110 kD catalytic component. It has been shown that in monocytes and neutrophils that Syk associates directly with the p85 regulatory domain of PI3-K via a pTyr-SH2 domain interaction [130] [131]. Subsequent effects include actin rearrangement and potential recruitment of myosin X required for pseudopod extension during phagocytosis [132], Akt and Erk activation, downstream NF-kB activation [133] [134] [135] and arachidonic acid (AA) release via the activity of PLA2 [136], among other processes.

FcγRIIB can also negatively regulate PI3-K dependent signaling by recruiting the Src homology 2 domain-containing inositol 5'-phosphatases (SHIP-1 and SHIP-2). These phosphatases dephosphorylate PIP3. This is an important mechanism of regulation by FcγRIIB at a level downstream of early activation signals such as Syk activation that prevents PI3-K dependent signaling from propagating. SHIP has been shown to downregulate B cell activation and inhibit FcγR-mediated phagocytosis in macrophages [137] and monocytes [138].

1.5.4. Akt pathway

3-phosphoinositides can trigger the recruitment of Akt to the plasma membrane and its subsequent activation [139]. Akt contains a N-terminal pleckstrin homology domain (PH-domain) and a C-terminal kinase domain and once activated by phosphorylation at its Ser473 and Thr308 site, translocates to the cytosol and nucleus to further phosphorylate substrates. This phosphorylation is mediated by phosphoinositide-dependent kinase 1 and 2 (PDK1 and PDK2), which are dependent upon the products of PI3-K activity. Akt is involved in a wide variety of signaling pathways, predominantly augmenting cell survival by regulating FoxO1, Bad and Bcl-xL pathways, blocking
apoptosis by regulating caspase-9 and p53, promoting protein synthesis, glucose transport, cell growth and in hematopoietic cells feeding into the JNK and NF-κB pathways [140]. In the context of FcγR signaling, it has been shown in murine macrophages that FcγR ligation increased Akt phosphorylation, which correlates with increased PI3-K activity [141].

The activation of NF-κB by Akt is probably carried out in two different ways. Activated Akt phosphorylates IκB kinase (IKK) resulting in phosphorylation of IκB, which dissociates from the p65/RelA-p50 heterodimer of NF-κB and is ubiquitylated and subsequently degraded [142]. This allows NF-κB to translocate to the nucleus. The second mechanism that has been proposed is that Akt stimulates the trans-activation potential of the p65/RelA subunits through utilization of IKK to activate MAPK p38 [143]. The role of Akt as a major signaling component has also been highlighted in FcεRIIIB signaling on mast cells [144]. FcγRIIB can inhibit Akt phosphorylation, activation of MAPK Erk1/2, p38 and JNK in a SHIP-dependent way. Akt has also been shown to promote signaling required for phagocytosis in Raw 264.7 macrophage cells or murine bone marrow derived macrophages through the activation of p70S6 kinase [145].

1.5.5. MAP Kinase pathway

Signaling from FcγRs has been shown to initiate mitogen-activated protein kinase (MAPK) activation in a variety of hematopoietic cells including neutrophils [71] and monocytes [146] [147]. MAPKs are a large and diverse group of serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens), and regulate various cellular activities such as gene expression, mitosis, differentiation and cell
survival/apoptosis [148]. The classical MAPK pathway involved in signaling from growth factor receptors such as epidermal growth factor receptor (EGFR) [149] and platelet-derived growth factor receptor (PDGFR) [150] involves as intermediates Grb2 and Sos. The adapter protein Grb2 forms protein complexes through interactions of its SH2 and SH3 domains. One Grb2 effector molecule in the mast cell is Sos, which is a guanine nucleotide exchange factor that promotes GTP-loading, and hence activation of the small GTPase, Ras. Ras subsequently activates cRaf or Raf-1, a serine/threonine kinase of the MAPKKK family. Signaling from Fcγ receptors has been shown to activate Grb2/Sos in U937 cells [151]. Activated Raf-1 can phosphorylate to activate the dual specificity protein kinases MEK1 and MEK2, which in turn phosphorylates Erk1 and Erk 2 [152] [153] [154]. In mast cells, FcεRI engagement by allergens also has been shown to trigger signaling via Grb2/Sos [155].

1.5.6. Erk1/2 activity

Among the six distinct groups that have been characterized in mammals, extracellular signal regulated kinases (Erk 1/2), also known as classical MAPKs, have been shown to have specific roles downstream of FcγR signaling [106] [107] [71]. In neutrophils, the homotypic interactions of FcγRIIA or FcγIIIB induce significant increase in the level of phospho-Erk [156] [71] [157] [158] [159]. Similarly, studies with pre-monocytic U937 cells stimulated with IgG cross-linking [160] suggest that Erk phosphorylation is one of the vital downstream processes for inflammatory mediator release. Erk is believed to be a convergence step and the pleiotropic effector of more than one signaling cascade initiated at the level of the cell surface. Erk mediates activation of nuclear factors such as Elk and NF-kB, which are known to be important for cytokine
expression [147] [161]. In neutrophils, there is evidence that FcγR cross-linking and phagocytosis induces Erk1/2 phosphorylation via PKC activation of Ras and Raf-1 [162]. Syk phosphorylation can activate PKC directly, or via PLCγ/DAG, highlighting the complexity of the system. Another parallel cascade initiated by FcγRIIA and FcγRIIIB leading to Erk 1/2 and p38 MAPK phosphorylation is through activation of PI-3K, at least in neutrophils [163] and macrophages [106]. A role of Erk in phagocytosis is thought to be the activation of PLA2 and the production of AA [164]. In neutrophils and macrophages, but not in monocytes, it has been demonstrated that FcγR-induced cPLA2-mediated AA release requires Erk [157], [165].

A schematic representation of various signaling intermediates downstream of Fcγ receptors is shown in Figure 1.3.

1.6. Cytokine production: A consequence of FcγR signaling.

Most studies of FcγR signaling have focused on requirements for immediate events, in particular phagocytosis and production of reactive oxygen species [164] [166]. However, one of the most important functions of immune cells is the ability to produce inflammatory mediators and cytokines that have a wide variety of functions in normal host defense, immune cell chemotaxis, trans-activation and differentiation of other cells and induction and release of subsequent immune regulators and factors [167] [168]. Hematopoietic and many non-hematopoietic cells participate in this process and are part of a tremendous and complex regulatory network. Like many other receptors of innate and adaptive immunity, FcγRs trigger inflammatory response through synthesis and
Figure 1.3. Schematic representation of some of the known Fcγ Receptor signaling components leading to cytokine production. Activatory Fcγ Receptors (blue) clustering by multivalent immune complexes leads to signaling that results in inflammatory cytokine production. Black arrows indicate activatory signal transduction while red arrows indicate inhibitory signaling initiated by FcγRIIB (maroon). The role of different signaling components shown have not been evaluated for all cytokines.
secretion of inflammatory mediators [21] [169] [170] [53]. Among the various cytokines secreted by monocytes and macrophages, tumor necrosis factor α (TNF-α), interleukins 1, 4 and 6 (IL-1, IL-4, IL-6) have been studied extensively [171] [172] [173].

1.6.1. NF-κB signaling system.

NF-kB is a protein complex found in almost all animal cells, that acts as a transcription factor for various gene expression including production of cytokines such as TNF-α in response to stress, cytokines, ultraviolet radiation and bacterial and viral antigens [174] [175]. There are five proteins in the NF-kB family: p50, p52 (NF-kB1), p65/RelA, RelB and c-Rel (NF-κB2) that share a Rel homology domain in their N terminus. The subunits p65, RelB and c-Rel have transactivation domain in their C-termini. The NF-kB subunits form homodimers or heterodimers dependent on the signaling cascade and translocate to the nucleus to perform their role as transcription factors [176]. Studied extensively in the toll-like receptor (TLR) signaling pathway, the p50 and the p52 subunits are known to be inhibitory in function as homodimers, due to the presence of transrepressor domain [177]. However, when paired with p65, RelB or c-Rel as heterodimers, they activate kB site transcription [178]. The significance of NF-kB in regulating a cellular response is attributed to the fact that it belongs to the category of rapid-acting primary transcription factors; that is, their activity does not require new protein synthesis to be activated [174]. NF-kB also regulates its own activity by upregulating IkB expression that can further sequester NF-kB in the cytosol.

Several cell surface receptors including receptor activator of nuclear factor B (RANK) found on osteoclasts, TNF receptor and TLRs are among the many receptor
systems that activate NF-kB [179]. In resting cells, NF-kB dimers are sequestered in the cytoplasm by a family of inhibitory proteins called inhibitors of kB (IkB), by their ability to mask the nuclear localization signal on the NF-kB molecules [180]. Activation of NF-kB is initiated by signal-induced degradation of IkB proteins, by IkB kinase (IKK), through phosphorylation of two serine residues located in the IkB regulatory domain. When IkB molecules are tagged by serine phosphorylation, they are targeted by ubiquitin and subsequently degraded. This process allows the dimeric NF-kB subunits to translocate and bind to a kB site on the DNA, such as a TNF promoter site [181].

In the context of FcγR signaling, as mentioned earlier, several possible signaling intermediates are thought to activate nuclear translocation of NF-kB in order to initiate transcriptional activity of cytokines like TNF-α. FcγR signaling has been shown to mediate IKK activation and subsequent IkB degradation in THP-1 monocytic cells [182] [183] [147] [184].

1.6.2. TNF-α production and implications in Fcγ Receptor-mediated signaling

Many inflammatory diseases, including rheumatoid arthritis (RA), have been shown to involve different inflammatory mediators and cytokines such as IL-1β, IL-6, IL-15, TNF-α, various chemokines and enzymes that degrade extracellular matrix [185] [186]. Particularly in the case of RA, where the initiation of RA is a T cell mediated, antigen-specific process, sustained inflammation is at least equally dependent on cytokine production by synovial macrophages and fibroblasts, which may act on each other in an autocrine or paracrine manner [187]. TNF-α and interleukin-1 (IL-1) are the major monocyte [188] and macrophage-derived cytokines [189] present in the rheumatoid joint.
and both induce the synthesis and secretion from synovial fibroblasts of matrix-degrading proteases, prostanoids, interleukin-6 (IL-6), interleukin-8 (IL-8) and granulocyte-macrophage colony stimulating factor (GM-CSF). The role and involvement of FcγR-mediated TNF-α production in the persistence and progression of RA has been highlighted in numerous inhibitor studies and has been targeted in clinical applications [190] [191] [192] [193] [194].

TNF is synthesized as 212-amino acid pro-TNF molecule that is arranged as a stable homotrimer and remains bound to the cell surface [195] [196]. It is biologically active and can exert its effect via juxtacrine intercellular signaling. However, pro-TNF can be cleaved by TACE (TNF-α converting enzyme) to release a 17kD soluble protein called TNF-α [197]. The induction and expression of TNF-α is dependent on NF-κB activation as mentioned in the previous section.

1.7. Rationale for thesis

Low-affinity Fcγ receptors internalize soluble multivalent ligands by the process of endocytosis. Typically the receptor-ligand complex is targeted towards late endosomal-lysosomal compartment [198] [199]. Moreover, during this process, immune complexes may also dissociate from the receptors in endosomal compartments. While signaling from Fcγ receptors requires receptor cross-linking as the initiation step, it is not clear however, how signal termination occurs and what is the relationship between receptor trafficking and signaling kinetics. Studies in other receptor systems like epidermal growth factor receptor (EGFR) have shown that internalization and lysosomal
trafficking contribute to the termination of signaling from these receptors [200] [201] [202] [203]. In contrast, signaling from transforming growth factor β receptor (TGFβR) has been shown to require receptor internalization [204] [205] [206] [207] [208]. Moreover, there exists evidence from studies of phagocytosis of IgG-coated beads that Fcγ receptors can cause signaling from phagosomes that leads to targeting of lysosomes to phagosomes [209]. Therefore, we wanted to address the issue of the relationship of Fcγ receptor internalization to downstream signaling events.

We analyzed responses of Fcγ receptor bearing cells to two limiting and extreme cases of FcγR-IgG interactions, namely to soluble multivalent complexes that mimic immune complexes and to surface bound IgG, following on the “frustrated phagocytosis” paradigm [210] [211]. We hypothesize that inhibition of receptor internalization, as occurs in the frustrated phagocytosis model, would cause persistent receptor engagement, cellular stimulation and production and secretion of cytokines and inflammatory mediators.

Interestingly, in diseases such as RA and glomerulonephritis, immune complexes and antibodies are deposited on the surfaces of joints and renal tissues. The persistence of pathology observed in these diseases is attributed to local release of inflammatory cytokines and mediators [98]. We therefore wanted to highlight the possibility that the inability of monocytes and macrophages in the inflammatory site to internalize these immune complexes might lead to sustained Fcγ receptor engagement and signaling, causing persistent inflammatory damage that perpetuates disease.
II. MATERIALS AND METHODS

2.1. Media, Reagents and Chemicals.

Cell culture media: Dulbecco’s modification of eagle’s medium (DMEM), RPMI 1640 media and heat-inactivated fetal bovine serum (HI-FBS) were obtained from Wisent Bioproducts, Canada.

Reagents: Lympholyte solution was from Cedarlane Laboratories, Phosphate buffered saline (PBS) and donkey serum was from Wisent Bioproducts. EasySep Magnetic Cell Separation Kit for Monocyte enrichment and RosetteSep Kit for human monocyte enrichment were from StemCell Technologies. BSA, Human recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) and human recombinant macrophage colony-stimulating factor (M-CSF) were from Bioshop. BD OptEIA Human TNF ELISA set and Phosphoflow Cytofix Buffer III and Cytoperm Buffers were from BD Biosciences. Alexa 488 coupled concanavalin A and 4',6-diamidino-2-phenyldindole, dihydrochloride (DAPI) were from Invitrogen. SuperBlock solution, Western Blot Stripping Buffer and Supersignal Pico Solution set were from Thermo Scientific.

IgG and Antibodies: For most experiments, human IgG from Sigma-Aldrich (catalog # 14506); in some early experiments, IVIG (Bayer) was used. Cy3-coupled donkey polyclonal anti-human IgG antibody, Cy5-coupled donkey polyclonal anti-human IgG antibody, Cy3-coupled donkey anti-rabbit IgG antibodies, donkey anti-rabbit and donkey anti-mouse IgG HRP-conjugated antibodies were from Jackson ImmunoResearch.
Laboratories. Mouse anti-FcγRII monoclonal antibody IV.3 was obtained from the Sunnybrook Hybridoma Facility. Rabbit anti-human phospho MAPK 42/44 (Thr202/Tyr204) and rabbit anti-human total MAPK 42/44 antibodies were from Cell Signaling Technologies. Alexa 488-conjugated phospho-Erk 1/2 antibody was from BD Biosciences, Canada. Rabbit anti-p65 antibody and rabbit anti-p50 antibody were from Ebiosciences. Mouse anti-beta actin antibody was from Sigma.

**Chemicals:** Phorbol 12-myristate 13-actetate (PMA), sodium carbonate (pH 9.5), sodium phosphate, Cycloheximide, Bafilomycin, Dynasore, Triton X-100, Tris-HCl Ultrapure, Tris-Base Ultrapure, sodium dodecyl sulphate (SDS) and bromophenol blue were from Sigma-Aldrich, Canada. Ethylene Diamine Tetra acetate (EDTA), dithiothreitol (DTT), Glycine and Tris were from Bioshop. 5N HCl, beta-mercaptoethanol (BME) and Tween-20 were from Fisher Scientific. Glycerol, methanol and Ammonium Persulphate (APS) were from Merck, Acrylamide and TEMED were from BioRad, para-formaldehyde (PFA) from Canemco.

2.2. Cells

2.2.1. U-937 cells

U-937 cells were obtained from ATCC (CRL-1593.2), cultured in DMEM containing 10% HI-FBS at 37° C, 5% CO2. The U-937 cells were stimulated with PMA at a concentration of 16.39 nM (10ng/ml) in media for 48 hours at 37° C, 5% CO2 to induce terminal monocytic differentiation in a 10 cm tissue culture plastic dish (Sarstedt).
The resultant adherent cells were scraped off at 4° C and 1 X 10^5 cells were used per condition to determine TNF-α production.

2.2.2. PBMC extraction

Fresh blood was obtained from healthy human volunteers by collecting 30 ml of blood per draw, as approved by the Research Ethics Board, Sunnybrook Research Institute. The blood was directly drawn in 6.0 ml plastic vacutainers that are pre-coated with 90 USP of sodium heparin (BD Biosciences), shaken 7-8 times for anti-coagulation and subsequently used for peripheral blood mononuclear cell (PBMC) isolation.

To obtain PBMCs, the vacutainers were first centrifuged at 300g for 10 minutes at room temperature and the serum component discarded. The cells were resuspended in RPMI 1640 and pooled together in a 50 ml polypropylene Falcon tube using sterile 2.0 ml polypropylene transfer pipettes (Sarstedt), (polypropylene was used to avoid cellular activation) and mixed with equal volume of pre-warmed (37° C) RPMI 1640. 15 ml of Lympholyte solution was pre-warmed at 37° C and the cell suspension was layered over the Lympholyte solution without mixing the two layers. The tube was centrifuged without brake at 300g for 30 minutes at room temperature and the buffy layer between the media and the Lympholyte was extracted and washed 3 times with cold sterile PBS with centrifugation at 300g for 10 minutes at 4° C. After the final wash, the pellet was resuspended in RPMI 1640, counted with trypan blue staining for viability and used for subsequent monocyte enrichment steps. The number of PBMCs isolated varied among donors, averaging 1-2 X 10^6 cells/ml of blood collected.
2.2.3. Monocyte enrichment

PBMCs were enriched for monocytes in two different ways. Initially, monocytes were enriched by using the EasySep magnetic separation kit by negative selection, with depletion of cells expressing CD2, CD3, CD19, CD20, CD56, CD66B, CD123, glycophorin A and dextran as per manufacturer’s instructions. The yield and purity of the enriched monocytes were 9% and 75%, respectively, of total PBMCs as verified by flow cytometry (FACSCalibur, Becton Dickinson) for CD14+ cells using FITC-labeled anti-CD14 antibody.

It was discovered that the EasySep Negative Selection Kit antibody cocktail also contains an anti-FcR blocking antibody to block Fc-mediated binding of these antibodies to monocytes. Accordingly, we opted for a different protocol for monocyte enrichment from fresh whole blood in later experiments, in order to circumvent any possible interference of this antibody with FcyR-mediated signaling. Monocytes were subsequently enriched using the RosetteSep Kit for human monocyte enrichment. Anticoagulated whole blood from each donor was mixed with 1 mM EDTA and the RosetteSep antibody cocktail that cross-links erythrocytes with all other cells except monocytes, forming immunorosettes within 20 minutes at room temperature. Equal volumes of 1X PBS containing 2% FBS were added to the blood-antibody mix. The mixture was then layered on top of pre-warmed (37°C) Lympholyte solution and centrifuged without brake at 300g for 30 minutes at room temperature. The resultant buffy layer above the Lympholyte solution and below the serum and PBS layer was extracted using 2 ml polypropylene pipettes and washed 3 times with cold PBS+2%FBS at 300g for 10 minutes each at 4°C. The pellet was resuspended in 10ml RPMI 1640,
cells counted and used in experiments. The purity of the cells was 72% determined by flow cytometry for CD14+ cells using FITC-conjugated anti-CD14 antibody.

2.2.4. Type I and Type II macrophages

For differentiation into macrophages, monocytes were isolated by adherence (PBMCs were incubated on 25 mm glass cover slips in RPMI 1640 for 3 hours at 37° C, 5% CO2; after 3 hours, the cover slips were washed with RPMI 1640 pre-warmed at 37° C three times to discard the non-adherent lymphocytes.).

Adhered monocytes were incubated for 5 days in either 50 units/ml of GM-CSF or 50 ng/ml M-CSF to obtain either Type 1 or Type 2 macrophages, respectively [212] [213]. After the third day, a second dose of GM-CSF or M-CSF was added to the cells, and the growing media was kept unchanged. At the end of 5 days, the cells were washed with fresh RPMI 1640 containing 10% HI-FBS.

2.3. Human IgG, aggregated IgG and surface-bound IgG

**Heat-aggregated IgG:** Human IgG was aggregated at 10mg/ml concentration in PBS at 62° C for 20 minutes and centrifuged for 10 minutes at 4°C at 13,000 rpm to pellet insoluble large aggregates.

**Surface-immobilization of human IgG:** Surface coating of plates with human IgG was achieved in the following two ways. In early experiments using U937 cells, wells of 96-well Falcon plates (BD Biosciences, Canada) were incubated with 100 μl of 1mg/ml human IgG in coating buffer, containing 0.1M sodium carbonate, pH 9.5,
overnight, at 4°C, following the TNF ELISA kit protocol. The wells were subsequently washed three times with the coating buffer and used for cell stimulation. However, we observed that this technique was sub-optimal for stimulating cells for TNF production and necessitated better surface coating techniques as described next.

To obtain a better and more stable IgG immobilization, we used Nunc Immobilizer strips. The surfaces of the wells in these strips have been coated with a chemical linker that can covalently bind to proteins. IgG binding was achieved with 100 μl of 10 μg/ml IgG with 2 hours of incubation at room temperature in the presence of 100 mM sodium phosphate buffer, pH 8.0. The amount of IgG for maximal binding was optimized as shown in Figure 3.12.

2.4. TNF-α production kinetics

For assessing TNF-α production by enriched monocytes, 10^5 cells were resuspended in a 100 μl media and stimulated with aggregated IgG alone or immobilized IgG in the wells of a 96 well Nunc immobilizer plate at 37°C, 5% CO2. The supernatant was harvested after the stipulated length of stimulation, centrifuged at 600g for 10 minutes at 4°C to pellet contaminating cells, and the supernatant used for ELISA assay using the BD OptEIA Human TNF ELISA set. It was confirmed that presence of 100 μg/ml agIgG or 5 μg/ml LPS in the supernatant did not alter the TNF-α detection efficiency of the ELISA system.
As per the protocol, standard curves were generated based on a series of TNF-α dilutions provided in the set. Wells of 96-well Nunc immobilizer plates were coated with the provided capture antibody diluted in 100 µl of 100 mM sodium phosphate pH 8.0 coating buffer for 2 hours at room temperature. The wells were washed three times with PBS containing 10% FBS, pH 7.0. 100 µl of standard TNF dilution as well as supernatants from the experiment were put in the wells and incubated for 2 hours at room temperature. The wells were washed 5 times with 200 µl of PBS containing 10% FBS and the plates were incubated with 100 µl of streptavidin-HRP conjugated detection antibody (ELISA kit) for 1 hour at room temperature. After 1 hour, the wells were washed 7 times with PBS containing 10% FBS and 100 µl of substrate solution comprising of tetramethylbenzidine (TMB) and hydrogen peroxide (ELISA kit), added to each well and incubated for 30 minutes at room temperature in the dark. Finally, 50 µl of 5N HCl were added to each well to stop the reaction and the wells were read at 450 nm for absorbance corrected with absorbance values at 450 nm. The samples were prepared at least in duplicates. The values obtained for the optical densities at 450 nm were converted to TNF-α concentrations in pg/ml based on the standard TNF curve using the formula:

$$\text{TNF-α (pg/ml)} = (\text{OD at 450nm} - \text{background}) / \text{slope of the standard curve}$$

2.5. NF-kB signaling by type 1 and type 2 macrophages

Differentiated type 1 and type 2 macrophages were used to observe kinetics of NF-kB subunit (p65 and p50) translocation to the nucleus in response to 100µg/ml human
agIgG. The cells were stimulated for different lengths of time and washed three times with cold 1X PBS containing calcium and magnesium (PBSCM). The cell surfaces were stained with Alexa 488-coupled ConA for 10 minutes on ice. The cover slips were then washed and fixed using 4% PFA at room temperature for 30 minutes and permeabilized with ice-cold methanol at -20° C for 20 minutes. Cells were blocked for 45 minutes with 5% milk in PBSCM. The cells were stained with 5μg/ml of either rabbit anti-p65 antibody or rabbit anti-p50 antibody in 5% milk for 30 minutes on ice. After the primary antibody staining, the cells were washed 3 times with cold PBSCM and incubated with 5μg/ml Cy3-coupled donkey anti-rabbit IgG antibodies in 0.5% milk in PBS for 30 minutes on ice. Nuclei were stained with 0.1μg/ml DAPI. The cells were washed thereafter with cold PBSCM and the cover slips mounted on glass slides using Dako Cytomation mounting medium, dried and observed on a Zeiss Axiovert 200 microscope using standard fluorescence filter sets. The images were acquired using AxioVision software.

2.6. Studies on Erk 1/2 phosphorylation kinetics

2.6.1. Phospho-flow cytometry

The kinetics of Erk1/2 phosphorylation in PBMCs was initially determined by phospho-flow cytometry using Alexa 488-conjugated phospho-Erk 1/2 antibodies. We used Phosphoflow Cytofix Buffer III and Cytoperm Buffers for fixing and permeabilization steps, respectively. We could not use anti-CD14 antibodies to distinguish the CD14+ monocyte population as the Cytofix buffer III led to a loss of
surface labeling. The monocyte population within the PBMCs was therefore gated based upon forward and side scatter.

2.6.2. Western Immuno-blotting

10^6 PBMCs or 10^5 enriched monocytes were resuspended in a volume of 100 µl media after isolation. We used 96-well plates (Sarstedt) in some initial experiments; to achieve better IgG binding, in subsequent experiments we used Nunc Immobilizer strips. After stimulation for different periods in wells the cells were directly lysed using 5X Laemli buffer containing 1M Tris-HCl, pH 6.8, 50% glycerol, 10% sodium dodecyl sulphate (SDS), 3.2% beta-mercaptoethanol (BME) and 5% bromophenol blue. These whole cell lysates were boiled at 100 °C for 10 minutes in the presence of 0.1 M DTT. The whole lysates were resolved in 10% SDS-PAGE at 150 mV constant voltage for 70 minutes and subsequently transferred onto nitrocellulose membrane at constant voltage of 100 mV for 60 minutes. The membranes were blocked for 60 minutes at room temperature on a nutator with 15 ml SuperBlock solution. The membranes were subsequently blotted with rabbit anti-phospho MAPK 42/44 (Thr202/Tyr204), anti-total MAPK 42/44 antibodies or mouse anti-beta actin antibody in Superblock solution overnight at 4 °C (for anti-phospho and total MAPK) or for 60 minutes at room temperature (for anti-beta actin antibody) on a nutator.

The primary antibodies were detected with donkey anti-rabbit or donkey anti-mouse IgG HRP-conjugated antibodies in Superblock solution by 60 minutes incubation at room temperature on nutator. The wash buffer used to wash the membranes was 1X TBT with 0.05% Tween20. The membranes were stripped using Western Blot Stripping
Buffer for 30 minutes at 37° C. The efficiency of the stripping was verified by using secondary anti-rabbit or anti-mouse antibodies on the membranes. The membranes were exposed to enhanced chemi-luminescence solution Supersignal Pico Solution set for 5 minutes and visualized using Kodak XOMAT films or a Syngene Chemiluminiscence workstation with GeneSnap software (Synoptics Limited).

The protein bands on the membrane captured by the Syngene workstation were quantified using the GeneTools software (Synoptics Limited) for luminescence intensity and background subtracted against the membrane luminescence. The background subtracted values obtained from phospho-MAPK 42/44 bands were divided by those of either total MAPK 42/44 or beta-actin of corresponding lanes, to normalize for differences in the amount of total protein loaded in each lane of the SDS-PAGE gels. The values obtained are expressed as ratios.

2.7. **Aggregated IgG uptake and inhibition studies**

10⁶ PBMCs were treated with aggregated human IgG to study internalization in the presence or absence of Dynasore, a dynamin GTPase inhibitor, in suspension at 37° C 5% CO2. After different lengths of time of incubation with aggregated IgG, the cells were washed with cold PBS on ice, stained for surface-bound aggregated IgG with Cy3-coupled donkey polyclonal anti-human IgG antibody at 5μg/ml concentration for 30 minutes on ice. After 30 minutes, the cells were washed 3 times with cold PBS by centrifuging at 600g for 10 minutes each and fixed with 4% PFA for 30 minutes on ice and subsequently permeabilized with 0.1% Triton X-100 for 20 minutes on ice. The cells
were blocked by using 0.5% donkey serum for 30 minutes and stained with Cy5-coupled donkey polyclonal anti-human IgG antibody at 5μg/ml to observe total IgG present inside the cell and on the cell membrane. The cells were also stained with 0.1μg/ml DAPI nuclear stain. Once the staining was complete, the cells were loaded on top of a 2.5 cm glass cover slips mounted in an Attofluor chamber (Molecular Probes) and visualized using a Zeiss Axiovert 200 fluorescence microscope. Monocytes within the PBMCs were distinguished from lymphocytes by their size and ability to internalize agIgG.
III. RESULTS

To test the hypothesis that termination of signaling by Fcγ receptors depends on receptor internalization, we compared two distinct limiting scenarios. On one hand Fcγ receptor-bearing cells were stimulated with soluble heat-aggregated human IgG, which can be internalized upon Fcγ receptor engagement. At the opposite extreme, we used plate-bound immobilized human IgG to mimic tissue deposited immune complexes that cannot be readily internalized. We compared the kinetics of signaling outcomes under these two limiting conditions.

Although Fcγ receptor signaling, in particular signaling required for early events such as phagocytosis and degranulation, has been studied extensively in cell lines and primary cells such as neutrophils, signaling from Fcγ receptors leading to inflammatory cytokine production in primary monocytes has not been studied in detail. Monocytes and monocyte-derived cells have been implicated in various immunological diseases in the context of cytokine production. Our experimental model also necessitated use of suspension cells for purposes of stimulation with immobilized IgG. Therefore, we chose to use primary human blood-derived monocytes for the majority of our studies, based on their accessibility, biological relevance and consistency with experimental design.
3.1. Kinetics of TNF-α secretion

We first compared TNF-α release triggered by Fcγ receptors in cells stimulated with aggregated IgG versus those stimulated with surface-bound IgG. TNF-α secretion was chosen because of its relevance in inflammatory responses to immune complexes [214] [215] [216] [217]. TNF-α secretion by human primary monocytes in response to aggregated human IgG or covalently plate-bound human IgG was assessed by measuring TNF-α levels in cell supernatants by ELISA. Figure 3.1 illustrates the kinetics of TNF-α production, measured in supernatants, comparing the different conditions including PBS control during the first 5 hours after stimulation. The level of TNF-α harvested from monocytes stimulated with aggregated IgG showed a transient rise by 60 minutes and subsequently decreased by the 2-hour time-point. In contrast, monocytes that were stimulated with immobilized IgG showed a steady rise in TNF-α levels over the same time period. The higher initial TNF-α release in response to aggregated IgG may be due to greater Fcγ receptor occupancy in comparison to surface-bound IgG, since in the latter case, only one face of the monocyte contacts the plate. Moreover, the difference in magnitude of the TNF-α response could reflect the difference in the absolute IgG amount between the immobilized and soluble conditions. Nevertheless, the transient recovery of TNF-α with soluble aggregated IgG in contrast to the more sustained recovery with plate-bound IgG suggests that a more transient signal is delivered in case where Fcγ receptor internalization is possible.

The rapid release of TNF-α by cells in response to aggregated IgG suggested the possibility of Fcγ receptor-induced cleavage and release of TNF-α from intracellular
Figure 3.1. TNF-α secretion kinetics by enriched human monocytes in response to aggregated and immobilized IgG stimulation. $10^5$ human monocytes enriched using RosetteSep Monocyte Enrichment kit were suspended in 100 μl media and stimulated with either 10μg human agIgG (agIgG), 1μg human IgG immobilized on Nunc Immobilizer Strips (bound IgG), or with PBS (Unstim). The supernatant from each well was harvested and assayed for TNF-α by ELISA. The values are mean of triplicate assays within the experiment with standard deviation. This is a representative experiment of 4 independent experiments performed using monocytes from 4 different donors.
pools. To test whether Fcγ receptor induces release of pre-existing TNF-α, we assessed the effect of the translation inhibitor cycloheximide on the kinetics of TNF-α secretion by PMA-differentiated U937 cells in response to aggregated IgG. As observed in Figure 3.2, cycloheximide pretreatment diminished but failed to completely abrogate the 1-hour peak in TNF-α levels in the supernatant in both aggregated IgG and LPS (positive control) stimulated cells, suggesting that some of the early appearance of TNF-α may be due to release of pre-formed stores.

The drop in total TNF-α level in the supernatant after the initial peak with aggregated IgG stimulation was unexpected. It implies that after release there must be a loss of TNF-α over time, highlighting the fact that measured TNF-α level is a reflection of two competing processes of secretion and loss. The loss could be due to either protease-mediated degradation or re-uptake by the cells. The simplest explanation for the drop in TNF-α level at 2 hours in response to aggregated IgG, but not plate-bound IgG, is that receptor internalization causes a drop in the rate of TNF-α release. In the plate-bound condition the rate of cytokine release is probably more sustained and counterbalances the loss. The possibility cannot be excluded that the rates of TNF-α loss may also be different between the two conditions.

While the early time-points were of particular interest for examining the difference in kinetics during the time frame over which receptors would initially be internalized, we also measured accumulation of TNF-α at a longer time point (20 hours), as cytokine secretion is typically only assayed after prolonged incubation in most studies. As shown in Figure 3.3, TNF-α levels in response to both aggregated IgG and bound
Figure 3.2. Effect of Cycloheximide on agIgG and LPS induced TNF-α secretion kinetics by U937 cells. 10^5 PMA treated U937 cells stimulated with either 10 μg human aggregated IVIG (agIgG), 5μg LPS or with PBS (no stim) in the presence or absence of 5μg/ml Cycloheximide (CHX). The supernatant from each well was harvested and assayed for TNF-α by ELISA. The values are mean of triplicate assays within the experiment with standard deviation.
IgG stimulation reached significantly higher levels compared to that at 1 hour. At 20 hours, the TNF-α level measured in the supernatant of cells stimulated with bound IgG was higher than that from cells stimulated with aggregated IgG, despite the larger initial response to aggregated IgG at early times (1 hour). The substantially higher level of TNF-α measured with bound IgG stimulation in comparison to that with soluble aggregated IgG is consistent with our hypothesis of signal persistence. Of note, the substantial TNF-α level achieved with aggregated IgG by 20 hours indicates that over time TNF-α levels can rise again, as was seen between 3-5 hours in Figure 3.1. There are several possible explanations for this delayed increase. On one hand, with longer incubation new Fcγ receptors are expressed on the cell surface and could induce further TNF-α production. Secondly, TNF-α is known to induce autocrine effects through TNF receptors leading to both NF-κB induced TNF-α and TNF-receptor upregulation [218]. Thus, much of the TNF-α level at the late time points may result from this positive feedback loop.

3.2. Nuclear translocation of NF-κB in response to Fcγ receptor signaling

The transcription factor NF-κB regulates gene expression of different cytokines and inflammatory mediators and acts as a convergence point of numerous signaling pathways. As NF-κB is known to induce TNF-α transcription in monocytes/macrophages and mouse macrophages [219] [220] [221], and NF-κB activation is a more proximal event of Fcγ receptor signaling than cytokine synthesis or release [156], we wanted to compare its kinetics to that of downstream outcomes. NF-κB consists of different activatory and inhibitory subunits that form homodimers or heterodimers and upon
Figure 3.3. TNF-α secretion kinetics by enriched human monocytes in response to aggregated and immobilized IgG stimulation for 20 hours. 10^5 human monocytes enriched using RosetteSep Monocyte Enrichment kit were stimulated with either 100μg human agIgG (agIgG), 1μg human IgG immobilized on Nunc Immobilizer Strips (bound IgG), or with PBS (Unstim). The supernatant from each well was harvested and assayed for TNF-α by ELISA. The values are mean of triplicate assays with standard deviation. This is a representative experiment of 3 independent experiments done with monocytes from 3 different donors.
receiving specific signaling inputs, translocate to the nucleus and induce gene expression or suppression. We therefore, examined nuclear translocation of p65 and p50 subunits of NF-κB, which are activatory and inhibitory, respectively, in response to aggregated IgG stimulation as shown in Figures 3.4 – 3.7.

In order to have adherent cells for microscopy, we differentiated monocytes in to two different types of macrophages: Type 1 and Type 2. Monocytes were differentiated with either GM-CSF (Type 1 macrophages) or M-CSF (Type 2 macrophages) for this purpose. These two types are distinct from one another both functionally and morphologically. While Type 1 macrophages secrete IL-12 and other pro-inflammatory cytokines leading to a Th1 response, Type 2 macrophages have been shown to secrete IL-10 and play an anti-inflammatory role [222] [223] [224] [224]. Morphologically, Type 1 cells assume a ‘fried egg’ shape, distinct from spindle-shaped Type 2 macrophages. Thus, we thought it would be interesting to compare translocation of NF-κB subunits in these two cell types.

**Figure 3.4** illustrates p65 translocation kinetics in Type 1 macrophages. We carried out two different sets of experiments, looking at either early p65 translocation kinetics between 5-40 minutes (**Figure 3.4A**) or late p65 translocation kinetics between 1-3 hours (**Figure 3.4B**). While unstimulated cells showed obvious nuclear exclusion of p65, cells that were stimulated with aggregated IgG showed decreased nuclear exclusion starting at 30 minutes and the peak nuclear localization was observed between 40-60 minutes. However, the extent of p65 translocation was modest, remaining mostly peri-nuclear, compared to p50, which showed obvious nuclear localization at comparable times (**Figure 3.5A**). By 2 hours, the p65 subunit was observed to translocate back to the
Figure 3.4. Kinetics of p65 (NF-κB) nuclear translocation in Type 1 macrophages in response to aggregated IgG. Type 1 macrophages were stimulated for different lengths of time with A. 100 μg/ml human aggregated IVIG or B. with PBS (Unstim) or 100 μg/ml human agIgG. Cell surface was stained with Alexa488-coupled ConA (green), nuclear stain with DAPI (blue). p65 staining was achieved by using rabbit anti-p65 antibody (red) after fixation and permeabilization. A. & B. represent two different experiments with similar conditions.
Figure 3.5. Kinetics of p50 (NF-κB) nuclear translocation in Type 1 macrophages in response to aggregated IgG. Type 1 macrophages were stimulated for different lengths of time with A. 100 μg/ml human aggregated IVIG or B. with PBS (Unstim) or 100 μg/ml human agIgG. Cell surface was stained with Alexa488-coupled ConA (green), nuclear stain with DAPI (blue). p50 staining was achieved by using rabbit anti-p50 antibody (red) after fixation and permeabilization. A. & B. represent two different experiments with similar conditions.
cytoplasm, consistent with the transient nature of Fcγ receptor signaling induced by aggregated IgG. We wanted to look at the kinetics of nuclear translocation of p50 as well with similar experiments. We observed that p50 nuclear translocation begins by 30 minutes and peaks between 2-3 hours. p50 translocation was much more obvious than p65. The high level of p50 in the nucleus at 2 hours suggests negative regulation of NF-κB signaling predominating. This suggests that an independent means of negative feedback distinct from receptor downregulation may be arising at later times.

Next, we assessed the kinetics of p65 and p50 nuclear translocation in Type 2 macrophages in response to aggregated IgG, shown in Figures 3.6 & 3.7, respectively. While p65 began translocating by 40 minutes (Figure 3.6A), highest co-localization with nuclear stain was observed at 2 hours, persisting even at 3 hours (Figure 3.6B). The role of NF-κB transcription factors in Type 2 activated macrophages is not well understood, although it may, as in other cell types, contribute to processes of cell survival and homeostasis. Notably, with Type 2 macrophages, p50, the inhibitory NF-κB subunit, showed nuclear translocation beginning as early as 15 minutes (Figure 3.7A) and it remained co-localized with the nuclear stain for up to 3 hours (Figure 3.7B). This was somewhat expected, as p50 has been shown to promote production of IL-10, the principal regulatory cytokine secreted by Type 2 macrophages [225].

From these initial studies, we find that in Type 1 macrophages p65 translocation occurs at a very low level and remain mostly peri-nuclear even at its peak nuclear localization. In contrast, p65 in Type 2 cells showed sustained and obvious nuclear inclusion beginning at 30 minutes and persisting even at 3 hours. p50 translocation occurs
Figure 3.6. Kinetics of p65 (NF-κB) nuclear translocation in Type 2 macrophages in response to aggregated IgG. Type 2 macrophages were stimulated for different lengths of time with A. 100 μg/ml human aggregated IVIG or B. with PBS (Unstim) or 100 μg/ml human agIgG. Cell surface was stained with Alexa488-coupled ConA (green), nuclear stain with DAPI (blue), p65 staining was achieved by using rabbit anti-p65 antibody (red) after fixation and permeabilization. A. & B. represent two different experiments with similar conditions.
Figure 3.7. Kinetics of p50 (NF-κB) nuclear translocation in Type 2 macrophages in response to aggregated IgG. Type 2 macrophages were stimulated for different lengths of time with A. 100 μg/ml human aggregated IVIG or B. with PBS (Unstim) or 100 μg/ml human agIgG. Cell surface was stained with Alexa488-coupled ConA (green), nuclear stain with DAPI (blue), p50 staining was achieved by using rabbit anti-50 antibody (red) after fixation and permeabilization. A. & B. represent two different experiments with similar conditions.
faster in Type 2 macrophages but remains localized in the nucleus in both Type 1 and
Type 2 macrophages until 3 hours.

3.3. Fcγ receptor signaling and Erk1/2 phosphorylation

Erk 1/2 phosphorylation is a critical step in the MAP Kinase signaling cascade
that has been implicated in Fcγ receptor signal transduction [184] [71] [226] and is
upstream of NF-κB activation [107]. We therefore wanted to investigate kinetics of Erk
1/2 phosphorylation in response to aggregated IgG and immobilized IgG, as a more
proximal readout of Fcγ receptor signaling.

Initially, we assessed Erk 1/2 phosphorylation kinetics in purified PBMCs by
phospho-flowcytometry. PBMCs were used to enhance recovery of cells due to
constraints of low cell number working with primary human monocytes. We looked at
levels of phospho-Erk 1/2 in PBMCs stimulated with aggregated IgG for up to 40
minutes, gating for monocytes based on granularity and size. Figure 3.8 shows that
aggregated IgG stimulation for 20 minutes was able to cause a transient rise in the levels
of phospho-Erk 1/2 above that observed in cells treated with PBS. The phospho-Erk 1/2
level subsequently returned to unstimulated values by 40 minutes, suggesting possible
signal termination.

While phosphoflow showed some promise, the challenges with cell recovery
(with limited number of primary cells) and difficulty in recovering cells to analyze by
flow cytometry after stimulation by immobilized ligand (due to strong adherence) led us
to further assess the kinetics of Erk 1/2 phosphorylation by Western Blot. First, we
Figure 3.8. Kinetics of Erk1/2 phosphorylation in PBMCs in response to aggregated IgG and PMA stimulation. Human PBMCs were stimulated with PBS (a, b, c) or with 100 µg/ml human aggregated IgG for 20 and 40 minutes (d, e) or 10ng/ml PMA for 20 minutes (f). The cells were fixed and permeabilized and stained with either Alexa488-coupled anti-phospho Erk1/2 antibody (c,d,e,f), Isotype control antibody (b) or kept unstained (a). The cells were analyzed by BD FacsCalibur by gating on monocyte population by size and granularity as shown on the left panels while the right panel shows MFI of each samples. Representative of 2 similar experiments.
wanted to determine the optimal aggregated IgG concentration required to induce and observe maximal Erk 1/2 phosphorylation. In dose-response experiments, we stimulated either $10^6$ PBMCs or $10^5$ monocytes with a range of agIgG doses, as shown in Figure 3.9. We observed that a dose of 1-10 $\mu$g of aggregated IgG in 100 $\mu$l total volume would induce maximal Erk 1/2 phosphorylation in both PBMCs and enriched monocytes. Therefore, a dose of 10 $\mu$g aggregated IgG in 100 $\mu$l was used in all subsequent experiments.

Interestingly, phospho-Erk 1/2 level dropped when PBMCs were stimulated with aggregated IgG doses of 1 mg/ml or more. There could be a few possibilities to explain this inverse relationship at higher aggregated IgG concentration. High amounts of aggregated IgG could possibly better engage the low-affinity inhibitory receptors and co-cluster them with activatory receptors and thus dampen signaling [53] [227]. An even more likely possibility is that at very high aggregated IgG concentration, aggregated IgG binds in a one receptor: one complex stoichiometry, which leads to a decrease in receptor clustering and as a result, less signaling.

We also determined the maximal IgG binding capacity of the Nunc Immobilizer plates by using a range of IgG dilutions. The extent of IgG binding was quantified using Alexa 488 fluorophore conjugated anti-IgG antibody (Figure 3.10). 10 $\mu$g/ml IgG concentration was found to be sufficient to saturate the covalent linkers on these plates.

First, we observed Erk 1/2 phosphorylation kinetics in response to aggregated IgG in comparison to that to bound immobilized IgG in PBMCs. Consistent with phospho-flow findings and our hypothesis, aggregated IgG stimulation led to a transient rise in
Figure 3.9. Aggregated IgG dose-response of Erk1/2 phosphorylation in PBMCs and enriched monocytes (A). 10⁶ human PBMCs were resuspended in 100 μl media and stimulated with PBS (Unstim), different dilutions of human aggregated IgG, 5μg LPS or 10 ng/ml PMA for 15 minutes in wells of a 96-well plate, (B). 10^5 enriched monocytes resuspended in 100 μl media and stimulated with human aggregated IgG dilutions between 0.01 μg – 1.0 mg in Nunc Immobilizer strip. The cells were lysed in the wells directly with 5X Laemilli buffer and the lysates were resolved in a 10% SDS-PAGE gel. The proteins were transferred on a membrane and blotted with anti-phospho Erk1/2 antibody and subsequently, the membrane was stripped and re-probed with anti-actin antibody.
Figure 3.10. Titration of IgG-binding capacity of wells of Nunc Immobilizer strip. Wells of Nunc Immobilizer strips were incubated with 100 μl of PBS (empty and Ab only) or with the different indicated dilutions of human IgG, in 100μl PBS for 2 hours at room temperature. After 2 hours, the wells were washed and incubated with 5μg of Alexa 488-coupled anti-human antibody or with PBS (empty) for 30 minutes at room temperature, washed and fluorescence detected using BioTek fluorescence microplate reader with excitation at 494nm emission at 520nm. The values are mean of triplicate assays within the experiment with standard deviation. Alexa488 coupled anti-human antibody detection amount was titrated and saturated at 5 μg for 1μg of immobilized IgG (data not shown).
phospho-Erk 1/2 level within 15 minutes, which subsequently decreased beyond 30 minutes and returned to basal levels in unstimulated cells, while PBMCs that were stimulated with immobilized IgG showed a steady increase in phospho-Erk 1/2 level over time, remaining elevated even at 5 hours (data not shown). While the principal cells in PBMCs that express activating receptors and thus would be expected to respond to multimeric IgG are monocytes, we sought to confirm and extend our findings specifically with enriched monocytes. Human monocytes were enriched from whole blood using the RosetteSep Human Monocyte Enrichment cocktail to compare kinetics of Erk 1/2 phosphorylation in response to 10 µg of soluble aggregated IgG or 1µg of IgG immobilized by covalent linkage on Nunc Immobilizer plates. Figure 3.11 shows 2 representative Western Blots of 6 similar experiments on monocytes from 4 different donors. As was seen with PBMCs, aggregated IgG stimulation led to a transient rise in phospho-Erk 1/2 level at 15 minutes, which returned to baseline, unstimulated values by 30 minutes (Figure 3.11A). In contrast, immobilized IgG stimulation led to a persistent and significant increase in phospho-Erk 1/2 levels over unstimulated levels that lasted up to at least 7 hours (Figure 3.11B).

We observed variations in the manner in which levels of immobilized IgG-induced phospho-Erk1/2 rose between experiments. While for three of the six experiments we noted a gradual rise in phospho-Erk1/2 levels within the first hour, 2 experiments showed a lag-time of 60 minutes and another one experiment showed a lag time of 120 minutes before significant immobilized IgG-induced phospho-Erk1/2 levels were observed. These differences could be due to experimental variations, or signify donor variability. Nevertheless, in all of these 6 experiments, we observed signaling
Figure 3.11. Kinetics of Erk1/2 phosphorylation in enriched human monocytes in response to aggregated and immobilized IgG stimulation. 10^5 enriched monocytes (RosetteSep Kit) resuspended in 100 μl media were stimulated with either PBS (Unstim), 1μg immobilized human IgG (Bound IgG) or 10 μg human aggregated IgG. The cell lysates were resolved in 10% SDS-PAGE and transferred to nitrocellulose membrane and blotted with anti-phospho Erk1/2, anti-total Erk1/2 and anti-actin antibodies. A. and B. represent 2 of 6 experiments.
persistence with immobilized IgG in contrast to the transient signaling observed with aggregated IgG.

3.4. Contribution of FcγRIIA in Erk 1/2 phosphorylation.

Among the activating Fcγ receptors, human monocytes express primarily high affinity FcγRI and low affinity FcγRIIA [228]. A subset of monocytes (10%) also expresses FcγRIIIA [229]. Activatory signals induced by aggregated IgG could therefore result from any of these receptors. FcγRIIA in particular, has been shown to respond to aggregated IgG and induce cellular responses [230]. We therefore determined the contribution of FcγRIIA in Erk 1/2 phosphorylation in enriched monocytes, by stimulating cells with aggregated IgG in the presence or absence of anti-FcγRIIA blocking antibody IV.3. As shown in Figure 3.12A, IV.3 antibody partially inhibited aggregated IgG-induced Erk 1/2 phosphorylation, suggesting that FcγRI and/or FcγRIIIA on the CD16+ sub-population contribute to the Erk 1/2 phosphorylation when stimulated with aggregated IgG. We also determined the contribution of FcγRIIA in TNF-α secretion, by assessing stimulation of FcγRIIA by aggregated IgG in the presence of FcγRIIA blocking IV.3 antibody (Figure 3.12B). The one-hour peak of TNF-α secretion was largely blocked in the presence of IV.3 antibody, suggesting that most of the TNF-α secretion requires FcγRIIA-binding by aggregated IgG.
Figure 3.12. Contribution of FcγRIIA in aggregated IgG-induced signaling in enriched monocytes. (A). 10^5 enriched monocytes (Magnetic Separation Kit) stimulated with either PBS (Unstim), 10 μg human aggregated IgG alone or in the presence of 5μg IV.3 antibody. The cell lysates were resolved in 10% SDS-PAGE blotted with rabbit anti-phospho Erk1/2 antibodies and anti-rabbit HRP antibody. Subsequently, the membrane was stripped and re-probed with mouse anti-actin antibodies. (B). Kinetics of TNF-α secretion by 10^5 enriched monocytes (RosetteSep Kit) stimulated with PBS (Unstim), 10 μg aggregated IgG alone or in the presence of 5 μg IV.3 antibody, or with 1μg immobilized IgG. The secreted TNF-α is determined by ELISA. The values are mean of triplicate assays within the experiment with standard deviation.
3.5. Effect of Dynamin inhibition on signal termination

The preceding results are consistent with the hypothesis that Fcγ receptor internalization and degradation causes downregulation of signaling. We wanted to inhibit these processes and assess whether this would lead to altered kinetics of signaling. Previous findings in our lab have shown that in a transfected system, the GTPase dynamin is required for FcγRIIA endocytosis [231]. We wanted to test whether aggregated IgG internalization in human monocytes is similarly dependent on dynamin. The results in Figure 3.13 confirm that incubation of PBMCs with aggregated IgG leads to its rapid internalization within 15 minutes. Dynasore is a recently developed small molecule inhibitor of dynamin [232]. Treatment with 80 μM dynasore, a concentration typically used for inhibition of receptor endocytosis [233, 234], was able to inhibit Fcγ receptor-mediated aggregated IgG endocytosis significantly. Even after 40 minutes of incubation, little aggregated IgG internalization was seen. It should be noted that, as with other experiments using suspension cells, we were able to recover very few cells after multiple processing steps. Nevertheless, these results suggest that Fcγ receptor endocytosis in human monocytes is dynamin-dependent.

We then tested the effect of dynasore on aggregated IgG-inducible Erk1/2 phosphorylation in enriched human primary monocytes. Our expectation was that inhibition of receptor internalization might lead to persistent signaling, similar to that observed with immobilized non-internalizable IgG. However, as seen in Figure 3.14A, in the presence of dynasore, aggregated IgG-inducible Erk1/2 phosphorylation was completely blocked. Our interpretation is that dynasore, a GTPase inhibitor, might have off-target effects on components of the MAP Kinase pathway. Alternatively, disruption
Figure 3.13. Effect of Dynasore on aggregated IgG uptake by monocytes.
0.5 X 10^6 Human PBMCs in suspension were treated with 100 μg/ml aggregated human IgG either in the absence (A) or presence (B) of 80 μM Dynasore, for 15 and 40 minutes. The cells were washed and incubated with Cy3-coupled anti-human IgG (red) in the cold for 20 minutes to label surface-bound IgG. The cells were thereafter fixed, permeabilized, blocked and incubated with Cy5-coupled anti-human IgG (cyan) to label total IgG. Nucleus was stained with DAPI. The monocytes were identified in the PBMCs by their size and ability to internalize aggregated IgG. Scale bars indicate 5 μm.
Figure 3.14. Effect of Dynasore on aggregated IgG-induced signaling. A. Western Blot for phospho-Erk1/2 and β-actin of 10^5 enriched human monocytes (Magnetic Sep) stimulated with 10μg aggregated human IgG either in the absence or in the presence of 80 μM Dynasore. The cell lysates were resolved in 10% SDS-PAGE and transferred to nitrocellulose membrane and blotted with anti-phospho Erk1/2, anti-total Erk1/2 and anti-actin antibodies. Representative of four similar experiments. B. ELISA showing TNF-α secretion kinetics by 10^5 enriched human monocytes (RosetteSep) stimulated with PBS (Unstim), 1 μg surface-immobilized IgG and 10 μg aggregated human IgG either in the absence or in the presence of 80 μM Dynasore. The values are mean of triplicate assays within the experiment with standard deviation. Representative of three similar experiments.
of endosomal membrane trafficking by inhibition of dynamin may influence important components of the Fcγ receptor-signaling cascade, e.g., by preventing their delivery to cell surface. A third possibility is that receptor internalization is actually required to initiate Erk1/2 phosphorylation. However, this possibility is difficult to reconcile with the signaling seen in the case of immobilized IgG.

We also examined TNF-α secretion by enriched monocytes in the presence of dynasore. Consistent with the inhibitory effect on Erk1/2 phosphorylation, dynasore treatment reduced the amount of TNF-α recovered at 1 hour significantly, though it was not abolished (Figure 3.14B). This observation points to the possibility of Erk1/2 independent signaling from Fcγ receptors leading to TNF-α secretion.


To test whether endosomal acidification or proteolysis is important for termination of signaling in response to aggregated IgG, we stimulated enriched monocytes with aggregated IgG either in the absence or presence of bafilomycin, an inhibitor of vacuolar H⁺-ATPase and endosomal acidification, and examined Erk1/2 phosphorylation kinetics by Western Blot. The results in Figure 3.15 show that bafilomycin inhibited the aggregated IgG-induced peak Erk1/2 phosphorylation observed at 15 minutes. It is not obvious why bafilomycin has an inhibitory effect on Fcγ receptor signaling, but this may also be due an indirect effect on endosomal trafficking. Notably, while the signal was reduced, it also persisted longer than in the control condition, remaining elevated at 30 minutes. This suggests that endosomal acidification may
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**Figure 3.15. Effect of Bafilomycin on aggregated IgG-induced signaling.** Western Blot for phospho-Erk1/2, total Erk1/2 and β-actin of $10^5$ enriched human monocytes (RosetteSep) stimulated with either PBS (Unstim) or 10 μg aggregated human IgG in the absence or presence of 0.3 μM Bafilomycin, for 0 – 120 minutes. The cell lysates were resolved in 10% SDS-PAGE and transferred to nitrocellulose membrane and blotted with anti-phospho Erk1/2, anti-total Erk1/2 and anti-actin antibodies. Representative of two similar experiments.
contribute to, but not be essential for, termination of Fc\gamma receptor signaling. This may reflect a pH-driven dissociation of aggregated IgG from the receptors, or proteolysis of the ligand and/or the receptor.

3.7. Effect of simultaneous aggregated IgG and immobilized IgG stimulation.

From the results described so far, we observe that soluble aggregated IgG induces a transient rise in phospho-Erk1/2 level at 15 minutes, which subsides within 30 minutes. On the other hand stimulation with immobilized IgG leads to a persistent signaling that leads to a gradual increase in phospho-Erk1/2 level. Aggregated IgG likely engages all Fc\gamma receptors on the cell surface and induce a robust, yet transient signal. If the decline in signaling with aggregated IgG is due to receptor internalization, we would expect that with simultaneous treatment of both soluble aggregated and bound IgG, the kinetics of Fc\gamma receptor stimulation would be more similar to that observed with aggregated IgG alone. Thus, the internalizable ligand would have a dominant effect on the overall signal by triggering receptor loss. The results in Figure 3.16A show such an effect. In the presence of immobilized IgG, soluble multimeric IgG, while able on its own to trigger signaling, has a net inhibitory effect on signaling at later times, presumably by virtue of its ability to cause receptor downregulation.

We examined whether internalizable aggregated IgG would similarly have a dominant effect on TNF-\alpha secretion. As illustrated in Figure 3.16B, at 1-2 hours, the amount of TNF-\alpha recovered with aggregated IgG alone or in the presence of bound IgG were identical, implying that aggregated IgG engagement dominates signaling, consistent
Figure 3.16. Effect of simultaneous stimulation of aggregated and immobilized IgG on signaling in monocytes. A. Western Blot for phospho-Erk1/2, total Erk1/2 and β-actin of 10^5 enriched human monocytes (RosetteSep) stimulated with either PBS (Unstim), 10µg aggregated human IgG alone, 1µg immobilized IgG alone, or with 1µg immobilized IgG in the presence of 10µg aggregated IgG. The experiment is representative of two experiments. B. ELISA showing TNF-α secretion by 10^5 enriched human monocytes (RosetteSep) stimulated with PBS (Unstim), 1µg surface-immobilized IgG (bound), 10µg aggregated human IgG (agIgG) and 1µg of immobilized IgG in the presence of 10µg aggregated IgG (mixed). The values are mean of triplicate assays within the experiment with standard deviation. Representative of three similar experiments.
with the receptor-downregulation hypothesis. At 20 hours, however, released TNF-α was higher than that observed with aggregated IgG stimulation alone, but significantly lower than that secreted by monocytes with surface-bound IgG stimulation alone. These results support our hypothesis of decreased Fcγ receptor availability subsequent to aggregated IgG stimulation and receptor internalization. We postulate that over time, the TNF-α levels approach those seen with bound-IgG stimulation, but do not reach identical levels, probably due to the initial loss in receptors available for stimulation.
IV. DISCUSSION

Signaling pathways downstream of Fcγ receptors leading to cytokine production have not been extensively studied, at least in primary human monocytes. In this project we were able to highlight some of the involved signaling events downstream of Fcγ receptors including Erk1/2 phosphorylation, NF-κB translocation and TNF-α secretion. We examined the kinetics of signal transduction by Fcγ receptors in the presence or absence of receptor internalization. Figure 4.1 shows a schematic representation of the kinetics of Fcγ receptor mediated signal transduction events that we observed in our experiments.

From our experiments, we observed a clear difference in the kinetics of FcγR-mediated signaling leading to Erk1/2 phosphorylation and TNF-α secretion by enriched human monocytes in response to soluble aggregated IgG and surface-bound immobilized IgG stimulation. While soluble aggregated IgG led to a transient rise in levels of Erk1/2 phosphorylation, peaking by 15 minutes and dropping at 30 minutes and beyond, immobilized IgG led to phospho-Erk1/2 phosphorylation that persisted at least 7 hours. Aggregated IgG stimulation also caused robust TNF-α secretion at 1 hour that subsided thereafter, in contrast to stimulation by immobilized IgG, which led to steady rise in secreted TNF-α. The difference in response in these two limiting scenarios is striking at the early hours and is consistent with our hypothesis.
Figure 4.1. Kinetics of signal transduction by Fcγ Receptors. A. Kinetics of peak levels of signaling components induced by soluble aggregated IgG. B. Comparison of the kinetics of Erk1/2 phosphorylation and TNF-α accumulation between Fcγ Receptors stimulated with plastic-bound immobilized IgG to that with soluble aggregated IgG. The graphs show difference in trends and not actual values.
4.1. Interpretation of results

The difference in kinetics of signaling by Fcγ receptors leading to Erk1/2 phosphorylation and TNF-α secretion in the two distinct scenarios suggests regulation by receptor internalization. While aggregated IgG stimulation induced an acute increase in the level of Erk1/2 phosphorylation by 15 minutes, these returned to unstimulated levels after 30 minutes. This is probably because most of the receptors on the cell surface that engage soluble aggregated IgG become internalized within 30 minutes and cease signaling. TNF-α production is a downstream event of Erk1/2 phosphorylation and therefore requires more time for induction of protein expression and secretion. The transient rise in TNF-α levels at 1 hour is indicative of these delayed kinetics. Taken together with the inhibitory effect of cycloheximide on the secretion of TNF-α at 1 hour, we conclude that the robust release of TNF-α induced by aggregated IgG is probably a net result of both new TNF-α production and secretion from pre-existing cellular pools. Early LPS-induced TNF-α secretion was also observed in the presence of cycloheximide, indicating that both TLR-4 and FcγRs are able to induce release of pre-existent TNF-α independent of new protein synthesis. It is possible that Fcγ receptor signaling may influence cytokine production at the levels of transcription, translation and/or post-translational control of secretion.

The transient nature of the TNF-α secretion was evident from the drop in the levels by 2-3 hours. We think that the decrease of TNF-α during these time-points is because of a skewed balance of secretion and loss. Large-scale receptor engagement and internalization in response to soluble aggregated IgG probably leads to a net
downregulation and a dampened stimulation. Processes of reuptake and/or proteolytic
degradation can then favor loss. The possibility that soluble aggregated IgG stimulation
induces a robust release of proteases also cannot be ruled out. While cytokine release is
the ultimate functional outcome of the signaling cascade, it is challenging to interpret it as
a measure of signaling kinetics because of these multiple simultaneous effects.

Interestingly, beyond 20 hours, TNF-α levels induced by aggregated IgG reached
high values. Aggregated IgG may induce a gradual release of TNF-α by acting on Fcγ
receptors that come to the cell surface over time, resulting in the significantly
accumulated levels we observed in our assays.

In the absence of receptor uptake, as with immobilized IgG, we saw clear
indications of signaling persistence highlighting the possibility that Fcγ receptor
internalization is necessary for signal termination. Although TNF-α might positively
induce its own synthesis over time, the lack of signaling in unstimulated cells implies that
at least the initiation of its synthesis and secretion is Fcγ receptor-dependent. The initial
delay in rise of phospho-Erk1/2 levels may result from the structural accessibility of the
receptors to the IgG as well as a qualitative difference in signaling between the two
scenarios of soluble aggregated versus plate-bound IgG. Signaling is probably dependent
upon a threshold number of receptors clustered at a given time, which is reached easily
with aggregated IgG in its soluble form. However, in the plate-bound case, it may take
longer for a sufficient number of receptors to be engaged, limited by diffusion to the site
of contact between the receptors and the plate-bound IgG. We observed that there is a
delay in Erk1/2 phosphorylation with plate-bound IgG, which may reflect the time taken
for sufficient receptor engagement to ensue. While we considered it possible that negative
feedback loops in the signaling pathways downstream of the receptors might limit signaling even in the plate-bound situation, the persistent signaling we see in fact suggests receptor internalization is necessary for signal termination.

In the 'frustrated phagocytosis' model where phagocytes encounter large antibody-coated surfaces, one consequence is the release to the extracellular space of destructive granule contents that would normally be targeted to phagosomes [235] [211]. More recently, another suggested outcome of frustrated phagocytosis has been suggested, namely reactive oxygen species (ROS)-mediated activation of the inflammasome [236]. Our results suggest a third outcome: persistent signaling leading to high levels of cytokine production downstream of Fcγ receptor signaling. In diseases with deposited immune complexes on organs and tissue surfaces, these phenomena might together explain the mechanism of the disease pathology and establish an association of monocyte and macrophage-derived cytokines and inflammatory mediators to Fcγ receptor signaling.

In the presence of aggregated IgG, we observed a net downregulation of signaling by immobilized IgG. With simultaneous aggregated and plate-bound IgG stimulation, aggregated IgG likely out-competes immobilized IgG for receptor engagement. We therefore observe that the kinetics of TNF-α secretion is identical to aggregated IgG stimulation alone, suggesting a net downregulation of signaling due to receptor internalization. Studies on Erk1/2 phosphorylation at early time points post-simultaneous aggregated IgG and immobilized IgG stimulation confirm this hypothesis. Observations from these mixing studies beyond 20 hours reveal that accumulated TNF-α levels are lower than that observed with sustained stimulation with immobilized IgG, suggesting that the initial drop in TNF-α levels, possibly due to receptor down-regulation induced by
aggregated IgG may be responsible for this difference. These findings have clinical implications. Since, in the presence of aggregated IgG, we were able to observe a net downregulation of signaling by immobilized IgG, use of FcγRI-engaging agents to trigger receptor internalization might be a strategy for reducing inflammation in the presence of immobilized immune complexes. It is possible that lower valency agents, e.g. dimeric IgG, might cause even less overall signaling, if they are relatively more efficient at triggering internalization than signaling. This would be an interesting avenue for future exploration.

It was interesting to observe that FcγRIIA, the principal low affinity activatory receptor expressed by monocytes, is the main contributor to soluble multivalent aggregated IgG-induced signaling. This was not surprising, as the high affinity FcγRI probably remains largely bound by monomeric IgG from the serum after monocyte isolation, or to monomeric IgG present in the agIgG preparation. We observed that in the presence of IV.3 antibody, TNF-α secretion was greatly reduced, but not completely inhibited, suggesting that un-engaged FcγRI probably contributes to a minor extent in signaling as well. Furthermore, as mentioned earlier, a minor subset of monocytes also express FcγRIIIA, which may contribute to the modest level of TNF-α secretion seen.

Fcγ receptor endocytosis is dependent on dynamin, which is required for clathrin-coated endocytic pits to pinch off from the plasma membrane [237]. We therefore sought to inhibit receptor internalization by inhibiting the GTPase activity of dynamin by the inhibitor dynasore. We compared the signaling kinetics that accompanied stimulation with aggregated IgG in its soluble form, either in the presence or absence of dynasore. However, while we observed significant inhibition of aggregated IgG internalization by
dynasore, it also blocked Erk1/2 phosphorylation. Dynasore also inhibited aggregated IgG-induced TNF-α secretion significantly, though not completely. This suggests two possibilities. On one hand, inhibition of Erk1/2 phosphorylation and TNF-α secretion in the presence of Dynasore could imply that signaling from Fcγ receptors actually requires some degree of receptor internalization to induce strong signaling. However, immobilized IgG could induce signaling even in the absence of receptor internalization, albeit with a difference in magnitude and kinetics, arguing against this possibility. It is nonetheless possible that signaling mechanisms are qualitatively different in response to soluble aggregated IgG and to immobilized IgG. The second and simpler explanation of Dynasore-induced inhibition of Erk1/2 phosphorylation and TNF-α secretion could be related to disruption of other dynamin dependent signaling events. Nevertheless, the fact that there was reasonable TNF-α production in the absence of any detectable phospho-Erk1/2 suggests there must exist Erk1/2-independent routes to TNF induction, e.g. possibly signaling downstream of PI3-K via effectors such as mTOR.

As the exact level of Fcγ receptor trafficking at which signal termination occurs is not known, we wanted to determine if signaling could occur after the receptor-IgG complexes are internalized. During endocytosis, Fcγ receptors are transported in endosomes that later fuse with lysosomes where the IgG cargo undergoes degradation [238] [198] [239]. Our approach was to prevent endosomal acidification using bafilomycin and observe if preventing Fcγ receptor degradation would have an effect on signaling. We saw phospho-Erk1/2 levels go up by 15 minutes and persist for 30 minutes following aggregated IgG stimulation in the presence of bafilomycin, suggesting a slightly delayed kinetics. However, beyond 30 minutes, phospho-Erk1/2 levels dropped
to baseline suggesting that bafilomycin could delay but not prevent receptor degradation. One possible explanation for this is that the receptors dissociate from aggregated IgG even at higher pH, independent of endosomal acidification.

Transcriptional activation of the TNF-α gene is dependent upon the transcription factor NF-κB and an initial objective was to follow the translocation of NF-κB subunits to the nucleus in response to Fcγ receptor stimulation, as a readout of signaling. In order to obtain enriched monocytes from isolated PBMCs we needed to adhere monocytes to tissue culture plastic, which activates the monocytes during the process. We therefore decided to focus on two functionally distinct classes of monocyte derived macrophages, named Type 1 and Type 2, based on their morphology and differentiation protocols. We observed that p65 translocation to the nucleus in both these types of macrophages was modest and remained mostly peri-nuclear, even at peak translocation times of 40 to 60 minutes. Nuclear co-localization of p50, the inhibitory subunit of NF-κB, however, was much faster and more pronounced.

One interesting observation was that maximal phospho-Erk1/2 levels were induced by aggregated IgG doses between 1-10 μg for 10^6 PBMCs or 10^5 monocytes. Doses higher than 10 μg induced lower amounts of Erk1/2 phosphorylation. This was somewhat counter-intuitive, as we had expected responses to saturate at high doses of the aggregated IgG used for stimulation. This inverse dose-response relationship at higher concentrations may be attributed to two possibilities. Higher aggregated IgG could be instrumental in engaging more inhibitory FcγRIIB receptors than at lower doses, resulting in dampened signaling. The decreased signaling could be also a result of a decrease in
Fcγ receptor clustering at the surface due to a possible 1:1 receptor-aggregated IgG configuration.

4.2. Challenges with the project

The number of enriched monocytes that we could obtain per blood draw from donors limited our experimental setup. We were unable to use enriched monocytes in experiments such as phospho-flow and immunofluorescence microscopy with cells in suspension due to extremely low cell recovery after multiple processing steps. We initially tried to enrich monocytes from PBMCs by plastic adherence, but once in contact with the plastic surface, monocytes flatten and adhere very tightly and are difficult to harvest. We therefore decided to enrich monocytes by negative selection using magnetic antibody-bead separation. However, we later discovered that the antibody cocktail also contained an FcγR-blocking antibody. In order to avoid any possible interference with our assays, we finally used a RosetteSep monocyte enrichment kit for monocyte isolation. We found enriched monocytes clump when maintained overnight, which dictated that they be used for experiments immediately after their isolation. We were interested in differentiating monocytes into macrophages, but our experimental model required suspension cells that could be sedimented down onto surfaces that were coated with IgG.

We did observe minor variations in the extent of Erk1/2 phosphorylation or TNF-α production between experiments. However, the overall kinetic pattern between experiments remained the same. We considered using lymphoma and leukemic cell lines such as U937 and THP-1, which are pre-monocytic and have been studied by different
groups, but we thought *ex vivo* primary cells would be most informative in understanding physiologic Fcγ receptor mediated responses. Having established the basic signaling kinetics in human primary monocytes, one could further address questions using the aforementioned cell lines in which manipulations such as transfection are more feasible.

We wanted to study the kinetics of activation of other signaling intermediates such as Syk and Akt as readouts of Fcγ receptor signaling. Unfortunately we were unable to obtain conclusive results from preliminary experiments.

In order to mimic tissue deposited immune complexes, we initially dried IgG onto plastic surface overnight at 4°C, as instructed by the manufacturer for the ELISA kit. However, we could not achieve efficient surface binding of IgG, which resulted in a very low level of TNF-α secretion and modest pErk1/2 phosphorylation. We subsequently resolved this by covalently linking IgG to plastic surfaces, with significant improvement in signal intensities. Moreover, this approach presumably precluded potential IgG release from the plate over time.

In addition to inhibiting receptor internalization by engaging cells to immobilized IgG, we sought to prevent receptor internalization and degradation pharmacologically by using dynasore and bafilomycin, respectively. We did not observe Erk1/2 phosphorylation in response to aggregated IgG in the presence of dynasore. This could be due to non-specific inhibition of Erk 1/2-related signaling effectors. Bafilomycin treatment resulted in a loss of phospho-Erk1/2 levels beyond 30 minutes in response to aggregated IgG, and also had an unexpected inhibitory effect. These findings highlight the challenges of using inhibitors with primary cells.
4.3. Future Directions

In this project we established differences in the kinetics of signaling between Fcγ receptors stimulated with aggregated IgG or immobilized IgG. Based on the observations we highlighted here, additional studies are planned to establish the kinetics of activation of other signaling intermediates and pathways including activation of Syk, Akt, PI3-K, PLCγ and NF-κB etc., which are involved in Fcγ receptor signaling. It would be important to examine the kinetics of synthesis and secretion of other pro-inflammatory cytokines such as IL-1 and IL-6 in response to Fcγ receptor stimulation and compare the results with that of TNF-α. Different cytokines are differentially regulated and the kinetics of their synthesis and secretion may vary considerably. As signaling pathways are controlled by different mechanisms, it would be particularly interesting to find pathways that might be engaged from the endosomal compartment as opposed to the plasma membrane. It would also be interesting to investigate the possible role of Fcγ receptors in providing signals for caspase-1 mediated IL-1 secretion by inflammasome activation, particularly in the frustrated phagocytosis model, given that inflammasome activation has been seen in response to large particulate stimuli, such as silica particles [236].

We observed distinct kinetics of TNF-α secretion with aggregated IgG and immobilized IgG. Secreted cytokines may activate cell surface receptors or be degraded over time, so that the kinetics of extra-cellular accumulation may not directly reflect the signaling that induces them. With TNF-α secretion we observed a drop at 1 hour post-treatment with aggregated IgG stimulation. To explore TNF accumulation further, one
could block TNF-α receptor-mediated re-uptake, which would probably inhibit the positive feedback loop of TNF-α and TNFR upregulation. To limit degradation, protease inhibitors could be used. Observing TNF mRNA levels would be an important alternative approach to obtain a more direct readout of Fcγ receptor induced signaling.

It would be interesting to establish the relationship of signaling to the extent of FcγR-IgG interactions by systematically varying the size of the opsonized surface, e.g. with opsonized beads. This might reveal qualitative differences in signaling initiated by large opsonized particles in comparison to soluble immune complexes. If so, it would be interesting to define how signaling switches across the endocytosis-phagocytosis spectrum, to better define the potentially varying outcomes of Fcγ receptor engagement.

We observed that simultaneous stimulation with aggregated IgG and immobilized IgG led to a net downregulation of signaling, possibly because of the dominant effect of aggregated IgG-induced receptor downregulation. We hypothesized that aggregated IgG effectively competes with surface-bound IgG to exert its effects. To examine this possibility further, experiments would be conducted to alter the timing of co-incubation in the mixing experiment by adding soluble ligand after pre-binding cells to plate-bound IgG, to see if aggregated IgG can still effectively compete for pre-bound receptors. It would be highly relevant clinically to observe if a low valency agent, possibly a dimeric or trimeric IgG, could trigger internalization and a further net decrease in cytokine production due to less surface clustering. It is unclear how valency of agents affects the efficiency of signaling versus internalization. For example, a low valency aggregated IgG molecule (e.g. a dimer) may be sufficient to cause receptor internalization but relatively little signaling, thus tapping the aggregated IgG-induced Fcγ receptor down-regulation as
a strategy for inhibiting sustained Fcγ receptor engagement and inflammatory pathology seen in diseases that are similar to the frustrated phagocytosis model.

Further studies are required to examine the level at which signaling is terminated from Fcγ receptors. Whether signal termination occurs immediately after receptor internalization, or whether subsequent sorting steps are necessary, needs to be determined by studies that correlate receptor trafficking with signaling. We were able to identify preliminary readouts of Fcγ receptor signaling in response to internalizable vs. non-internalizable IgG in primary human cells, which are undoubtedly the most relevant cells in the context of physiology and disease. Further detailed mechanistic questions could be addressed in cell line and transfected cell systems where particular aspects of the trafficking machinery can be manipulated more selectively, such as by expression of dominant negative mutants, such as those of dynamin (for inhibiting Fcγ receptor internalization) and various Rab proteins (for inhibiting endosomal maturation).

4.4. Conclusion

Fcγ receptors play a pivotal role in health and in disease. They influence the functions of immune cells and translate the pathogen recognition potential of IgG into cellular responses. Upon being clustered by multivalent soluble immune complexes, low affinity Fcγ receptors initiate signaling that leads to a variety of effector functions, including production and secretion of inflammatory mediators and cytokines. Signal termination probably occurs upon receptor internalization and degradation.
In this study, we hypothesized that the failure of Fcγ receptors to internalize would prevent signal termination from occurring. In order to address this hypothesis, we compared the kinetics of Fcγ receptor mediated signaling in response to soluble aggregated IgG and immobilized IgG, and examined Erk1/2 phosphorylation, NF-κB translocation and TNF-α production. We observed that aggregated IgG was able to induce a transient rise in Erk1/2 phosphorylation and TNF-α secretion in primary human monocytes and cause p65 and p50 translocation in monocyte-derived macrophages. We observed clear difference in the kinetics of Erk1/2 activation and TNF-α secretion in situations where internalization was prevented. Immobilized IgG caused signaling persistence by sustained engagement to Fcγ receptors. This strongly suggests that receptor internalization is a key step in the regulation of signal induction by Fcγ receptors, consistent with our hypothesis.

Physiologically, signal termination is important as it limits the inflammatory damage caused by immune cells, mediated by cytokines and other mediators. In the absence of receptor internalization, as with Fcγ receptor interactions with deposited immune complexes in rheumatoid arthritis and glomerulonephritis, signaling may persist, possibly contributing to a sustained inflammatory and pathologic state. Through this study, we also highlighted the possibilities of modulating Fcγ receptor to obtain a net downregulation in signaling, as a potential therapeutic approach.
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