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Determinant of g-chain mediated Fc Receptor Signaling

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DETERMINANTS OF γ-CHAIN MEDIATED FC RECEPTOR SIGNALING

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ABSTRACT

Fc receptors provide an interface between humoral immunity and cellular immunity by binding to the Fc portion of immunoglobulin and mediating effector functions. Cells expressing Fc receptors play a major role in immune complex clearance via phagocytosis, leading to a context dependent pro- or anti-inflammatory environment.

Signaling by multi-chain Fc receptor complexes, including FcγRI, FcγRIII, FcεRI and FcαRI is mediated by the ITAM-containing common FcεRIγ (γ-chain) subunit. However, despite the use of a common γ-chain, different Fc receptor complexes elicit specific cellular programs, suggesting additional layers of regulation of tyrosine based signaling by α-chain. We hypothesized that the unique α-chain cytoplasmic domains differentially modulate Fc receptor complex functions. In order to test our hypothesis, we established P388D1 and RBL-2H3 cell lines stably expressing either WT or Tail minus (TL) mutant FcγRI α-chains. Data from these systems suggest that α-chain cytoplasmic domain (CY) indeed influences certain Fc receptor signaling pathways such as phagocytosis, degranulation and IL-6 production, but not others such as TNF-α and IL-1β production.

Furthermore, serine and threonine residues in the γ-chain are reportedly phosphorylated after Fc receptor stimulation. We hypothesized that putative phosphorylation of serine/threonine sites fine-tune Fc receptor signaling, and provide the additional layer of regulation. To test this hypothesis, we stably transfected WT human γ-chain or mutants carrying serine/threonine to alanine changes into RBL-2H3 cells lacking the endogenous γ-chain expression. Using these cells, we observed that serine/threonine residues in the γ-chain ITAM influence γ-chain signaling pathways such as IL-4 production and intracellular calcium flux. Additionally, using mass spectrometry, we observed that Serine 51 in the ITAM is phosphorylated in stimulated samples. Studies with Serine 51 to Alanine and Aspartic Acid mutants suggest an inhibitory role for Serine 51 phosphorylation in γ-chain signaling.

Taken together, our data identified two layers of regulation of signaling by Fc receptors that utilize the common γ-chain. Identification of these regulators and, more importantly, identification of their interacting proteins, will provide attractive therapeutic targets for pharmacological interventions in Fc receptor mediated diseases.

Key Words: Fc Receptors, γ-chain, ITAM, Serine/Threonine phosphorylation, Kinase

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CHAPTER II

LIST OF ABBREVIATIONS

Ab	Antibody
ABP-280	Actin Binding Protein-280
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
Ag	Antigen
BCR	B Cell Receptor
DAG	Diacylglycerol
DAP10/12	DNAX-Activating Protein 10/12
EC	Extra Cellular
FACS	Fluorescent Activated Cell Sorting
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPI	Glycosyl Phosphotidyl Inositol
IBD	Inflammatory Bowel Disease
IC	Immune Complex
ITAM	Immunoreceptor Tyrosine based Activation Motif
ITIM	Immunoreceptor Tyrosine based Inhibition Motif
ITP	Immune Thrombocytopenia
LPS	Lipopolysaccharides

NK	Natural Killer
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ	Phospho Lipase C γ
PMA	Phorbol Myristate Acetate
PP1/2A	Protein Phosphatase 1/2A
PPL	Periplakin
PRR	Protein Recognition Receptors
RBL-2H3	Rat Basophilic Leukemia-2H3
SH2	Src-Homology 2
SLE	Systemic Lupus Erythematosus
TCR	T Cell Receptor
TLR	Toll-Like Receptors

INTRODUCTION

Humoral Immune Response

In order to evade constantly invading and evolving pathogens, higher vertebrates have developed several layers of defense which comprise our immune system. The first layer of defense is the skin which provides a direct physical barrier against many pathogens such as bacteria and viruses from even entering the host organism. All plants and animals have an additional layer of defense called the innate system, a system of cells that express pattern recognition receptors (PRRs) which recognize molecules on pathogens. Cells of the innate system include mast cells, monocytes, macrophages, dendritic cells, natural killer cells, and neutrophils. Macrophages and dendritic cells express a kind of PRR known as Toll-Like Receptor (TLR), which specializes in recognizing bacterial Lipopolysaccharides (LPS) and unmethylated CpG islands of bacterial DNA. Cells expressing other PRRs recognize bacterial proteins such as peptidoglycans, lipoteichoic acid, flagellin, pillin and double stranded RNA from many viruses. PRRs are also known as primitive pattern recognition receptors since they developed earlier in evolution.

Vertebrates have yet another layer of defense called the adaptive immune system, which is also called the acquired immune system. While the innate immune response is rapid and the recognition non-specific, the adaptive system response is longer lasting and more specific. The adaptive system specializes in tailoring the immune response to improve the recognition of the pathogen. Additionally, the adaptive system retains an ‘immunological memory’ of the infection for faster response during subsequent attacks. Cells of the adaptive system include T cells and

B cells, which are also known as T and B lymphocytes. The function of the T cells and B cells is to become 'educated' so as to recognize and distinguish between the self-antigens (host's owns proteins) and the foreign antigens, and to initiate an immune response against the latter. Through the process of somatic hyper-mutations, each individual T cell and B cell is able to express a highly specific receptor that recognizes either self or foreign antigens.

The innate system and the adaptive system both comprise a humoral component and a cell mediated component. The major component of the humoral response is secreted antibodies in bodily fluids (humours). Antibodies bound to an antigen is called an immune complex (IC) and the effector functions of these antibodies are mediated by Fc receptors located on the cell surface of mast cells, macrophages, neutrophils, etc (Figure 1).

Although their purpose is to distinguish between self and non-self-antigens and mount an immune response against the non-self-antigens, occasionally T and B lymphocytes nevertheless initiate an immune response to host tissues, creating an autoimmune condition. The antibodies produced by the autoreactive B lymphocytes bind to self-antigens creating autoantibody immune complexes, and, similar to immune responses against non-self-antigens, autoimmune responses to self-antigens are also mediated by Fc receptors expressed on effector cells. Studies in human autoimmune diseases and murine models of these diseases suggest that Fc receptors are critical components of human autoimmune disease.

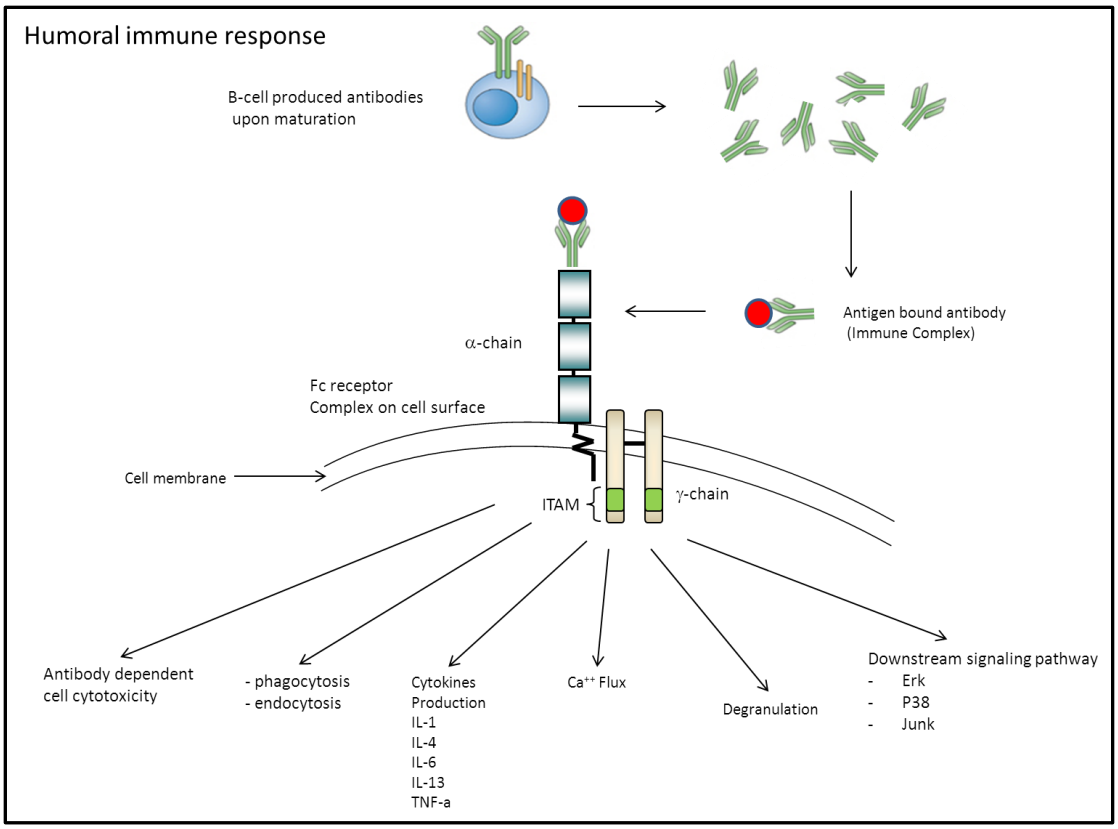


Figure 1: Fc Receptors on effector cells mediate humoral immune response

Fc Receptors in autoimmune SLE

Systemic Lupus Erythematosus (SLE) is a prototypic chronic, systemic autoimmune disease characterized by the presence of autoreactive B cells. Although environmental [1, 2] and other factors [3] have been implicated, several genetic factors also play a major role in SLE [4, 5]. Studies of monozygotic twins show a 25%-40% concordance rate for SLE confirming the presence of a strong genetic component to SLE [6]. Genome-wide association searches for SLE candidate genes in murine lupus models and human populations have identified the chromosome 1q21.1-24 locus [7-12], which encodes FcγR receptors that recognize the constant portion (Fc) of the IgG antibody. FcγRs on mononuclear phagocytes clear antigen-

antibody immune complexes (IC's) from the body, and the inability to clear IC's efficiently leads to autoimmune diseases such as SLE. Such diseases have highlighted the importance of Fc receptors in autoimmune disease pathogenesis [13-16], and the fact that functionally relevant single nucleotide polymorphisms in the FcγRs have been linked to SLE underscores this importance. Fc receptors have also been implicated in other diseases such as rheumatoid arthritis, multiple sclerosis (Reviewed in [17, 18]), and infectious diseases such as AIDS (reviewed in [19]).

Fc Receptor Structure

In humans, three different classes of FcγRs are expressed: FcγRI (CD64), the high affinity IgG receptor, and FcγRII (CD32) and FcγRIII (CD16), which have lower affinity. FcγRI is expressed as a receptor complex consisting of the ligand binding α-chain non-covalently linked to a signaling γ-chain homodimer. The γ-chain homodimer is also shared by other Fc receptors including FcαRI, FcεRI, and FcγRIIIa, in addition to other immunologic receptors (Figure 2). Signaling is initiated by cross-linking the surface receptors with IC's or with antibodies that recognize specific Fc receptors.

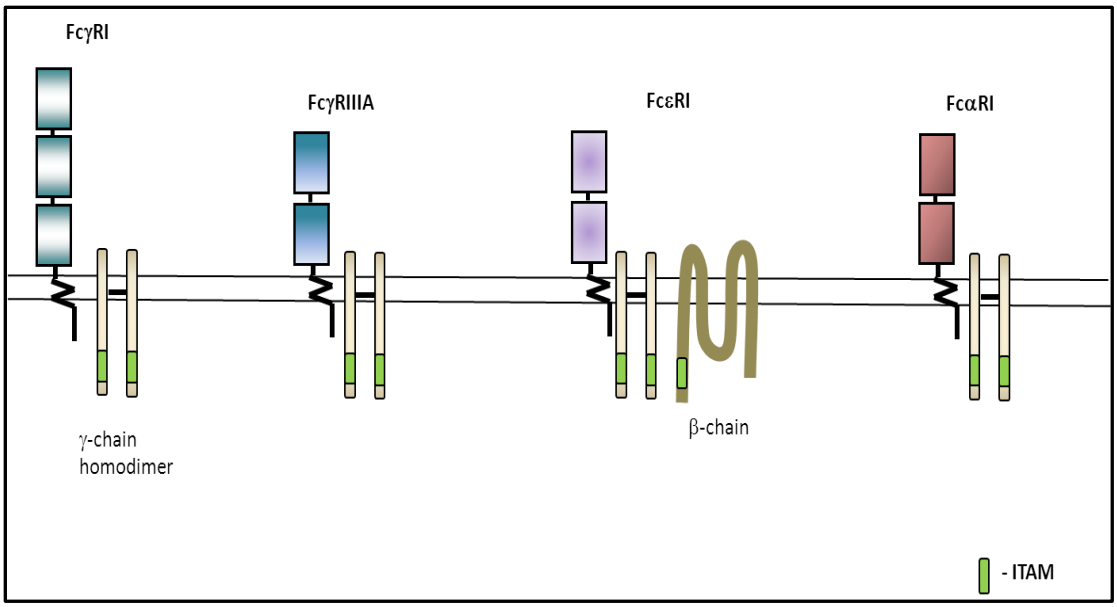


Figure 2: Structure of the FcRγ-associated receptors

FcγRI

Three genes (FCGRIA, FCGRIB and FCGRIC) located on chromosome 1q21 encode the FcγRI receptor alpha chains family; however, only FCGRIA is known to produce a full length protein [20]. FcγRI is the only high affinity receptor in the FcγR family with three Ig-like domains in the extracellular portion of α-chain, as opposed to two Ig-like domains for other Fc gamma receptors. The presence of a third EC domain explains the high affinity of FcγRI for monomeric IgG antibody molecules [21]. In humans, the binding affinity of FcγRI towards IgG1 and IgG3 is around 10^8 M^{-1} to 10^9 M^{-1} (reviewed in [22]), which is at least 100-1000 fold higher than the affinity of other Fcγ receptors. Due to this high affinity, FcγRI is constitutively saturated with monomeric IgG present in the serum. Activation however, is dependent on the cross-linking of bound IgG's via multivalent antigen [23]. The

associated γ-chain plays an integral role in FcγRI signaling via the Immune Receptor Tyrosine- based Activation Motif (ITAM) in the cytoplasmic domain. γ-chain not only associates with FcγRI α-chain [24], but was also found to be indispensable for the high surface expression of the α-chain and for effector functions such as phagocytosis [25]. Additionally, observations indicate that γ-chain is required for increased binding affinity of FcγRI α-chain for antibodies [26]. FcγRI is constitutively expressed on monocytes and macrophages, but it's expression can be up-regulated on neutrophils stimulated with IFN-γ [27] or GM-CSF (Granulocyte macrophage colony stimulating factor) [28].

FcγRII

Three genes (FCGRIIA, FCGRIIB, and FCGRIIC) encode the low affinity FcγRII receptors which are capable of binding only aggregated IgG (Kd approx. 10^6 M^{-1}). Unlike FcγRI and FcγRIIIa, which are expressed as receptor complexes with FcRγ-chain, required for surface expression and signaling, the FcγRII receptors are single chain molecules that incorporate both ligand binding and signaling components within the single receptor molecule, and they do not associate with γ-chain. FcγRIIa and FcγRIIc are both activating receptors consisting of two Ig-like extracellular domains, a transmembrane domain, and identical cytoplasmic domains that contain ITAMs (Li X, et al, 2013; unpublished observations). FcγRIIb, which contains extracellular domains identical to those of FcγRIIc, is an inhibitory molecule that is

expressed only on B cells and contains an Immune Receptor Tyrosine-based Inhibition Motif (ITIM) in its cytoplasmic domain. FcγRIIa and FcγRIIc are more widespread in their expression and are found on myeloid cells. FcγRIIc is also expressed on B cells (Li X, et al, 2013; unpublished observations).

FcγRIII

Two genes (FCGR3A and FCGR3B) encode the two receptors of the FcγRIII family, namely, FcγRIIIA and FcγRIIIB. Only FcγRIIIA associates with γ-chain and like FcγRI, is dependent on γ-chain for stable cell surface expression, activation and signaling. FcγRIIIB, which lacks an intracellular cytoplasmic domain, is expressed on neutrophils and eosinophils as a GPI-anchored protein [29]. Both FcγRIIIA and FcγRIIIB are considered to be intermediate affinity receptors and can bind monomeric IgG and immune complexes with lower affinity than FcγRI. FcγRIIIa is expressed on monocytes [30], NK cells [31], and NKT cells [32]. FcγRIIIB was thought to be expressed solely on neutrophils [33], however, recent evidence suggests basophils might also express FcγRIIIB [34].

FcγRIIIA is the key mediator of ADDC (antibody-dependent cell-mediated cytotoxicity) in NK cells, thus rendering FcγRIIIA expression on NK cells imperative for IC clearance. A nucleotide substitution of T to G at position 559 in the extracellular domain that changes phenylalanine (F) to valine (V) defines two allotypes of FcγRIIIA with different binding affinities: FcγRIIIA-158V/V (also

known as 176V/V, including the leader sequence) binds IgG1 and IgG3-containing IC's with higher efficiency in contrast to 158F/F (176F/F), and is also capable of binding IgG4. In addition, the higher-binding 158V/V allotype mediates stronger downstream cellular signaling effects such as higher calcium flux, higher CD25 expression and faster NK cell apoptosis [35, 36]. The low-binding allotype, 158F/F, has been associated with susceptibility to SLE in several case-control studies, especially in patients with lupus nephritis, suggesting FcγRIIIA-158F/F is a risk factor for SLE susceptibility or severity [35, 37, 38]. FcγRIIIA- 158FV/V was also shown to be related to faster and worse progression of end-stage renal disease [39].

FcεRI

FcεRI, which binds IgE antibody, is one of the better characterized Fc Receptor molecules. FcεRI is also unique amongst the γ-chain associating Fc receptors in that the FcεRI/α-chain complex also includes another ITAM-containing molecule, the β-chain (Figure 2). FcεRI has a very high affinity (Kd 10^9 to 10^{10} M^{-1}) towards IgE molecules [40]. As with FcγRIIIa, FcεRI requires FcRγ-chain for surface expression and signaling [41]. Published reports also indicate that the β-chain recruits signaling molecules and serves as a 'signal amplifier' [42]. In humans, FcεRI has been found to be expressed on monocytes [43], eosinophils [44], platelets [45], dendritic cells [46] and also Langerhans' cells [47, 48]. In addition, FcεRI appears to regulate circulating IgE levels as genome-wide association studies for factors regulating total serum IgE levels have identified FCER1A as a susceptibility locus

[49]. Finally, single nucleotide polymorphisms in the associated β-chain have been associated with atopic asthma and bronchial hyper-responsiveness [50-53], suggesting an important role for the β-chain in these disease processes.

FcαRI

A single gene coding for FcαRI (CD89) is located on the q-arm of chromosome 19 (19q13.4) [54]. While FcαRI belongs to the Ig gene superfamily due to the similarity of its molecular structure to FcγR and FcεR [55], it is more homologous to another receptor family, the leukocyte receptor cluster (LRC, reviewed in [56]). FcαRI is considered a low affinity receptor with approximate Kd of 10^6 M^{-1}. Like several other receptors described above, signaling via FcαRI is also dependent on the FcRγ-chain [57]. However, unlike FcγRI and FcγRIIIa, the FcαRI α-chain does not require FcRγ-chain for surface expression. In neutrophils, FcαRI α-chain has been found in both γ-chain associated and non-associated forms [58], and recent studies of transfected cells expressing γ-chain mutants incapable of associating with FcαRI α-chain showed no significant difference in surface expression of the α-chain in the absence of γ-chain association [59]. In monocytes however, the level of FcαRI-γ-chain association can be up-regulated using phorbol esters and interferon-γ [60]. Surprisingly, transgenic mice carrying the human FcαRI α-chain, but deficient in γ-chain fail to express FcαRI [61] thus suggesting the possibility of differential requirement for γ-chain to support FcαRI α-chain surface expression in different cell

types. Alternatively spliced variants of FcαRI cDNAs, including a shorter version missing 66 bp (EC2), are expressed on alveolar macrophages, neutrophils and eosinophils [62-67]. However, the role of FcRγ-chain in the surface expression of these alternatively spliced FcαRI variants remains unexplored. In neutrophils from pneumonia patients compared to those from healthy controls, a significant reduction in the EC2 splice variant was observed. This observation suggests a physiologic relevance of FcαRI splice variants in IgA-mediated host defense. Allelic variations in CD89 cytoplasmic domain have also been found to play an important role in SLE [59].

Fc receptor Signaling

Fc receptor signaling is initiated by cross-linking the receptors with multivalent immune complexes or by super aggregation of the receptors using secondary antibodies against primary antibodies already bound to the receptors. Upon cross-linking the receptors, Src kinases phosphorylate the two tyrosines of the γ-chain ITAM and the phosphorylated ITAM becomes a high affinity docking site for the Src Homology 2 domains (SH2) of Syk kinase [68-71]. ITAM-recruited Syk is then phosphorylated and activated, and activated Syk phosphorylates downstream target proteins to activate signaling pathways that culminate in cellular effector functions (reviewed in [72]). The two SH2 domains of Syk bind to phosphorylated tyrosines of ITAMs with different affinities, and ITAM tyrosines therefore have differential potential to activate Syk and downstream signaling pathways [73].

In some FcεRI models, β-chain seems to play an important role as it constitutively binds Lyn kinase, which phosphorylates both the β-chain ITAM and the γ-chain ITAM [74]. However, others have reported that FcεRI mediates signaling independent of β-chain [75]. In FcγR, early tyrosine phosphorylation of the γ-chain is attributed to Src kinases [76], and of the six Src kinases identified, namely Fgr, Fyn, Lck, Lyn, Hck and Yes, four have been found in immunoprecipitates of FcγR complexes. For example, Hck and Lyn have been identified in complexes with FcγRI in monocytes [77], while Lck has been found associated with FcγRIIIa in NK cells [78]. Similarly, the Src kinase, Fgr, was coimmunoprecipitated with FcγRIIa in neutrophils [79]. Since FcγRs do not associate with β-chain, the mechanism by which Src kinases are recruited to γ-chain in the FcγR complex is currently unclear. It has been proposed that a low level of constitutive association occurs between Src kinases and γ-chain, which can then be cross-phosphorylated upon receptor aggregation [80, 81], but direct association between Src kinases and γ-chain has not been demonstrated. In addition, while recruitment and activation of Src's are established, the exact role of each Src kinase is not well understood because of the high level of apparent functional redundancy among Src kinases [82, 83].

Role of the FcγRI α-chain CY in FcR γ-chain mediated signaling

It is well established that FcγRI signals through the non-covalently associated FcRγ chain and that the γ-chain is indispensable for signaling. However, our previous

observations indicate that the α-chain of FcγRI modulates γ-chain signaling. FcγRI mutant receptors lacking the α-chain CY domain mediated inefficient endocytosis, IL-6 production, antigen presentation [84, 85] and differential gene expression [86]. Similarly, FcγRIIIa α-chain CY domain-deleted receptors mediated lower levels of tyrosine phosphorylation of PLC-γ1, ZAP-70 and Syk kinase, and were unable to induce an intracellular calcium flux [87]. Together, these data indicate that the CY domain of Fcγ receptors, and possibly the CY domains of other immune receptors that signal through γ-chain, modulate receptor complex function.

The mechanism by which α-chains of Fc receptors modulate γ-chain signaling is not well understood. We have documented that, in resting cells of the U937 human monocytic line, FcγRI α-chain CY is constitutively phosphorylated on serine residues, but is dephosphorylated upon receptor cross-linking, followed by tyrosine phosphorylation of the γ-chain and cellular activation [85]. Incubation of these cells with Okadaic acid, a PP1/PP2A phosphatase inhibitor, results in persistent FcγRI CY serine phosphorylation despite receptor cross-linking, and importantly inhibits both tyrosine phosphorylation of γ-chain and downstream signaling. Therefore, FcγRI CY serine phosphorylation is an important "off" switch in receptor signaling, and dephosphorylation is an important "on" switch that regulates tyrosine phosphorylation of γ-chain thereby regulating FcγRI-initiated downstream cellular events.

ITAM structure and function in receptor signaling

In humans, the FcR γ-chain ITAM consists of two Y-X-X-L motifs separated by a spacer region of seven amino acids. The number of amino acids is conserved between human, mouse, and rat γ-chain ITAMs, but the amino acid sequence surrounding the ITAM and in the spacer region show differences (Figure 3).

Human γ-chain ITAM	…**YT**^{48}**GL** S^{51}T^{52}RNQET^{57}**YET**^{60}**L**…
Murine γ-chain ITAM	…**YT**^{48}**GL** NT^{51}RSQET^{57}**YET**^{60}**L**…
Rat γ-chain ITAM	…**YT**^{48}**GL** NT^{51}RNQET^{57}**YET**^{60}**L**…

Figure 3. Sequence alignment of murine, rat, and human FcR γ-chain ITAMs

For example, while phophorylatable threonine residues at positions 48, 52, 57 and 60 are conserved among the three species, only the human γ-chain possesses a phosphorylatable serine at position 51 (S^{51}), while murine γ-chain has a serine at position 54 (Figure 3). However, the fact that previous reports have shown that T^{52} alters the phenotype of the rat γ-chain in the context of FcεRI [87] suggests the possibility that T^{52} and the adjacent S^{51} in the human γ-chain may also influence γ-chain signaling.

Traditional bipartite motif ITAMs (**YXXL**-X_{7-12}-**YXXL**) and variations on this theme are found in other transmembrane signaling proteins that associate with ligand binding receptors, and are present in the CY domains of some single chain receptors (Table 1). FcγRIIa, a single chain lower affinity activating receptor widely expressed on myeloid cells, contains an unusual ITAM structure with a twelve amino acid long spacer region in the CY domain. Nevertheless, FcγRIIa cross-linking results in

phosphorylation of the ITAM tyrosines by a Src kinase, recruitment and activation of Syk, and phosphorylation of downstream signaling molecules (reviewed in [22]). The Igα and Igβ signaling chains of the B Cell Receptor each contain ITAMs with the traditional YXXL structural motif, and the ITAMs have been shown to be critical for BCR signaling and recruitment of additional signaling molecules [68, 88]. Interestingly, serine and threonine residues in the Igα ITAM have recently been shown to be important for signaling [68, 88].

ITAM adapters	Sequence of ITAM	Expression	Couple to receptors	Recruit Syk or ZAP70
Classical ITAMs (Consensus: Yxxl/Lx $_{(6-12)}$ Yxxl/L)				
DAP12	ESPYQELQGQRSDVYSDL	Myeloid and NK cells (less abundant on some T and B cells, osteoclasts, microglia)	NKG2D-S, KIR-2D, Ly49, TREM1, 2, 3. (integrins, plexin A1)	Syk or ZAP70
FcRγ	DGVYTGLSTRNQETYETL	Broad hematopoietic expression	FcεR, FcγR, OSCAR, PIR-A, Dectin-2, GPVI, TCR (integrins)	Syk
Igα, Igβ	ENLYEGLNLDDCSMYEDI DHTYEGLDIDQTATYEDI	B cells	BCR, MHC class II	Syk
CD3ζ	DGLYQGLSTATKDTYDAL	T cells	TCR, CXCR4	ZAP70
Nonclassical ITAMs				
Moesin	VLEYLKIAQDLEMYGVNYFSI	Broad expression, hematopoietic, endothelial, epithelial	PSGL-1	Syk
Dectin-1	MEYHPDLENLDEDGYTQL	Myeloid cells (less abundant in DCs and T cells)		Syk
CLEC2	MQDEDGYITL	Myeloid cells, DCs, platelets		Syk
RhoH	PLSYQQADVVLMCYSVA	Hematopoietic cells (most abundant in thymus and T cells)		ZAP70
MMTV Env	AYDYAAIIVKRPPYVLL	Viral envelope protein of MMTV		Syk
EBV LMP2A	HSDYQPLGTQDQSLYLGL	Infected B cells (required for latency)		Syk

Table 1. Summary of ITAM-containing adaptor molecules. Adapted from Abram CL and Lowell CA, 2007 (*Cell Signaling Technology*).

The CD3 ζ-chain in T Cell Receptor contains three somewhat traditional ITAM motifs, which are phosphorylated during TCR signaling [89]. However, in

vitro studies show that the CD3 ζ-chain ITAMs differ in primary amino acid sequence and structure (the spacer region in the membrane-proximal ITAM 1 and membrane-distal ITAM 3 are seven amino acid residues long, but that of ITAM 2 is eight residues long), and in their ability to recruit and activate ZAP70, an SH2 domain-containing kinase similar to Syk [89]. This suggests that differences in the primary sequence and structure of the spacer region might contribute to differences in ITAM function. In addition, only the CD3 ζ-chain ITAM 3 spacer contains phosphorylatable serines and threonines and may therefore have additional layers of regulation that are absent from ITAM 1 and ITAM 2 [89].

DAP10 and DAP12 proteins which associate with Integrins, Ly49, NKp44, and Siglec H are also signaling chains that possess traditional ITAMs. DAP10 and DAP12 are expressed on myeloid cells and their signals are mediated by Syk recruitment and activation, as described above for other ITAMs [90, 91]. The major β-Glucan receptors Dectin-1 and Dectin-2, which are also expressed on myeloid cells, and which bind and internalize zymosan, also signal via ITAMs. However, Dectin-1 is a single chain signaling protein where the traditional N-terminal Y-X-X-L ITAM motif has been replaced by a Y-X-X-X-L motif in the CY domain. Despite this unusual motif, the ITAM of Dectin-1 has been shown to be phosphorylated by Src kinases, and upon phosphorylation its ITAM recruits and activates Syk, which in turn activates downstream pathways including NF-κb activation [92]. On the other hand, similar to other FcRγ-chain associating receptors, Dectin-2 has been shown to associate with and signal through γ-chain [93].

Regulation of FcR γ-chain ITAM function

While it is clear that the serine dephosphorylation of the FcγRI α-chain is an important step in γ-chain signaling, the mechanism underlying phospho-serine regulation of γ-chain (tyrosine phosphorylation-based) signaling is unclear. Following FcεRI stimulation in mast cells the γ-chain is not only phosphorylated on ITAM tyrosines, but is also phosphorylated on threonine residues in the ITAM [94]. Swann *et al.* showed that phosphorylation of threonine at position 60 near the C-terminal YXXL motif was required for complete activation of Syk and efficient signaling for downstream cellular events [87]. Others have observed threonine phosphorylation in the γ-chain ITAM and have shown that threonine phosphorylation precedes tyrosine phosphorylation, suggesting that threonine phosphorylation might be a prerequisite for tyrosine phosphorylation [95]. However, in human γ-chain protein, a single phosphorylatable serine residue at position 51, and several phosphorylatable threonines residues are located at positions 48, 52, 57 and 60 in the ITAM (Figure 4), which suggest the possibility of complex regulation by multiple phosphorylation events.

...RLKIQVRKAAITSYEKSDGVYT^{48}GLS^{51}T^{52}RNQET^{57}YET^{60}LKHEKPPQ

Figure 4. Human FcR γ-chain sequence showing S/T residues within and upstream of the ITAM.

It is currently unclear whether any of these phospho-acceptor residues including T^{60} are important in γ-chain function and in FcγRI signaling. However, in a study of FcγRI stimulation in U937 cells, phospho-amino acid analysis of a lower mobility γ-chain species immuno-precipitated from stimulated cells showed

prominent serine phosphorylation, while γ-chain from resting cells was predominantly phosphorylated on threonine residues [96]. The serine-phosphorylated lower mobility γ-chain species was also induced by stimulation of U937 cells with phorbol myristate acetate (PMA), a phorbol ester that stimulates cellular serine/threonine kinases of the protein kinase C (PKC) family, suggesting the possibility that PKC might play a role in phosphorylating γ-chain [96]. The exact site and role of the serine phosphorylation was unclear since other non-ITAM serine residues are also present in γ-chain (at positions 39 and 43; a non-ITAM threonine residue is also present at position 38; see Figure 4). However, since it is unclear whether serine phosphorylation of γ-chain is required for activation, the authors speculated that serine phosphorylation may also play an inhibitory role in γ-chain signaling [96].

Protein Kinase C (PKC) serine/threonine kinases in Fc receptor signaling

While the role of PKC serine/threonine kinases in Fc receptor signaling is not entirely clear, Fc receptor engagement activates several isoforms of the PKC superfamily which translocate to the membrane and may potentially phosphorylate FcRγ-chain upon activation. Several PKC isoforms have been identified that belong to three subgroups: The Classical PKC's (PKCα, PKCβ, PKCγ) require Ca^{2+}, diacylglycerol (DAG), and phospholipids (PL) for activation; the Novel PKC's (PKCδ, PKCε, PKCθ, PKCη) are calcium-independent and require only DAG and PL for activation; and the Atypical PKC's (PKCζ, PKCι) which do not require the co-

activators, leaving their mode of activation unclear (reviewed in [97]). Studies show that several PKC isoforms are activated in response to Fc receptor stimulation. Lang et al., [98] showed that PKCε is activated following FcαR stimulation and is transiently translocated to the membrane. Similary, other studies showed that PKCα, PKCβ, and PKCε are activated and rapidly associate with the cell membrane following FcγR engagement [99-101], and inhibitors of PKC also inhibit FcγR-mediated phagocytosis [102]. In RBL-2H3 cells (the model system used in this thesis work) PKCα, PKCβ, PKCδ, PKCε and PKCζ [103] are expressed and are rapidly activated [104]. However, all but PKCζ translocate to the plasma membrane after FcεRI activation, suggesting that PKCα, PKCβ, PKCδ, and PKCε play important roles in the signaling process and that one or more of them could possibly phosphorylate FcRγ-chain. In addition, only PKCα and PKCδ were able to reconstitute FcεRI signaling in vitro [103]. Furthermore, treatment of RBL-2H3 cells with inhibitors of PKC resulted in inhibition of FcεRI-mediated functions in mast cells including hydrolysis of inositol phospholipids, release of arachidonic acid, and initiation of exocytosis following stimulation with antigen [102]. In other studies, Germano et al. [105] showed that PKC-δ physically associates with the FcεRI β-chain and phosphorylates γ-chain on threonine at position 60 (T^{60}) in the ITAM, indicating a direct role for PKCδ in γ-chain signaling, and suggesting the possibility that other PKC isoforms that translocate to the membrane might play a similar role in human FcRγ-chain-mediated signaling. However, whether PKC's associate with and/or phosphorylate human γ-chain in vivo or in vitro has not been reported.

SCOPE OF THESIS

The overall aim of the research described in this thesis was to elucidate the role of serine/threonine phosphorylation of the FcRγ-chain ITAM in Fc receptor signaling, which might lead to the identification of novel therapeutic targets in autoimmune diseases. Accordingly, the work in this thesis focuses on three main questions:

I. Does the unique cytoplasmic tail of the Fc receptor α-chain influence γ-chain mediated signaling in the RBL-2H3 model system?

II. Is serine/threonine phosphorylation evident in human FcR γ-chain ITAM?

III. Do putative serine/threonine phosphorylation sites in γ-chain ITAM influence γ-chain function?

CHAPTER I

THE CYTOPLASMIC TAIL OF THE FC RECEPTOR α-CHAIN INFLUENCES γ-CHAIN MEDIATED SIGNALING

By

SPANDAN SHAH, ANDREW GIBSON, XIAOLI LI, JEFFREY EDBERG AND ROBERT KIMBERLY

ABSTRACT

Signaling by multi-chain FcR complexes, including FcγRI, FcγRIII, FcεRI and FcαRI is mediated by the ITAM-containing common FcR γ-chain subunit and previous observations indicated that γ-chain was indispensable for receptor-mediated functions. However, despite the use of a common γ-chain, different Fc receptor complexes elicit specific cellular programs. This suggests a role for the unique α-chain cytoplasmic domains in modifying the Fc receptor complex functions. Based on these observations, we hypothesized that the unique α-chain cytoplasmic domains differentially modulate Fc receptor functions and we have documented that, in P388D1 transfectants, the FcγRI α-chain CY modulates γ-chain tyrosine-based functions. One possible mechanism by which the α-chain modulates γ-chain function is by influencing serine/threonine modification of γ-chain. Therefore we wished to establish a system in which modifications of serine/threonine residues that might play a role in signaling could be studied in transfected human γ-chain. Using a γ-chain negative RBL-2H3 cell line, we first wished to show that transfected FcγRI α-chain can influence human γ-chain function as had been observed in the P388D1 system. In order to confirm our results in P388D1 system, we analyzed the RBL-2H3 cell line stably co-expressing human FcR γ-chain and FcγRI WT or a cytoplasmic domain–lacking (TL) α-chain mutant for the capacity to carry out receptor-mediated endocytosis, phagocytosis, F-Actin polymerization, and cytokine protein and mRNA expression. Results indicate that the α-chain cytoplasmic domain is required for efficient phagocytosis, IL-6 and IL-4 production, and F-Actin polymerization, but not

for TNF-α expression. These results demonstrate that, as in P388D1 murine cells, the unique FcγRI α-chain CY domain modulates γ-chain function in RBL-2H3 and is required for efficient FcγRI-mediated effector cell functions. These data also indicate that similar signaling mechanisms are mediated by FcγRI.γ-chain complex in both systems and that our RBL-2H3 system is therefore suitable for investigating the role of serine/threonine modification in γ-chain function.

INTRODUCTION

Fc receptors play an important role in immune complex clearance, regulation of inflammatory responses and antibody secretion. Tyrosine phosphorylation in the Immuno-receptor Tyrosine Based Activation Motif (ITAM) following receptor cross-linking [22] is critical to these functions. Additionally, involvement of the γ/ζ subunits to Fc receptor signaling pathways lead to the possibility of redundant roles of Fc receptor in signaling pathways. However, recent evidence suggests that these receptors are not redundant. For example, FcγRIIIa is necessary for Arthus inflammatory reaction [106, 107] while FcγRIa and FcαRI can down-regulate inflammatory responses by initiating the secretion of IL-10 and IL-1ra, respectively [108, 109]. While the mechanism underlying these differences is not clear, both receptors signal through the common γ-chain suggesting that the unique α-chain cytoplasmic domains play an important role in signaling.

FcγRI is expressed on the cell surface in association with the γ-chain subunit, [24, 110]. This association is necessary for stable expression [25, 111]. The γ-chain cytoplasmic domain contains an ITAM and evidence suggest that the γ-chain cytoplasmic domain is both necessary and sufficient for FcγRIa mediated functions [112-114]. Biochemical studies have shown that cross-linking of the Fc receptor complex results in activation of a Src family kinase(s) and the tyrosine kinase Syk (reviewed in [22, 115]). Activation of these kinases results in tyrosine phosphorylation of the γ-chain and the initiation of a signaling cascade that can culminate in the induction of degranulation, phagocytosis, an oxidative burst and

antigen-dependent cell cytotixicity (ADCC). The association between FcγRIa and γ-chain may also be important in the formation of a higher affinity receptor complex through the recruitment of two ligand binding chains to the γ-chain homodimer [26].

The cytoplasmic domain of FcγRI does not contain an ITAM or other tyrosine containing signaling motifs. However, murine FcγRI on J774 cells is constitutively phosphorylated on serine and after phorbol 12-myristate 13-acetate stimulation the level of phosphorylation increases [116]. We and others have shown that the cytoplasmic domain of FcγRI, FcγRIIIa, and FcαRI modulate γ-chain tyrosine-based signaling by an as yet unclear mechanism [84, 85, 117, 118]. The cytoplasmic domain of the α-chain also associates with actin-binding protein-280 (also known as non-muscle filamin) in the absence of ligand [119]. Receptor engagement by ligand apparently abrogates this association, although its functional significance is not clear. Both of these observations suggest that the cytoplasmic domain of FcγRI may be actively involved in the biologic phenotype of FcγRI cross-linking. We have recently shown that FcγRI interacts with the cytoskeletal protein 4.1G in a serine phosphorylation-dependent fashion, which targets the receptor to lipid microdomains [117], that FcγRIIIa associates with S100A4 in a calcium-dependent fashion, which regulates phosphorylation of the cytoplasmic domain by PKC [120], and that FcαRI interacts with Lyn kinase in an allele-dependent fashion and modulates receptor pro-inflammatory status [59]. Interestingly, in one study FcγRI in the absence of the γ-chain was shown to flux calcium in COS-1 cells and the transmission of this calcium signal required the FcγRIa cytoplasmic domain [121].

Published observations with the FcεRI.γ-chain system indicate that, in addition to ITAM tyrosine phosphorylation, γ-chain is phosphorylated on threonine [94], and that PKCδ phosphorylates threonine at position 60 in the ITAM, which is required for efficient receptor function [87]. Additionally, studies using the human monocytic cell line, U937, show that human γ-chain is both serine and threonine phosphorylated in a stimulation-dependent fashion [96]. Based on these observations, we hypothesized that the serine/threonine residues in γ-chain provide an additional level of regulation and that, perhaps, the α-chain cytoplasmic domains of the γ-chain–associated receptors can regulate γ-chain signaling by modulating serine/threonine phosphorylation in addition to the obligatory tyrosine phosphorylation.

To begin to study the role of γ-chain serine/threonine modification in signaling, we used the rat basophilic leukemia (RBL-2H3)-derived γ-chain null cell line transfected with human γ-chain [122] . We first wished to show that this cell line could carry out human FcγRI-γ-chain mediated effector functions and that the cytoplasmic domain of FcγRI could modulate receptor complex signaling as we had previously shown for P388D1 murine macrophages expressing human FcγRI with endogenously expressed murine γ-chain [84]. By directly comparing the function of wild type human FcγRI with a cytoplasmic domain deletion mutant of FcγRI, both expressed at comparable levels in stable transfectants of the RBL-2H3, we recapitulated our previous observations with P388D1 cell lines, suggesting that similar FcγRI-γ-chain mediated signaling pathways are at work in our two systems. More importantly, these data suggest that our RBL-2H3 system could be used for

future studies to examine the role of γ-chain serine/threonine residues in the function of the γ-chain ITAM.

MATERIALS AND METHODS

Cell Lines and Reagents

RBL-2H3 γ-chain negative line was stably transfected with a cDNA encoding human FcγRIa or a mutant form of FcγRIa containing a stop codon after the first amino acid of the cytoplasmic domain (Lys^{315} → Stop 315) were prepared as described previously [112]. The FcγRI/FcγRIIIA chimeric receptor constructs comprising human FcγRI extracellular and transmembrane (EC-TM) domains fused to FcγRIIIa cytoplasmic domain (CY) was made using overlapping PCR to generate a *Kpn*I/*Bam*HI-flanked fragment. First, the FcγRI EC-TM cDNA fragment was amplified from human cDNA using the primers 5'TAA CGG **GGT ACC** GGA GAC AAC ATG TGG TTC TTG ACA ACT-3' (*Kpn* I site in bold) and 5'-GCT TCG AAT GTT TGT CTT TAT TGT CAC CCA GAG AAC-3', and the FcγRIIIa CY cDNA fragment was amplified using primers 5'-GTT CTC TGG GTG ACA ATA AAG ACA AAC ATT CGA AGC-3' and 5'-TTA CTA **GGA TCC** TCA TTT GTC TTG AGG GTC C-3' (*BamH* I site in bold). The amplified fragments were gel-purified using QIAquick Gel Extraction Kit (Qiagen), mixed together with upper primer 5'-TAA CGG **GGT ACC** GGA GAC AAC ATG TGG TTC TTG ACA ACT-3') and lower primer 5'- TTA CTA **GGA TCC** TCA TTT GTC TTG AGG GTC C-3' and the annealed chimeric receptor cDNA amplified and gel-purified. After digesting with *Kpn*I and *Bam*HI, the cDNAwas ligated to similarly digested pcDNA3.1 plasmid.

The rat basophillic leukemia (RBL-2H3) derived γ-chain negative cell line [122] was a kind gift from Dr. Juan Rivera at NIAMS, Bethesda, MD. Cells were maintained as adherents in DMEM medium supplemented with 10%FBS, 1X penicillin-streptomycin, 1mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES, and 2.5μM Plasmocin mycoplasma antibiotic (complete medium). Transfected RBL-2H3 were maintained in complete medium with 1mg/ml G418 (with 500uM hygromycin added to media for double transfectants). Primers used to amplify IL-6, TNF-α, and β-actin cDNA were designed using Oligo software (Molecular Biology Insights, Inc) pruchased from Life Technologies Corp (Carlsbad, CA) (See table 1). Human and mouse IgG were obtained from Sigma. Mouse $F(ab)'_2$fragments and $F(ab')_2$ goat anti-mouse IgG (GαM) were obtained from Jackson ImmunoResearch (West Grove, PA). The anti-FcγRI mAb 32.2 was obtained from the UAB Hybridoma facility, and $F(ab)'_2$ fragments were made by Rockland Inc, Gilbertsville, PA. All other reagents were from Sigma Aldrich Corp. (St. Louis, MO). Quantitative huFcγRI expression was matched for cells expressing the wild type (WT) and the cytoplasmic domain deletion mutant (MUT) by FACS analysis using anti-FcγRI mAb 32.2 and FITC-labeled goat anti-mouse IgG 2^o Ab (Jackson ImmunoResearch, West Grove, PA). Polyclonal anti-γ-chain Abs were prepared in rabbits immunized with a C-terminal peptide sequence that is shared by both human and murine γ-chain exactly as described [41] while a monoclonal antibody (7D3) that recognized the C-terminal 8 amino acids was prepared by the UAB hybidomal facility.

Immunoprecipitation and Phosphotyrosine Analysis

FcγRI was immunoprecipitated from the transfected lines using either mAb 32.2 or mAb 197 (kindly provided by Dr. Paul Guyre, Dartmouth University Medical School) [123] pre-bound to protein G-agarose (GE Healthcare Lifesciences, Pittsburgh, PA). γ-chain from transfected cells was immunoprecipitated by donkey anti-goat bound to protein G-agarose (that targeted the goat anti-mouse 2^o Ab used to crosslink the primary Ab bound to the HA-tagged γ-chain). Cells ($10–20 \times 10^6$/ml) were lysed in PBS containing either 1% Nonidet P-40 or 1% digitonin (Sigma) and protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL). For immunoblotting analysis, immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Membranes were blocked with 5% BSA followed by incubation with either polyclonal anti-γ-chain Ab or anti-phosphotyrosine mAb 4G10 (UBI, NY). Blots were washed 3 times with PBS, 0.1% Tween 20 and bound Ab was detected with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Jackson ImmunoResearch). Following 3 more washes, bound Ab was detected using ECL (Thermo Sicentific) according to the manufacturer's directions. Membranes were stripped by incubation with Tris-HCl, pH 2.3, for 30 min at room temperature and then re-probed as described above.

Endocytosis and Phagocytosis

Endocytosis of transfected huFcγRIa was determined by monitoring the disappearance of cell surface-associated anti-FcγRI mAb 32.2 $F(ab')_2$ upon cross-linking with $F(ab')_2$ GαM [124]. Cells (50μl, 5×10^6/ml) were incubated with a

saturating concentration of mAb for 15 min on ice. Following two washes in PBS, $F(ab')_2$ GαM was added, and cells were incubated for an additional 15 min on ice. Cells were then placed at 37 °C for varying periods of time, rapidly pelleted, and washed with PBS, containing azide at 4 °C. Remaining cell surface-associated receptor was quantitated with FITC-conjugated $F(ab')_2$ donkey anti-goat IgG by flow cytometry.

Cytokine Analysis

RBL-2H3 cells were opsonized with 12CA5 (anti-HA) mAb for 30 minutes on ice, followed by two washes with PBS. Cells were then stimulated with F(ab')2 goat anti-mouse IgG for 30 minutes at 37^0 C, washed, and then plated in complete media in 24-well culture plates for 15 hours at 37^0 C**.**

F-Actin Polymerization

RBL-2H3 cells expressing FcγRI WT or a mutant lacking the cytoplasmic domain (FcγRI TL) were stimulated for the indicated times with F(ab')2 fragments of FcγRI-specific mAb, 32.2, and F(ab')2 fragments of goat anti-mouse IgG secondary antibodies. Cells were then fixed with formaldehyde for 15 mins on ice, washed, permeabilized, and stained overnight for F-actin content using a mixture of 0.01% Triton X-100 and AlexaFluor 488-conjugated Phalloidin (which binds filamentous actin only). After washing, cells were analyzed by FACS analysis.

mRNA Purification

RBL-2H3 transfectant cells were stimulated with 32.2 mAb and GαM 2^0Ab for the indicated times (as above). Cells were then immediately lysed in Trizol reagent (Invitrogen, Carlsbad, CA) and RNA extracted according to manufacturer's protocols. cDNA was synthesized from the purified RNA using the Super-Script III First-strand cDNA synthesis kit (Invitrogen) according to manufacturer's instructions, and PCR amplification of cytokine cDNAs was performed using standard reagents and conditions provided (Invitrogen). PCR-amplified bands were electrophoresed on 2% agarose containing ethidium bromide and visualized under UV-light.

Flow Cytometry

50μL aliquots of cells at 5×10^6 cell/ml were incubated with saturating concentrations of primary mAb for 30 min on ice, washed, and then incubated with saturating concentrations of FITC-conjugated $F(ab')_2$ goat anti-mouse IgG on ice for another 30 min. After washing, the cells were analyzed immediately for immunofluorescence using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Statistical Analysis

Analysis of flow cytometry data was done using CellQuest software (Becton Dickinson). Statistical comparisons were performed with the paired *t* test. A probability of 0.05 was used to reject the null hypothesis that there is no difference between the samples.

RESULTS

The huFcγRI CY Domain Alters the Magnitude and Kinetics of FcγRI Phagocytosis in P388D1 Cells

Since γ-chain associated receptors mediate distinct cell programs, we considered the possibility that the CY domain of huFcγRIa may contribute to the functional properties of the receptor complex. To investigate the functional significance of the cytoplasmic domain of human FcγRIa, we previously utilized P388D1 cells stably and equally expressing wild type FcγRIa (WT) or a cytoplasmic domain deletion mutant form of FcγRIa (MUT) and tested their ability to internalize erythrocytes coated with the anti-human FcγRIa mAb 22.2 $F(ab')_2$. Our data showed that while both WT and MUT huFcγRIa mediated receptor-specific phagocytosis, the WT receptor consistently displayed a significantly higher phagocytic index despite identical levels of receptor expression (Figure 1).

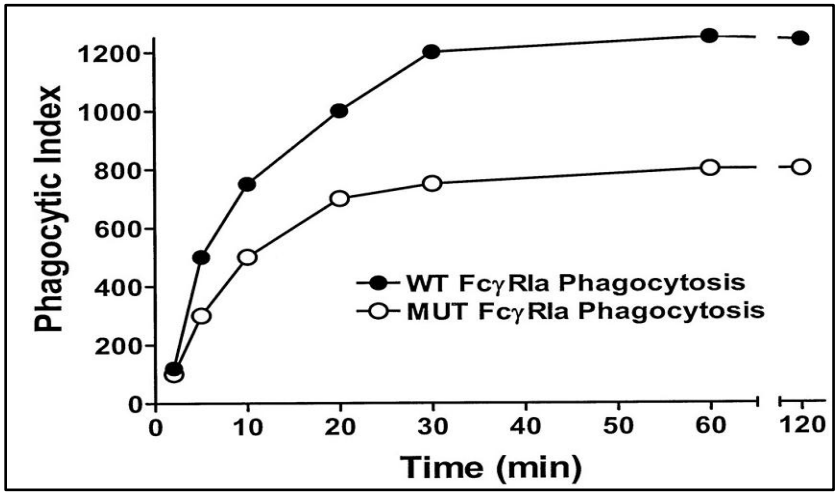


Figure 1. Receptor-specific phagocytosis by WT and MUT human FcγRI. Kinetics of phagocytosis of E-22 F(ab')2 by WT (●) and MUT (○) human FcγRI P388D1 stable transfectants. $p < 0.001$, FcγRI-specific phagocytosis by MUT versus WT (Edberg et al, 1999).

Requirement of the FcγRIa α-Chain for the Induction of IL-6 Secretion in P388D1

In addition to its role in internalization and phagocytosis, we also documented that FcγRI CY domain can modulate the induction of cytokine secretion thereby modulating the immune response. We had shown that P388D1 expressing the WT and MUT forms of huFcγRI were capable of eliciting comparable levels of IL-1β secretion, while cross-linking WT huFcγRI, but not MUT FcγRI, induced the secretion of IL-6 (Figure 2). These results showed that the α-chain CY of the FcγRI receptor complex was required for the induction of the IL-6 production and that the pathways leading to IL-6 secretion and IL-1β secretion are distinct.

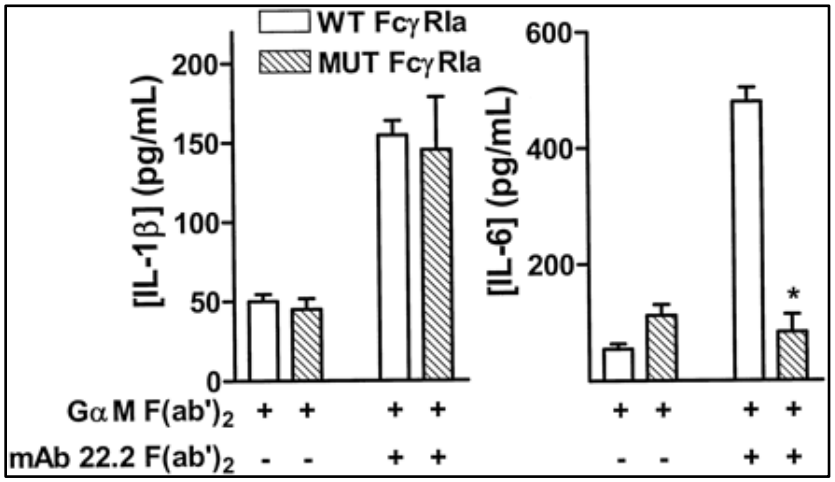


Figure 2. IL-6 release, but not IL-1β release, requires the cytoplasmic domain of FcγRI. P388D1 stable transfectants were cultured for 8 h in tissue culture wells that had been pretreated with F(ab')2 G αM (XL) or mAb 22.2 F(ab')2 + XL. Data are expressed as the mean pg/ml cytokine produced ± S.D. (n = 6). *, $p < 0.01$ relative to the XL alone control (Edberg et al, 1999).

Equivalent expression of γ-chain and α-chain in RBL-2H3 system

In order to establish an RBL-2H3 system in which to study the signaling role of γ-chain modification, we used RBL-2H3 derived cell lines lacking endogenous γ-

chain expression [122], and stably transfected with a fusion gene consisting of hemagglutinin (HA)-tagged WT human γ-chain. Expression of the γ-chain fusion was followed using anti-HA mAb, 12CA5. Cells stably expressing the γ-chain fusion were then stably transfected with WT huFcγRI α-chain or a cytoplasmic domain tailless (TL) mutant. Transfected cell lines were sorted to enrich for clones with identical levels of α-chain and γ-chain expression and were used in subsequent studies (Figure 3).

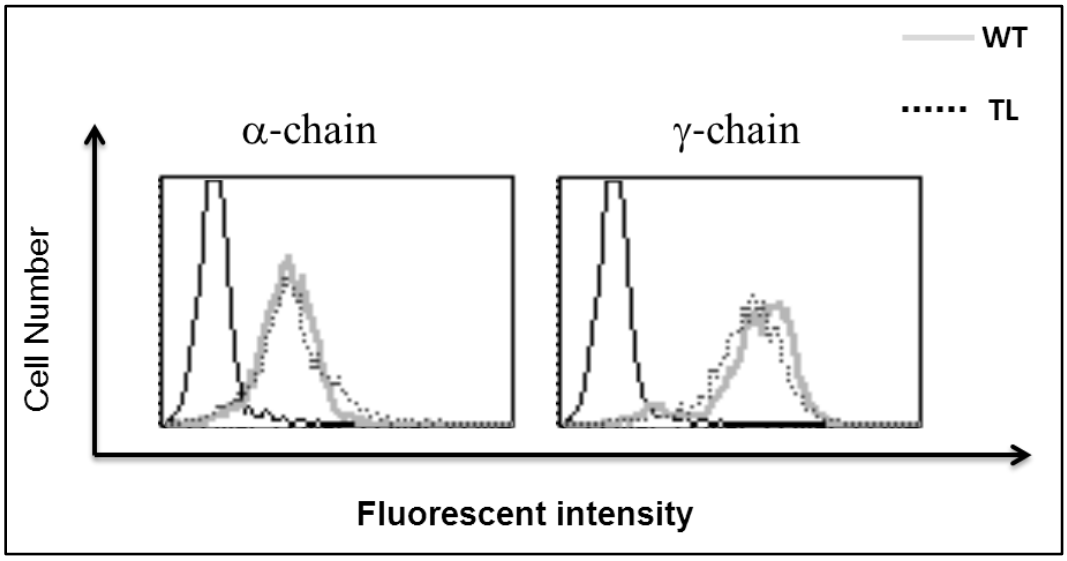


Figure 3. Comparable expression of human α-chain and human γ-chain in RBL-2H3 cells. RBL-2H3 cells stably expressing α-chain and HA-tagged γ-chain were stained with anti-α-chain antibody (F(ab)'2 mAb 32.2) or anti-HA-antibody (mAb 12CA5) to determine the cell surface expression.

RBL-2H3 cells expressing FcγRI TL mutant produce less IL-4 cytokine

In order to test whether the cytoplasmic domain of the FcγRI α-chain influences γ-chain mediated functions in RBL-2H3 cells, we cross-linked FcγRI WT and TL mutant α-chains using a receptor specific antibody (mAb 32.2 F(ab)'2). As

controls, FcεRI receptors were also cross-linked using murine IgE to test the equivalent capacity of both cell lines to produced IL-4. Our data show that, compared with FcγRI WT, the FcγRI TL receptor consistently induced significantly lower levels of IL-4 cytokine (Figure 4). In addition, stimulation of the cell lines with IgE showed no difference in IL-4 production, suggestion that while both cell lines have equivalent capacity to produce IL-4, the cytoplasmic domain of FcγRI is required for efficient FcγRI-γ-chain receptor complex mediated IL-4 production. These data are consistent with previous observations in P388D1 cells which demonstrated a requirement for FcγRI CY domain in efficient signaling for production of IL-6 cytokine.

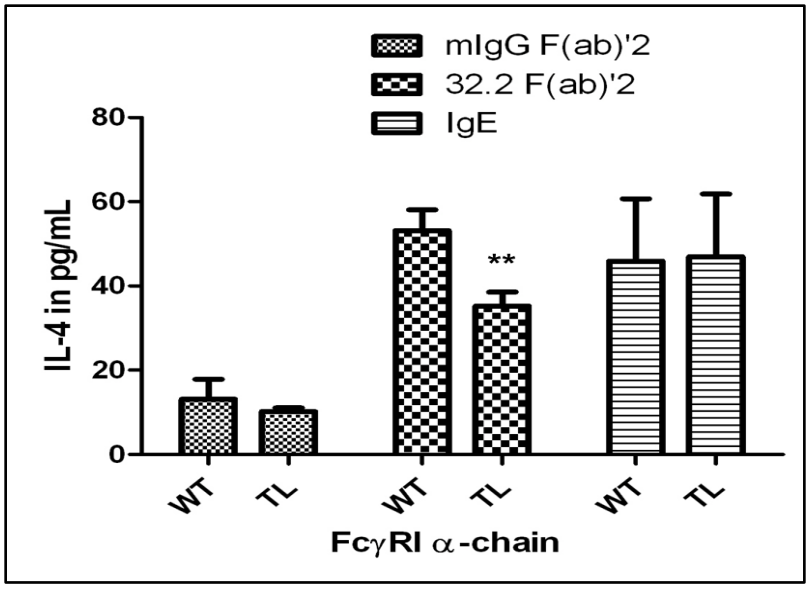


Figure 4. Reduced capacity of FcγRI TL mutant to signal for efficient IL-4 expression. FcγRI WT or TL mutant-expressing RBL-2H3 cells were incubated with receptor specific antibodies that were then super cross-linked using a goat-anti-mouse F(ab')2) secondary antibody. **P < 0.001 FcγRI TL vs FcγRI WT cross-linked with mAb 32.2 F(ab')2.

Reduced F-Actin polymerization by FcγRI TL mutant in RBL-2H3 cells

FcγR-mediated phagocytosis of particles requires cytoskeletal reorganization which requires polymerization of F-Actin [125]. To determine whether the FcγRI CY domain is required for efficient F-actin polymerization, we determined the ability of human FcγRI WT or TL mutant receptors to signal for F-Actin polymerization at different time points following stimulation of RBL-2H3 transfectants. Our results showed that, at each time point, the TL mutant produced significantly lower levels of polymerized F-Actin compared to WT cells (Figure 5). These data are consistent with observations in P388D1 transfectants expressing both receptor forms [117] and further suggest that similar FcγRI-γ-chain complex signaling pathways are utilized in both murine P388D1 and RBL-2H3 cell lines.

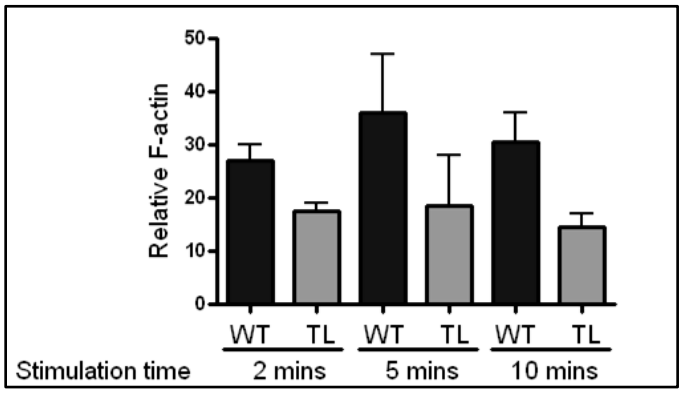


Figure 5. Reduced F-Actin polymerization by FcγRI TL mutants. RBL-2H3 cells expressing either WT or TL α-chain were stimulated with anti-α-chain antibody (mAb 32.2 F(ab)'2) and F-Actin polymerization was measured in resting cells or cells stimulated for 2, 5 or 10 minutes as described in materials and methods.

Reduced IL-6 cytokine mRNA levels in FcγRI TL mutant RBL-2H3 cells

While we were unable to detect IL-6 protein produced by RBL-2H3 derived cell lines expressing FcγRI WT or TL α-chain and HA-tagged γ-chain, we examined

the levels of IL-6 mRNA expression over time following FcγRI stimulation of the two cell lines. Our data showed that, over an 8 hour period following receptor stimulation, FcγRI TL-expressing cells produced lower levels of IL-6 mRNA (Figure 6A, top panel).

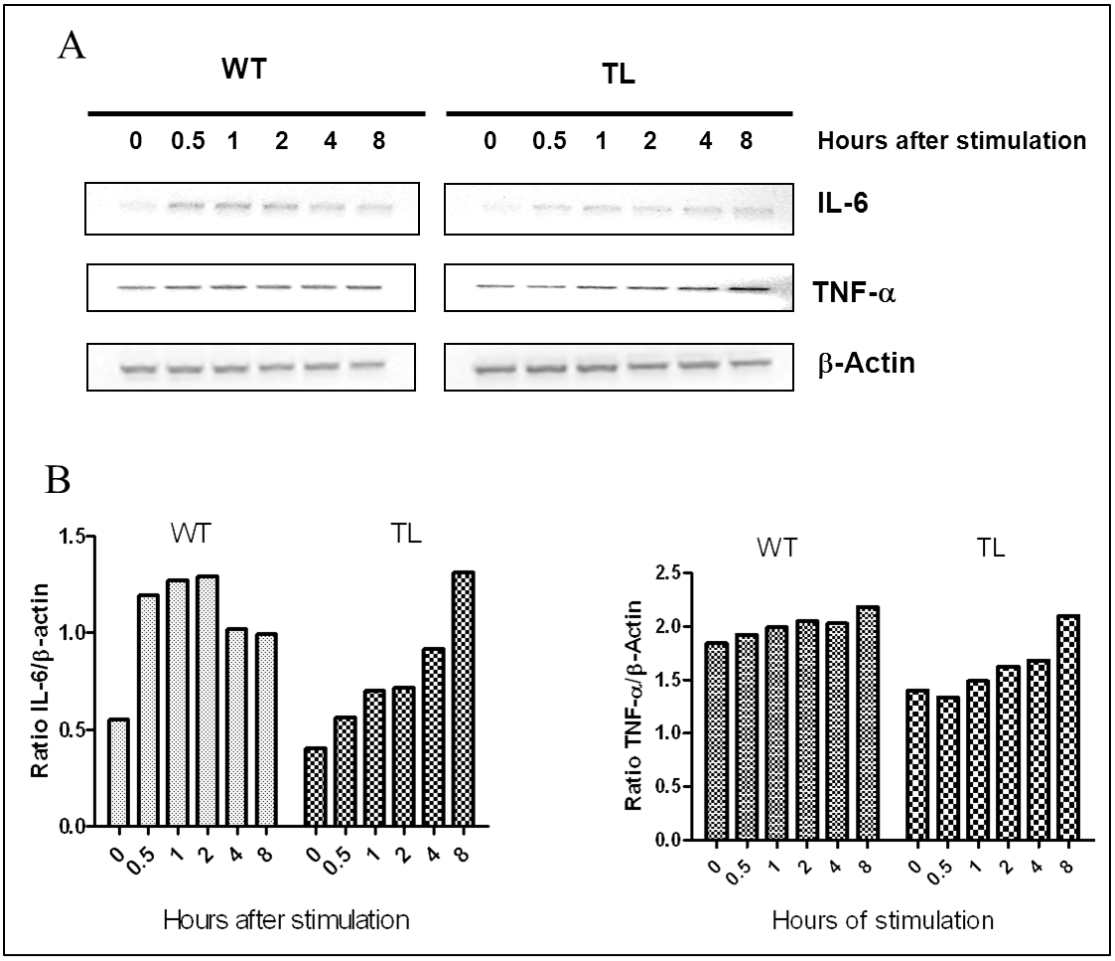


Figure 6. FcγRI CY domain is required for efficient IL-6, but not TNF-α mRNA production. RBL-2H3 derived cells stably expressing FcγRI α-chain WT or TL mutant and human FcRγ-chain were stimulated with receptor specific antibody (mAb 32.2 F(ab)'2) for indicated times. **A.** mRNA levels of IL6, TNF-α and β-Actin. **B.** Graph of the ratio of IL-6 or TNF-α mRNA vs β-actin mRNA at time points in A.

When compared to β-Actin mRNA levels, the difference in IL-6 mRNA production between the two cell lines was consistently greatest between 0.5 hrs and 2

hrs following stimulation (Figure 6B). These data indicate that the FcγRI CY domain is required for efficient IL-6 production and are consistent with our previous observations in P388D1 transfectants (Figure 2). In addition, there was no difference in TNF-α mRNA production between RBL-2H3 FcγRI WT and TL expressing cells at any time point indicating that, as with our previous observations in P388D1 cells, the FcγRI cytoplasmic domain is not required for efficient production of some cytokines.

FcγRI, but not FcγRIIIa α-chain CY fails to signal for Degranulation

It is well established that several FcR's signal through the common γ-chain, and our previous work indicated a requirement for the CY domain of the FcγR α-chains in efficient γ-chain based signaling. However, since different receptor-specific antibodies which possess different binding affinities and avidities are used in these studies, it remained unclear whether the α-chain CYs possess equivalent capacities to influence γ-chain signaling or whether differences in the binding properties of the receptor-specific antibodies contributed to observed differences in cellular outcomes. To address this question and eliminate the influence of differential antibody binding, we created chimeric FcγRI α-chains consisting of the extracellular and transmembrane domains of FcγRI fused to the cytoplasmic domain of FcγRIIIa (WT = FcγRIA and chimeric receptor = FcγRIA/FcγRIIIA) and tested their signaling capacity using the same anti-FcγRI-specific mAb, 32.2. FcγRI WT and FcγRIa/FcγRIIIa chimeric receptors were transfected into RBL-2H3 cells and sorted to enrich for cells with equivalent surface expression (Figure 7).

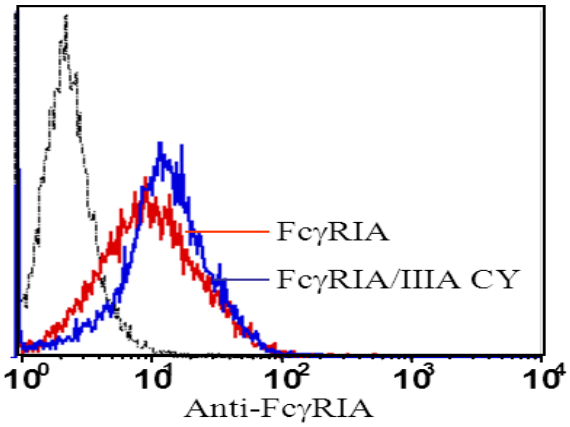


Figure 7. RBL-2H3 cells expressing equivalent levels of FcγRI WT or chimeric FcγRI-IIIa. Stable expression of either WT FcγRIA α-chains or chimeric α-chains (FcγRIA CY substituted for FcγRIIIA CY) were sorted for equivalent cell surface expression by staining for receptor specific antibody (mAb 32.2 F(ab)'2).

Transfected cells expressing equivalent levels of either the FcγRIA WT α-chain or chimeric FcγRI/FcγRIIIa α-chain were stimulated with 32.2 mAb and the primary antibody was super cross-linked using goat anti-mouse IgG secondary antibody. Following stimulation, the ability of the cells to signal for internalization of the surface receptor was measured by staining for levels of receptor remaining on the surface at various time points, and release of β-hexoseaminidase (degranulation) was determined as described in materials and methods. Data show that while both FcγRI CY and FcγRIIIa CY domains have equivalent capacity to signal for receptor internalization (Figure 9A), only FcγRIIIa CY had the capacity to signal efficiently for degranulation (Figure 9B). These data suggest that the two cytoplasmic domains of γ-chain associated receptors have equivalent capacity to signal for some functions, but in some cases possess unique signaling properties.

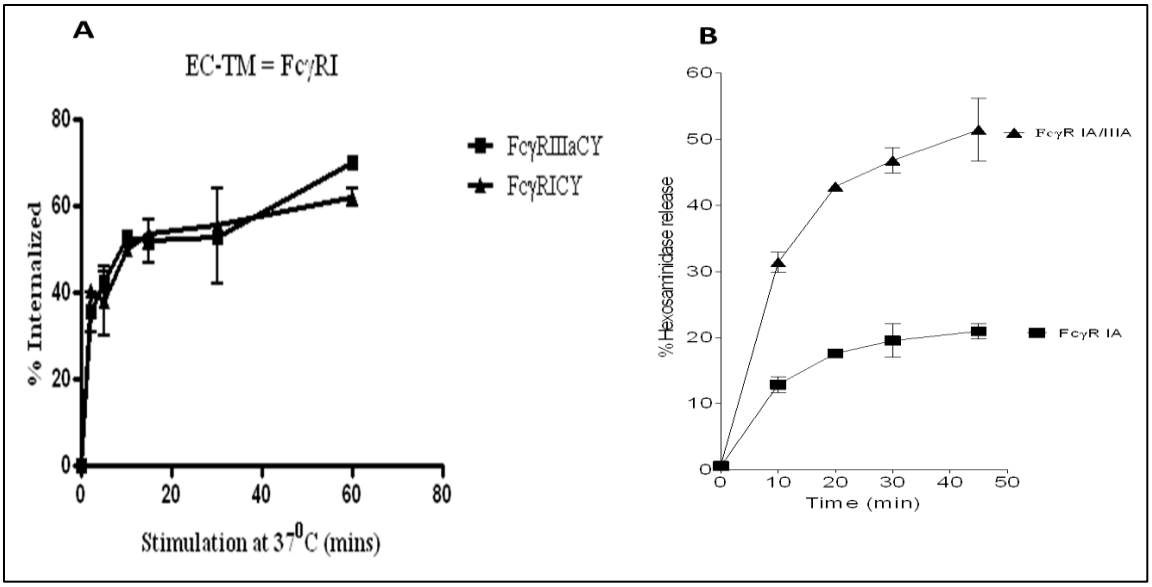


Figure 8. α-chain cytoplasmic domains influences Fc receptor functions. A. RBL-2H3 cells were analyzed for their ability to internalize receptor targeted antibody (mAb 32.2 F(ab)'2) from cell surface after stimulation for various time-points. **B.** Kinetics of β-Hexoseaminidase released by WT FcγRI α-chain or chimeric FcγRIA/FcγRIIIA after FcγRIA stimulation.

DISCUSSION

Our previous observations with murine P388D1 transfectants expressing human FcγRI α-chain documented that the cytoplasmic domain of FcγRI is required for efficient receptor signaling and that the cytoplasmic domain modulated tyrosine-based signaling by the associated, endogenously expressed murine γ-chain. We now show that, in a γ-chain negative RBL-2H3 rat cell line transfected with both human γ-chain and human FcγRI α-chain, the FcγRI CY domain is required for efficient receptor complex signaling, and that the CY domains of γ-chain associated receptors possess differential capacities to modulate γ-chain based signaling for different cellular outcomes. More importantly, these data using the RBL-2H3 γ-chain null lines validate an experimental system in which we can begin to probe the role of specific γ-chain amino acid residues in FcR signaling. Observations from these future studies may be of value in identifying targets for therapeutic modification of FcR-mediated diseases.

Previous studies of FcγRI show that γ-chain is required for phagocytosis [112-114]. The role of γ-chain in FcγR signaling, and the central role of the tyrosine kinase Syk, which docks via SH2 domains to the tyrosine-phosphorylated γ-chain, have been well established [83, 126-128]. Although the mechanism is unclear, we have observed that cellular outcomes are modulated by the FcγRI α-chain cytoplasmic domain regardless of in different cell types (Figure 1, 2, 4, 5 and 6).

The ability of the cytoplasmic domain of FcγRI to modulate intracellular signals mediated by the FcγRI-γ-chain receptor complex suggest the possibility that the α-chain CY recruits signaling molecules which might modify γ-chain on

serine/threonine residues thereby favoring particular cellular outcomes. Similarly, the CY domains of other γ-chain associating α-chains may uniquely alter γ-chain signaling capacities. Consistent with this idea, studies of the high affinity receptor complex for IgE which includes a ligand binding α-chain, a β-chain, and a γ-chain homodimer indicate that the β-chain is constitutively associated with the Src kinase Lyn and recruits protein kinase C-δ. Lyn is a tyrosine kinase and PKCδ is a serine/threonine kinase, and have been shown to modify the γ-chain ITAM and facilitate γ-chain mediated signaling [74, 105, 129, 130]. Although the role of serine phosphorylation of γ-chain is not yet established, PKCδ has been shown to phosphorylate a threonine residue at position 60 (T^{60}) in the γ-chain ITAM, a modification shown to be required for efficient Syk recruitment and efficient TNF-α production [87]. The presence of a putative phosphorylation sites – a serine residue at position 51 (S^{51}) and threonines at positions 48, 52, 57, and 60 (T^{48}, T^{52}, T^{57}, T^{60}) within the human γ-chain provide attractive targets for regulation of signaling. Studies have shown that, indeed, FcγRI cross-linking on U937 cells results in the serine phosphorylation of the γ-chain [96]. Additionally, protein kinase C-β and protein kinase C-ε are recruited to the membrane by Fcγ receptors during phagocytosis [100] and may play a role in γ-chain modification. It is currently unclear whether following receptor stimulation the cytoplasmic domain of human FcγRI α-chain and those of other FcγR's that signal through γ-chain differentially recruit γ-chain modifying enzymes directly or indirectly. However, that the unique α-chain cytoplasmic domains of γ-chain associated receptors interact with different cellular proteins is well established [117], [120]. Interestingly, subtle differences in

the ITAM sequences used by Fc receptors may also contribute to distinct biological properties. The ITAM-like sequence in FcγRIIa differs from the ITAM in the γ-chain in primary sequence and structure (12-residue spacer in FcγRIIa vs 7-residue spacer in γ-chain) and this difference, or other adjacent sequences, may influence the capacity to signal for specific cellular outcomes. These differences, along with the presence of serine/threonine residues within the ITAM may allow for differences in some of the functions between FcγRIIa and FcγRI/FcγRIIIa.

Although it contains no ITAM, the mechanism(s) by which the cytoplasmic domain of FcγRI alters receptor function is not clear. The modulation of phagocytosis by the cytoplasmic domain of the α-chain may be due to an association of cytoskeletal elements or signaling molecules with the cytoplasmic domain, resulting in efficient phagocytosis. Indeed, FcγRI α-chain has been shown to associate with Actin-binding protein (ABP-280) constitutively and α-chain dissociates from ABP-280 upon receptor engagement [119]. Additionally, PPL (periplakin) [118], and protein 4.1G [117, 131] also bind to the α-chain cytoplasmic domain and influence receptor function. It is therefore possible that the α-chain CY's differentially recruit γ-chain modifying molecules directly or indirectly, resulting in modulation of γ-chain function.

Our previous observations with P388D1 murine macrophage cells expressing endogenous murine γ-chain and transfected human FcγRI show that FcγRI CY is required for efficient IL-6 production, efficient receptor internalization, and efficient polymerization for F-actin [84, 85, 117], thus establishing that the CY domain modulates γ-chain tyrosine-based signaling. In this current work using rat RBL-2H3

transfected with both human γ-chain and human FcγRI α-chain, we show that FcγRI CY is required for efficient production of IL-4 and IL-6 cytokines, and for efficient F-actin polymerization, thus recapitulating our finding in P388D1. Importantly, the RBL-2H3 data suggest that signaling pathways similar to those in P388D1 are used following FcγRI-mediated stimulation. Therefore, by co-expressing site-specific γ-chain mutants with FcγRI, the RBL-2H3 system will allow us to query the role of potential phospho-acceptor sites in the γ-chain ITAM function. Unique sequences of the α-chain cytoplasmic domains of the γ-chain-associated Fc receptors suggest unique capacities to regulate γ-chain mediated signaling perhaps by differential modification of γ-chain.

Understanding the detailed mechanisms by which the unique α-chain CY's facilitate γ-chain function, including the identification of modified sites in γ-chain, their functional roles in signaling, and the identification of the responsible modifying enzymes will provide opportunities to selectively target specific receptor functions. These potential therapeutic targets will be of value in modifying FcR-mediated diseases like autoimmune diseases. In summary, in this work we have developed an RBL-2H3 human FcγRI/γ-chain double transfection system that will allow us to carefully and methodically identify specific functional sites in human γ-chain, and to identify potentially therapeutic targets for interrupting specific FcR-mediated cellular outcomes in disease processes.

CHAPTER II

SERINE/THREONINE PHOSPHORYLATION SITES IN γ-CHAIN ITAM INFLUENCE γ-CHAIN SIGNALING

By

SPANDAN SHAH, ANDREW GIBSON, CHARLIE JI, KYOKO KOJIMA, JAMES MOBLEY AND ROBERT KIMBERLY

ABSTRACT

FcγRI, FcγRIII, FcαRI and FcεRI are multi-chain receptors that utilize the common FcRγ-chain adaptor molecule to initiate signaling cascades in response to ligands. Signaling via the γ-chain requires phosphorylation of two tyrosine residues in the ITAM motif which provides a docking site for recruitment and activation Syk, a critical signaling intermediate. While the requirement for tyrosine phosphorylation is well established, the functional importance of putative serine/threonine phosphorylation sites in the ITAM is unclear. Reports in the rat FcεRI system have identified threonine 60 in the ITAM as a site critical for function, and have shown that threonine 60 is phosphorylated by PKCδ. Studies using the human U937 monocytic cell line have also suggested that human γ-chain is phosphorylated on serine and threonine residues in a stimulation-dependent fashion; however the exact sites of phosphorylation and their functional importance were not addressed. In this report, we used a RBL-2H3 γ-chain negative cell line expressing the human γ-chain to begin to address the functional importance of serine and threonine residues in the human γ-chain ITAM. Using mutational analysis of serine and threonine residues we show that these residues differentially regulate the γ-chain ITAM signaling. Using mass spectrometry, we have identified Serine 51 as a major site of phosphorylation in stimulated samples. Importantly, we show that this modification negatively regulates γ-chain mediated signaling at the level of tyrosine phosphorylation. These data suggest a significant contribution by Serine 51 in γ-chain signaling pathways and identifies a potential therapeutic target.

INTRODUCTION

Several Fc receptors including FcγRI, FcγRIII, FcεRI and FcαRI, associate with γ-chain, a signaling molecule containing an immunoreceptor tyrosine based activation motif (ITAM) [132, 133]. Cross-linking the Fc receptors using immune complexes or specific antibodies initiates γ-chain signaling with phosphorylation of the two ITAM tyrosines by a Src kinase, recruitment and activation of Syk and phosphorylation of downstream signaling intermediates which result in cellular effector programs.

Despite signaling through the common γ-chain molecule, Fc receptors initiate different cellular programs [106-109] suggesting additional layers of regulation beyond phosphorylation of the ITAM tyrosines in γ-chain. Previously, we [84-86] and others [134, 135] showed that the cytoplasmic domain of the Fc receptor α-chains in part regulate γ-chain ITAM-mediated signaling pathways. However, the presence of other putative serine and threonine phosphorylation sites within the ITAM provide attractive targets for additional regulation of γ-chain signaling, and might provide attractive targets for therapeutic intervention in FcR-mediated diseases. Indeed, in rat FcεRI signaling a threonine residue (T^{60}) located in the C-terminal YxxL motif of the γ-chain ITAM was shown to be phosphorylated by PKC-δ and to be required for efficient FcεRI function [87]. Several published reports have also identified serine and threonine phosphorylation in the homologous murine and human γ-chain [94, 96, 136]. However, the location of the phosphorylated residues and their role in ITAM signaling has not been explored.

The role of serine/threonine phosphorylation in signaling has been studied in other ITAM's and in other proteins. In the B cell receptor, phosphorylation of serines and/or threonines in the Igα subunit results in negative regulation of tyrosine phosphorylation of the ITAM [137, 138]. Negative regulation by serine/threonine phosphorylation has also been observed in the Insulin Receptor Substrate-1 (IRS-1), where phosphorylation of IRS proteins inhibit IRS docking to IR [139].

In this study, we sought to identify the sites of phosphorylation in human γ-chain ITAM and to explore their role in γ-chain-mediated cellular responses. Using HA-tagged human γ-chain stably expressed in γ-chain-deficient RBL-2H3 cells, we identified serine at position 51 (S^{51}) as a site that is predominantly phosphorylated in non-stimulated cells. Using S51A and S51D mutants, we show that S^{51} plays an inhibitory role in the production of IL-4. Finally, we show that S^{51} alters tyrosine phosphorylation of the γ-chain ITAM, and subsequent Syk and SHP-1 activation, suggesting an inhibitory role in Src kinase phosphorylation of γ-chain. Therefore, our results indicate that S^{51} in the human γ-chain plays a negative role in receptor tyrosine-based signaling and suggests that other serine and threonine residues around γ-chain ITAM may also regulate ITAM signaling. Serine and threonine residues in the γ-chain ITAM and their modifying enzymes might therefore provide therapeutic targets for interrupting receptor signaling in inflammatory diseases.

MATERIALS AND METHODS

Cells Culture and Reagents

The murine monocytic cell line U937 cells were obtained from ATCC and cultured in RPMI complete medium (as described in Chapter 1). The day before stimulation, the medium was replaced with complete medium with 10% (low IgG-containing) FBS. RBL-2H3 γ-chain-deficient cells [122] were used to derive stable transfectants. To obtain cell populations expressing equal numbers of receptors, stable transfectants were stained with FITC-12CA5 and sorted for the top 10% of the cells with the highest expression (fluorescence signal). HA-tagged human γ-chain plasmid constructs encoding serine/threonine to alanine mutations (T48A, S51A, T52A, T57A and T60A) and serine to aspartic acid (S51D) were created by site directed mutagenesis of WT γ-chain using mutagenic primers and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by DNA sequencing with the ABI Prism BigDye terminator cycle sequencing performed in the UAB Heflin Sequencing Core Facility. Anti-HA (12CA5) antibody was prepared in the UAB Hybridoma Core Facility and used at 20μg/mL for all assays. For stimulation, 12CA5-opsonized FcRγ was super cross-linked for various times using F(ab')2 goat anti-mouse 2^0 antibody at 20μg/mL.

ELISA, Calcium Flux and mRNA Analysis

Cells were opsonized with 12CA5 or isotype control for 30 mins at 4^0C. Cells were then stimulated with goat-anti-mouse secondary antibody for 30 mins at 37^0C then plated and incubated for 15 hours at 37^0C. For Calcium flux assays, cells were

first loaded with Indo-1 AM dye (Molecular Probes, Carlsbad, CA) for 40 mins at 37^0C, opsonized with 12CA5, and then stimulated with goat-anti-mouse IgG Ab. Excitation of Indo-1 AM at 305nm, and signal emission at 405nm (Ca^{2+} unbound) and 490nm (Ca^{2+} bound) was measured using a TECAN Infinite M200 PRO instrument (TECAN Systems Inc, San Jose, CA). mRNA was isolated using Trizol (Life Tech. Inc.), and cDNA was generated from the mRNA using the First Strand cDNA kit (Life Tech. Inc) according to instructions. PCR amplification was performed as previously described using standard protocols (see Chp 1 materials and methods).

Immunoprecipitation and Western Blotting

γ-chain was immunoprecipitated using mAb 12CA5 or mAb 7D3 prebound to protein G-agarose (Pharmacia Corp. Piscataway, NJ). Cells ($10–20 \times 10^6$ cells/ml) were lysed in PBS containing 0.9% NP-40 (Sigma), 0.1% Digitonin, 1X HALT protease inhibitor cocktail and 1X phosphatase inhibitors (Pierce, Rockford, IL). Immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting. For immunoblotting analysis, immunoprecipitates were transferred onto nitrocellulose membranes; membranes were blocked with 10% nonfat milk or 3% bovine serum albumin and then incubated with the blotting antibodies. Blots were washed three times with phosphate-buffered saline-0.1% Tween 20 and probed with HRP-conjugated anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences or Jackson ImmunoResearch). After three more washes, bound Ab was detected using ECL (Amersham Biosciences) according to the manufacturer's directions. Membranes

were stripped by incubation with Tris-HCl, pH 2.3, for 30 min at room temperature and then reprobed as described above.

In-Vitro Kinase Assays

Glutathione beads loaded with 20 μg GST protein, GST-FcRγ CY fusion protein, or serine-to-alanine mutants of FcRγ CY were incubated with 2 μCi ^{32}P-ATP and 0.5 U PKA or PKC (Signal Chem, Richmond, BC, Canada), according to the manufacturer′s suggestions. Beads were washed twice in 50 mM Tris-HCl/5 mM EDTA, pH 8.5/0.5 M NaCl, resuspended in 2X SDS buffer, electrophoresed on 15% agarose gels. 1μg Myelin Basic Protein (MBP), which is phosphorylated by the enzymes, was used as a positive control. Following incubation with PKA or PKC, MBP was precipitated using 50% TCA, resuspended in 50μL 2X SDS loading dye, and 5μL loaded on gels. Gels were then dried and exposed to film.

Mass Spectrometry

HA-γ-chain was cross-linked using 12CA5 and goat anti-mouse IgG 2^{o}Ab, then immunoprecipitated from cell lysates using donkey anti-goat IgG Ab pre-adsorbed to protein G beads. Following SDS-PAGE purification and digestion, phosphate group modifications in γ-chain ITAM were detected at UAB Bioanalytical and Mass Spectrometry Shared Facility using the Orbitrap Velos Pro as previously described [140]. Data were normalized for spectral count to compare phosphorylated vs non-phosphorylated peptides and analyzed by Scaffold PTM software with A-scoring capabilities.

RESULTS

Equivalent expression of WT and mutant γ-chain by RBL-2H3 cells

To analyze the roles of S^{51} and T^{48}, T^{52}, T^{57} and T^{60} in γ-chain mediated function, a γ-chain null RBL-2H3 cell line [122] was used. Transfectants expressing equal levels of HA-tagged human γ-chain WT or mutants in which the serines and threonines had been changed individually to alanines (Figure 1A, 1B) were sorted for the top 10% cells until all cell lines expressed γ-chain at equivalent levels.

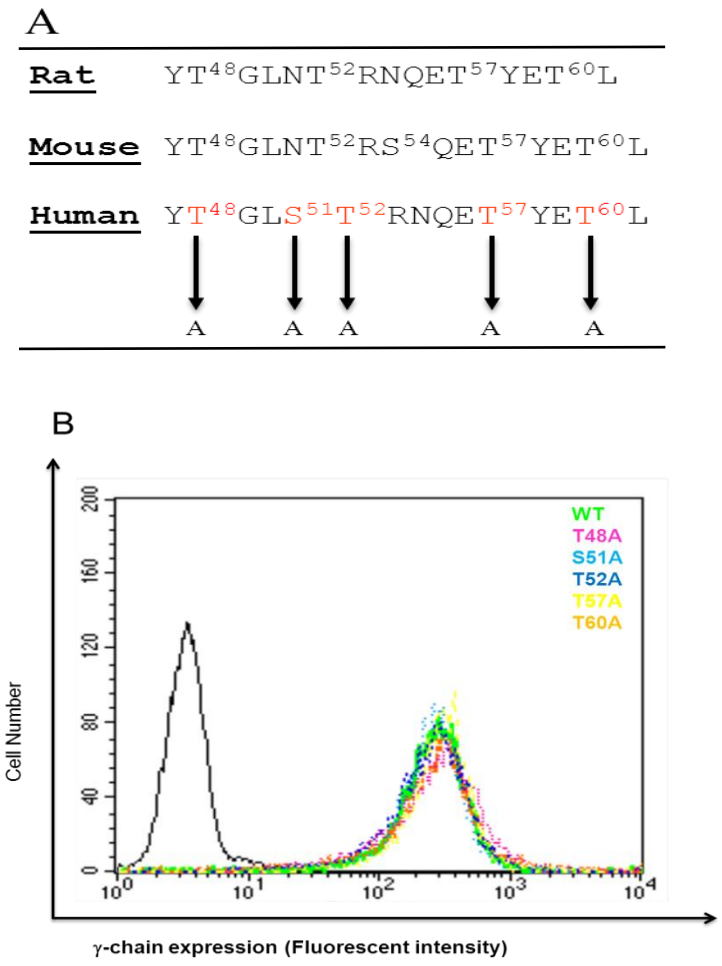


Figure 1. RBL cells expressing equivalent levels of human HA-γ-chain WT or mutants. A. Alignment of rat, murine and human γ-chain ITAMs. ITAM residues that were changed to alanines are in red and numbers indicate residue positions in the γ-chain sequence. **B.** FACS analysis of RBL-2H3 cells expressing HA-tagged human γ-chain WT and mutants. Cells were stained with mAb 12CA5 (20μg/mL) followed by FITC-conjugated goat-anti-mouse 2^0Ab (20 μg/mL).

ITAM Serine/Threonines influence γ-chain mediated signaling pathways

RBL-2H3 cells expressing equivalent levels of WT or mutant HA-tagged human γ-chains were opsonized with 12CA5 (anti-HA) and stimulated with goat anti-mouse IgG as described in materials and methods. IL-4 production was measured by ELISA and intracellular calcium release was measured using Indo-1 AM calcium-sensitive reagent (Figure 2A, 2B). Figure 2A shows that mutations of T^{48}, T^{57} and T^{60} in the γ-chain resulted in significantly reduced IL-4 levels compared to WT levels, while S^{51} and T^{52} mutations resulted in significantly increased levels of IL-4 (Figure 2A). These data suggest that specific serine/threonine residues in the γ-chain provide either positive (T^{48}, T^{57} and T^{60}) or negative (S^{51} and T^{52}) signaling for IL4 production. An examination of the ability of the mutant γ-chains to signal for intracellular calcium release also showed differential regulation by γ-chain ITAM residues. Compared with WT levels, T^{57}, T^{52}, and T^{60} mutations resulted in lower levels of calcium release upon stimulation (Figure 2B). However, mutations of T^{48} and S^{51} did not affect the ability of γ-chain to signal for calcium release as there was no difference when compared with wild type levels. These data suggest that serine and threonine residues in the γ-chain ITAM differentially influence γ-chain mediated signaling pathways, and that the same residue may play different regulatory roles in differently pathways.

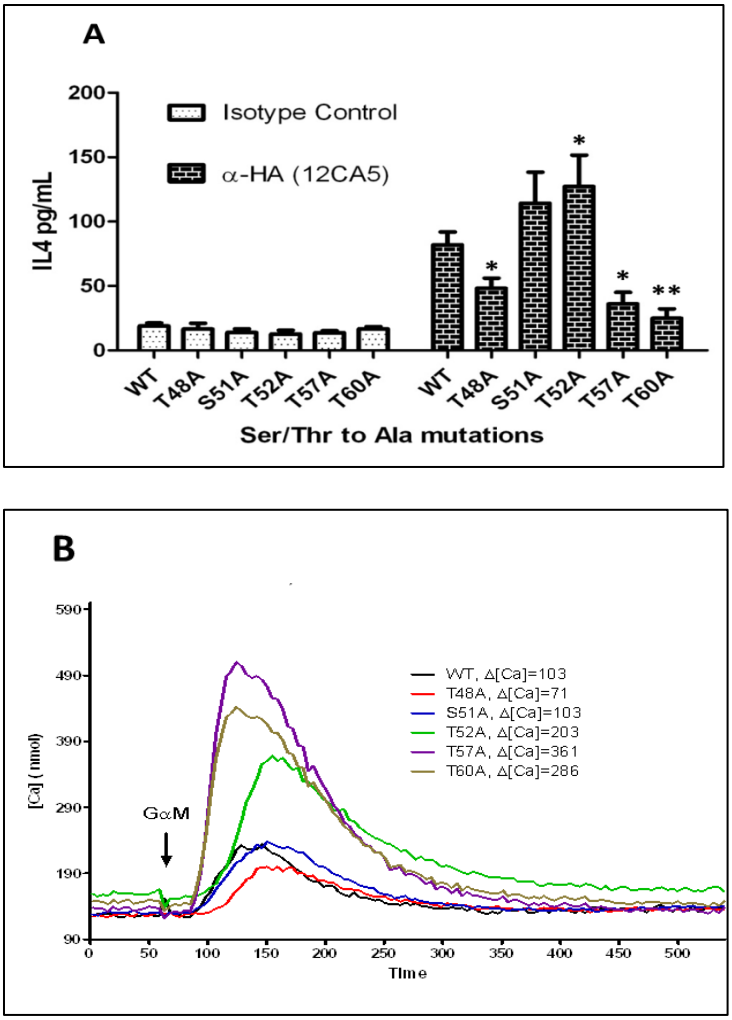


Figure 2. Serine/Threonine residues in the ITAM influence γ-chain functions. **A.** WT or mutant γ-chain expressing RBL-2H3 cells were opsonized with saturating concentrations of anti-HA-antibody (mAb 12CA5, 20 μg/mL) and stimulated with 20 μg/mL goat-anti-mouse F(ab)'2 for various times. **A.** Graphical representation of IL-4 produced by RBL cells after 15 hours of incubation **B.** Calcium flux levels induced by the stimulated cells as measured by the ratio Ca^{2+}-bound Indo-1 (405 nm) to unbound Indo-1 (490 nm).

The Serine/Threonine phosphatase inhibitor, Okadaic Acid (OA), alters γ-chain tyrosine phosphorylation

Our data from mutational analysis of the γ-chain ITAM serine/threonine residues suggested a role for these residues in Fc receptor mediated signaling. One possibility

is that phosphorylation of these residues by serine/threonine kinases is required for regulation of γ-chain phosphotyrosine-based function. Our previous observations using human FcγRI-expressing P388D1 cells, which had been pre-treated with okadaic acid (OA), a PP1/PP2A serine/threonine phosphatase inhibitor, showed that inhibition of serine dephosphorylation of α-chain CY resulted in decreased tyrosine phosphorylation of γ-chain, suggesting that phosphorylation of the α-chain CY negatively regulates γ-chain tyrosine phosphorylation. However, it is possible that phosphatases affect γ-chain independently of the effects due to the α-chain CY. To test whether the inhibitory effects of OA alter γ-chain signaling in the absence of α-chain, we pre-treated RBL-2H3 cells expressing the HA-tagged WT human γ-chain with OA. Cells were incubated with 1μM OA for 15 minutes followed by stimulation of cells with 12CA5 (anti-HA) as previously described. Following stimulation, cells were lysed and tyrosine-phosphorylated proteins were immunoprecipitated using anti-phospho-tyrosine Ab's (4G10/pY20) pre-adsorbed to protein G beads. Western blot analysis of immunoprecipitates using 4G10p showed that, compared to untreated cells in which γ-chain tyrosine phosphorylation peaked at 2.5 mins after stimulation (Figure 3, lane 4), OA treated cells showed a kinetic shift in γ-chain tyrosine phosphorylation such that tyrosine phosphorylation peaked at 1 min after stimulation (Figure 3, lane 10). In addition, subsequent stripping and immunoblotting with anti-HA (12CA5) showed more intense multiple γ-chain bands in the OA-treated cells (Figure 3, lower panel, lanes 8 to 13), suggesting that more tyrosine-phosphorylated γ-chain species were immune-precipitated from the treated cells. These data suggest that serine/threonine phosphatases indeed play a role in γ-

chain tyrosine phosphorylation independent of their effects on the α-chain CY. Interestingly, unlike the negative role of serine phosphorylation of the α-chain CY, these data suggest that serine/threonine phosphorylation positively regulates γ-chain tyrosine phosphorylation.

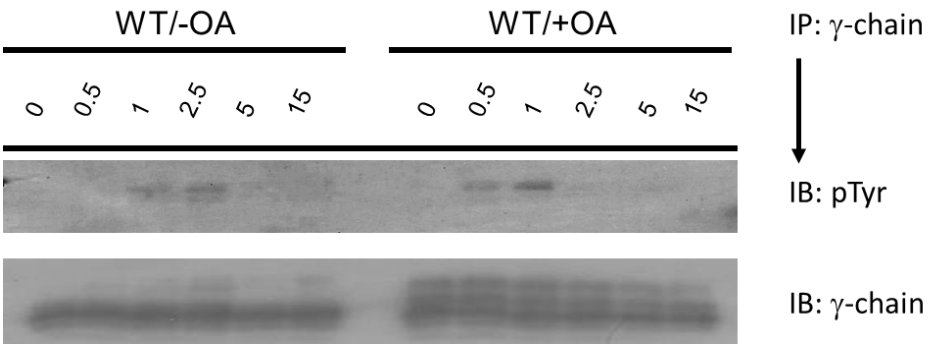
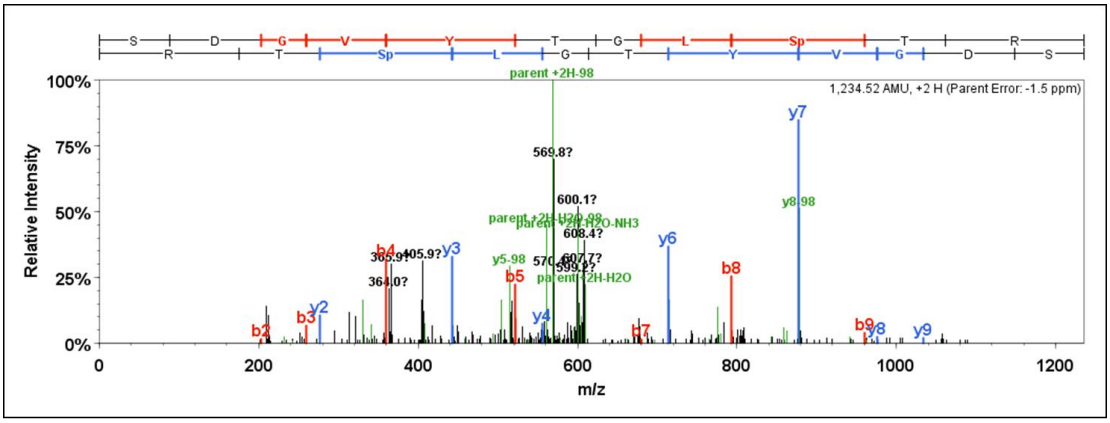


Figure 3. OA treatment of RBL-2H3 cells alters the kinetics and amount of γ-chain tyrosine phosphorylation. Cells expressing WT γ-chain were stimulated for the indicated times with 12CA5 and goat anti-mouse IgG with or without OA pretreatment. Western blot analysis was performed on phospho-tyrosine (4G10p) immunoprecipitates from each time point. Blots were probed with 4G10p, then stripped and re-probed with anti-HA antibodies (for total γ-chain).

Serine51 is predominantly phosphorylated in receptor-stimulated cells

To identify the site(s) of phosphorylation in human γ-chain ITAM, γ-chain was immunoprecipitated from resting and stimulated RBL cells, purified on SDS-PAGE, and the bands analyzed by Mass Spectrometry. Data show that, besides phosphorylation of the two ITAM tyrosine, S^{51} was predominantly phosphorylated in stimulated samples (Figure 4) suggesting that this site might play a prominent role in γ-chain function. In addition, phosphorylation at other sites (T^{48}, T^{52}) was observed at lower levels in γ-chain from stimulated cells while phosphorylation at T^{57} was observed in both unstimulated and stimulated samples (data not shown).



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	88.0			70.0	S	1,235.5	618.3	1,218.5	1,217.5	11
2	203.1			185.1	D	1,148.5	574.8	1,131.5	1,130.5	10
3	260.1			242.1	G	1,033.5	517.2	1,016.4	1,015.5	9
4	359.2			341.1	V	976.4	488.7	959.4	958.4	8
5	522.2			504.2	Y	877.4	439.2	860.4	859.4	7
6	623.3	312.1		605.3	T	714.3	357.7	697.3	696.3	6
7	680.3	340.6		662.3	G	613.3		596.2	595.3	5
8	793.4	397.2		775.4	L	556.2		539.2	538.2	4
9	960.4	480.7		942.4	S+80	443.2		426.1	425.2	3
10	1,061.4	531.2		1,043.4	T	276.2		259.1	258.2	2
11	1,235.5	618.3	1,218.5	1,217.5	R	175.1		158.1		1

Figure 4. Serine 51 in human γ-chain ITAM is phosphorylated in stimulated cells. WT γ-chain was immunoprecipitated from lysates of unstimulated cells or cells stimulated with 12CA5 at 20μg/mL, then purified and analyzed for phospho-site modifications in Velos Orbitrap tandem MS/MS. Ions with phosphate group modification on Serine 51 only are shown.

Equivalent expression of S51A and S51D mutants in RBL-2H3

In order to understand the role of Serine 51 in γ-chain mediated function, we created Serine 51 to aspartic acid (S51D) mutants to mimic phospho-serine, and Serine 51 to alanine (S51A) γ-chain mutants and transfected these into RBL-2H3. Transfectants were stained using 12CA5 and sorted for those expressing equivalently high levels of γ-chain (Figure 5). Equivalently expressing transfectants were then used in the following experiments to determine the ability of Serine 51 mutations to signal for various γ-chain mediated cellular functions, including IL-4 cytokine production and release of intracellular calcium. Transfected cells were also used to

probe the molecular mechanism underlying differences in the functional capacities of the γ-chain mutants.

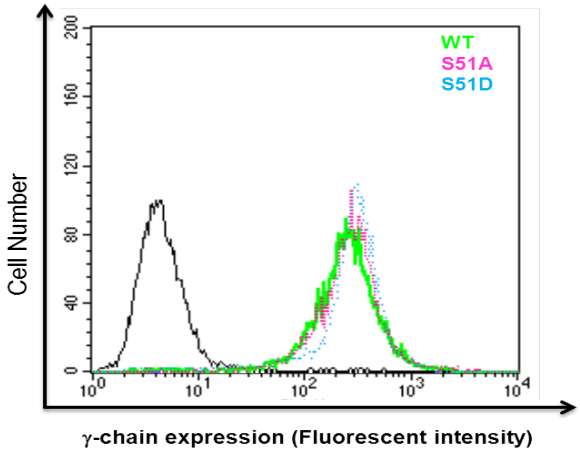


Figure 5. RBL cells expressing equivalent levels of WT, S51A and S51D mutants. Human HA-tagged WT or mutant γ-chain with Serine 51 to Alanine (S51A) or Serine 51 to Aspartic Acid (S51D) were expressed in γ-chain null RBL-2H3 cells and transfectants were sorted for the top 10 % expressers matched for expression levels. Cells were stained with anti-HA-antibody (mAb 12CA5, 20 μg/mL), washed and further stained for FITC-conjugated goat-anti-mouse (20 μg/mL).

Phosphorylation of Serine 51 inhibits γ-chain mediated functions

In order to elucidate the role of serine phosphorylation in γ-chain mediated signaling, we analyzed IL-4 production and intracellular calcium release by RBL-2H3 transfectants expressing equal levels of γ-chain WT, S51A, or the S51D mutation that mimics a constitutive serine phosphorylation state in the ITAM. Results show that S51A cells produced significantly more IL-4 cytokine than WT γ-chain and S51D expressing cells, suggesting that S^{51} plays a negative regulatory role in IL-4 production (Figure 6A). There was no significant difference in IL-4 levels between WT γ-chain and the S51D mutant. Interestingly, in intracellular calcium release

assays the S51D phospho-serine mimic mediated reduced levels of calcium release compared to WT γ-chain (Figure 6B), consistent with a negative regulatory role for phosphorylation of S^{51}. There does not seem to be a significant difference in calcium flux between stimulated cells expressing γ-chain WT and the S51A allele.

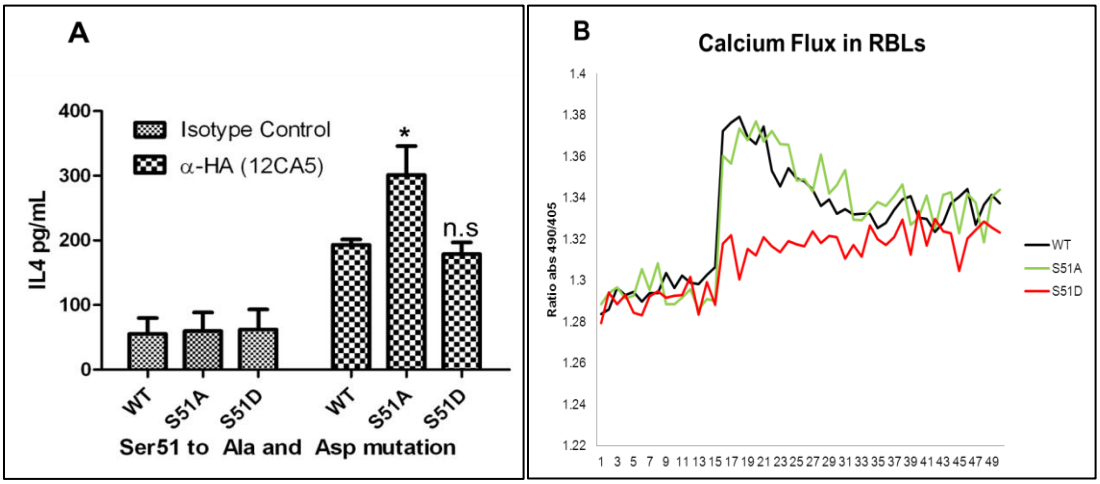


Figure 6. Serine 51 plays an inhibitory role in γ-chain mediated signaling. **A.** Cells expressing HA-tagged human γ-chain WT, S51A and S51D were stimulated using 12CA5 (20 μg/mL) for 30 mins on ice, followed by goat-anti-mouse F(ab')2 for 30 minutes at 37^0C. Supernatants were collected after 15 hours and analyzed by ELISA for IL-4 levels. **B.** Intracellular calcium flux was analyzed in cells expressing γ-chain WT, S51A and S51D after loading with Indo-1dye followed by stimulation with 12CA5 and GAM as decribed in materials and methods.

Serine 51 modulates tyrosine phosphorylation of the ITAM and subsequent Syk recruitment:

The mechanism by which γ-chain mediates its functions is dependent on tyrosine phosphorylation of the ITAM, and subsequent Syk recruitment and activation are essential first steps. To elucidate the mechanism by which Serine 51 exerts its effect on the ITAM signaling pathways, we analyzed γ-chain ITAM tyrosine

phosphorylation in stimulated cells expressing γ-chain WT, S51A and S51D mutations. Immunoblot analysis showed that upon stimulation, S51A resulted in a modest increase in tyrosine phosphorylation (Figure 7, lower panels, lanes 2-3 vs lanes 6-7) while S51D dramatically reduced the tyrosine phosphorylation (Figure 7, lower panels, lanes 2-3 vs lanes 10-11). Additionally, there was a corresponding increase in Syk co-immunoprecipitation and activation (phospho-Syk) in S51A (Figure 7, upper panels, lanes 1-4 vs 5-8) and corresponding reductions in S51D cells (Figure 7, upper panels, lanes 1-4 vs 9-12).

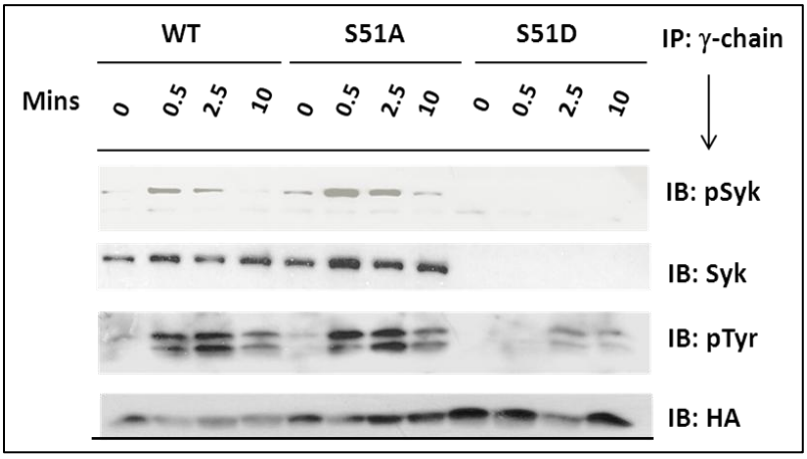


Figure 7. Phosphorylation of Serine 51 results in reduced γ-chain tyrosine phosphorylation and Syk activation. Cells expressing γ-chain WT, S51A and S51D were stimulated for the indicated times with 12CA5 and goat anti-mouse IgG, and western blot analysis performed on phospho-tyrosine (4G10p) immunoprecipitates from each time point. Blots were first probed with 4G10p, then sequentially stripped and reprobed with anti-phospho-Syk, anti-Syk and anti-HA antibodies.

Serine 51 is phosphorylated in vitro by PKA and PKC

To determine which kinase phosphorylates Serine 51 we initially performed an *in silico* search for putative kinase motifs in the amino acid sequence of the γ-chain ITAM. Motif search results suggested that S^{51} could be targeted by PKA and PKC,

and that several other serines and threoinines within the region of the γ-chain ITAM could be targeted by various other protein kinases (Figure 8).

RKAAIT[38]S[39]YEKS[43]DGVYT[48]GLS[51]T[52]RNQET[57]YET[60]LKHEKPPQ

Kinase and Predicted site \ Website	Phospho Motif finders	Kinase Phos	Phosida	Prosite	NetPhos K	Minimotif miners
T38 - CK2	Y	Y	Y	Y	Y	Y
S39 – GSK3β	Y		Y			Y
S43 – CK1			Y			
T48 - CDK		Y				
S51 – PKC/PKA	Y	Y		Y	Y	Y
T52 - PKC					Y	
T57 - CK1	Y					
T60 - PKC		Y				Y

Figure 8. FcR γ-chain predicted kinase target sites identified in motif searches. Websites used are listed in top row and predicted kinases with numbered amino acid target sites in the γ-chain sequence are shown in the first column. A partial sequence of the γ-chain with reference positions is shown above the figure. Y = kinase target site was identified using specific website software listed above the Y.

In order to test the whether PKA and/or PKC isoforms could phosphorylate Serine 51, we created GST-γ-chain fusion constructs where all serine and threonine sites within the ITAM, except S^{51}, had been mutated to alanine (see Figure 4). To avoid the possibility of confounding phosphorylations occurring on S/T residues outside the ITAM, we also mutated S^{38}, T^{39}, and S^{43} immediately upstream of the ITAM to alanine residues. In addition, previous observations indicated that, of the 15 known PKC isoforms, only PKCα, PKCβ, PKCδ, PKCε, and PKCζ are expressed by RBL cells [103], and of these, all but PKCζ are recruited to the membrane upon FcεRI activation (and therefore, would be correctly localized to possibly phosphorylate γ-chain). Accordingly, we limited our analysis to PKCα, PKCβ, PKCδ, PKCε, in addition to PKA. Using recombinant PKC enzymes and GST-γ-chain

fusion proteins purified from bacteria, we tested the ability of PKA and the PKC isoforms to phosphorylate γ-chain on S^{51} in *in vitro* kinase assays as described in materials and methods. Results showed that PKA robustly phosphorylated γ-chain S^{51} (Figure 9A). In addition, PKCβ and PKCδ also phosphorylated S^{51} (Figure 9B, panels 2-3), while PKCα was inefficient at phosphorylating S^{51} and PKCε failed to phosphorylate S^{51} (Figure 9B, panels 1 and 4).

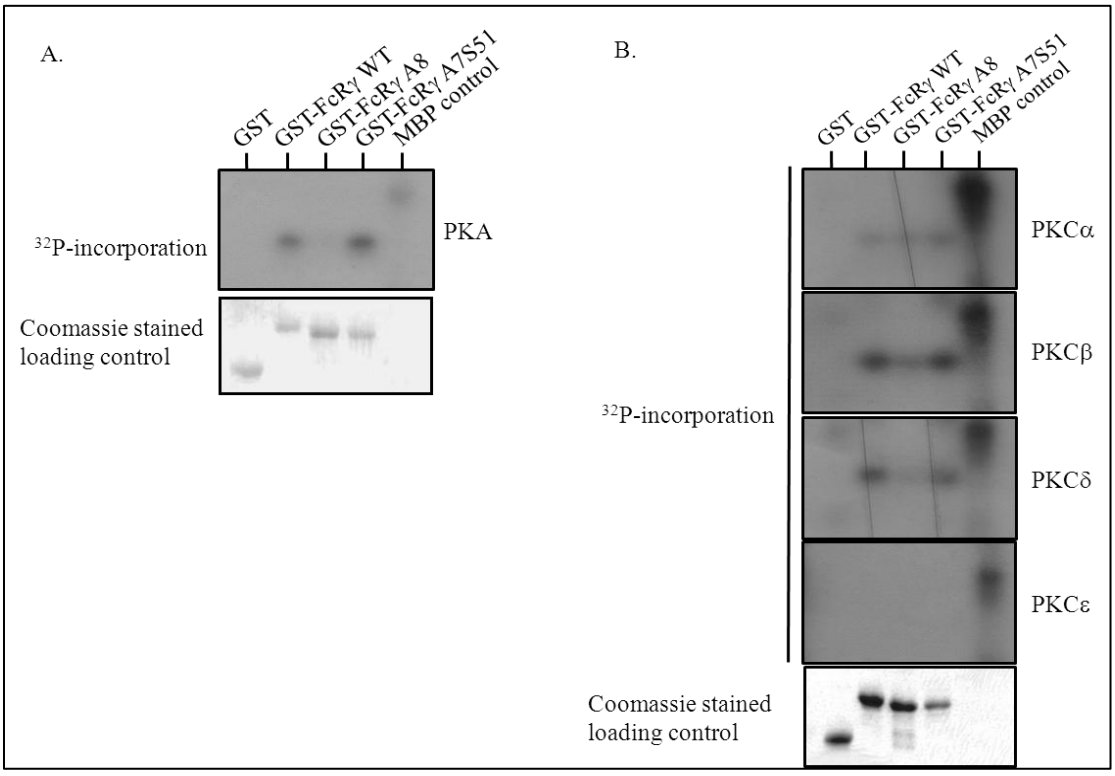


Figure 9. Phosphorylation of human γ-chain by PKA, PKCβ, and PKCδ. **A.** FcRγ-chain S^{51} phosphorylation by PKA (autoradiograph is from 2 hrs exposure to film). **B.** FcRγ-chain phosphorylation by PKCα, PKCβ, PKCδ, and PKC ε (autoradiographs are from 24 hrs exposure to film). FcRγWT = FcRγ-chain wild type; FcRγA8 = FcRγ-chain with all S/T residues in ITAM region changed to alanines; FcRγA7S51 = FcRγ-chain with all S/T residues except S^{51} changed to alanines.

DISCUSSION

In this study we showed that the serine and threonine residues in the human γ-chain ITAM affect ITAM-mediated function of the γ-chain. We further showed that Serine 51 in γ-chain ITAM is phosphorylated, that phosphorylation occurs, at least in vitro, by PKA or by PKC, and that phosphorylated S^{51} has a negative regulatory effect on γ-chain signaling. Our observations also indicate that the mechanism(s) by which S^{51} exerts its negative effects include reduced tyrosine phosphorylation of γ-chain, and reduced recruitment and activation of Syk kinase leading to overall reduced signal transduction.

It is well established that, despite being expressed on the same cell and signaling through the common γ-chain, the γ-chain-associated receptors, which include FcγRI, FcγRIIIa, FcαRI, and FcεRI, elicit distinct cellular programs [84, 85, 117]. We and others have documented that the unique α-chain CY domains of these receptor complexes modulate tyrosine-based γ-chain function, but the mechanisms by which this modulatory effect is exerted is not entirely clear [84, 85, 117]. However, we and others have shown that the CY domains of the α-chains are capable of recruiting intracellular proteins which may facilitate signaling. For example, we have shown that FcγRI in tethered to lipid microdomains by interaction with protein 4.1G in an activation-dependent fashion, and that FcγRIIIa reruits S100A4 in a calcium-dependent fashion to modulate receptor function [117]. However, the mechanism by which these molecules affect signaling is unclear, and whether they affect γ-chain modification is unexplored. In other work, it has been documented that the β-subunit of FcεRI recruits PKCδ, and that PKCδ phosphorylated γ-chain on threonine 60,

which is critical for Syk recruitment to the ITAM and downstream TNF-α production, and our mutational studies confirm a requirement for T60 in production of IL-4 by human γ-chain. While the method by which γ-chain modifying enzymes are recruited or regulated is unclear, published reports indicate that human γ-chain is phosphorylated on serine and threonine residues [96], and it is clear from our mass spectrometry data that γ-chain ITAM serine/threonine residues are phosphorylated. It is also clear that, at least *in vitro*, several PKC isoforms and PKA can phosphorylate S^{51}. In addition, based on our mutational studies S^{51} exerts a negative effect on signaling (reduced IL-4 and Ca^{2+} release by the S51D phospho-serine mimic), and other serine/threonine residues are also important in γ-chain signaling (Figure 5A and 5B). A negative role for phospho-S^{51} is consistent with published observations that ITAM phospho-serines inhibit tyrosine phosphorylation of the Igα subunit ITAM of the BCR [137, 138] as well as other inhibitory functions mediated by phospho-serines in other proteins [141, 142].

Evidence of the mechanism underlying the negative influence of S^{51} is seen in the S51D mutant which results in reduced tyrosine phosphorylation of the γ-chain ITAM, less Syk recruitment and activation, and less overall signaling. That S51D results in lower levels and S51A in higher levels of ITAM tyrosine phosphorylation suggests that the negative charge of phosphorylated S^{51} and that of S51D inhibit targeting of the ITAM tyrosines by Src kinases. However, these data are also consistent with increased recruitment of phosphatase(s) to the ITAM. Therefore, S51A and dephosphorylation of S^{51} would facilitate ITAM targeting by Src's and allow efficient downstream signaling. This suggests a mechanism of action different

from that of the T^{60} mutation previously described [87]. Swann et. al., had shown reduced Syk recruitment by the γ-chain T60A mutant, however there was no observed effect on ITAM tyrosine phosphorylation [87]. Therefore, while the T60A mutation appears not to affect Src kinase phosphorylation of the ITAM tyrosines, it inhibited subsequent Syk recruitment, suggesting requirement for a negative charge at ITAM position 60 (Y*ET^{60}L) to facilitate Syk-SH2 binding to the phospho-tyrosine* residue. It is therefore interesting to speculate that the stimulation-dependent role of the serine/threonine residues in the ITAM is to block or facilitate initial Src kinase phosphorylation of the ITAM tyrosines, and/or to block or facilitate subsequent recruitment of Syk and additional signaling molecules to the ITAM phospho-tyrosines thereby fine tuning the γ-chain signaling response. Therefore, recruitment of various proteins to γ-chain, including Src kinases, Syk kinase, PI-3 Kinase, and Vav could be the target of modulation by serine/threonine residues in the ITAM. It is also interesting to speculate that different γ-chain-associated α-chain CY's might modulate γ-chain function by differentially recruiting specific modifying enzymes that target specific ITAM serine/threonine residues. This in turn would alter the complement of downstream signaling molecules that are recruited by γ-chain and result in receptor-specific cellular outcomes (for example, stimulation of FcγRIIIa would result in unique γ-chain modifications and recruitment, resulting in efficient signaling for degranulation, as in Figure 9B).

The precise mechanism of α-chain CY modulation of γ-chain signaling is unclear. Nevertheless, our data show that serine/threonine residues within the human γ-chain ITAM influence γ-chain function and that at least one mechanism by

which this is accomplished is by altering the ability of ITAM tyrosines to be phosphorylated and to recruit the critical signaling intermediate, Syk kinase. Therefore, our studies further advance the understanding of γ-chain signaling and provide a framework to better understand the fine tuning of γ-chain signaling pathways, which may lead to the identification of potential therapeutic targets of importance in modifying FcR-mediated diseases.

GENERAL DISCUSSION

Fc Receptor targeted therapies

Due to their ability to bind to antibodies, Fc receptors provide an interface between components of humoral immunity and cellular immunity (effector cells). Fc receptors are involved in various cellular responses such as degranulation, antigen presentation, ADCC (antibody-dependent cellular cytotoxicity), phagocytosis, intracellular calcium flux, endocytosis and secretion of reaction oxygen species. Through these responses, Fc receptors directly or indirectly play a role in clearance of immune complexes such as opsonized allergens, infectious agents and even elimination of tumor cells (reviewed in [143]). Since activating Fc receptors play such a prominent role in autoimmune host tissue destruction and pathophysiological processes, they have been utilized as targets for pharmacological intervention. Therefore, in terms of therapy, Fc receptors have been targeted for 3 different purposes:

1. In an autoimmune disease, Fc receptors can directly be targeted to block the hyper-activation of immune system pathways involving pro-inflammatory signals.
2. In diseases involving unwanted cells and/or pathogens, Fc receptor effector cells can be directed to eliminate virus-infected or tumor cells via ADCC.
3. Effector cell responses can be fine-tuned by targeting parts of activating or inhibitory Fc receptors and associated signaling proteins.

Several therapeutic strategies have been employed to block Fc receptor mediated functions. In a study of patients with rheumatoid arthritis, it was shown that

stimulation of blood monocytes with solid-phase immune complexes induced secretion of IL-1β, TNF-α and IL-8. Secretion of these cytokines was inhibited upon FcγRIIA blockade [144]. An effort to employ soluble Fc receptors as decoy competitors has shown promising results at least in animal studies. The goal of this approach is to target immune complexes rather than Fc receptors in an attempt to blockade binding of ICs to Fc receptors thus preventing phagocytosis and receptor mediated signaling in effector cells [145-148].

Though the importance of Fc receptors in autoimmune diseases has been known for decades, the development of anti-Fc receptor antibodies as therapeutic targets has been slow. Kimberly et al. [149] had shown the positive effect of using an anti-FcR antibody as a therapeutic agent in chimpanzees more than 20 years ago . In humans, anti-FcR antibody was used to analyze the effect on refractory immune thrombocytopenic purpura (ITP) [150]. These studies utilized mouse mAb 3G8 that targets FcγRIII. Interestingly, a study of the molecular mechanism of 3G8 showed that while it depleted antibody coated cells quite efficiently, the same was not true of soluble immune complexes [151]. Antibody against high-affinity FcγRI, mAb 197, has also been tested in a clinical setting for treatment of immune thrombocytopenic purpura (ITP). The results showed that while the antibody had limited therapeutic benefits, it did influence the FcγRI expression on circulating monocytes [152]. Another antibody against FcγRI, H22, had a similar effect on level of FcγRI expression and phagocytosis in vivo [153], but to our knowledge, this antibody has not been tested against ITP.

The use of competing peptides that bind to Fc receptors or the Fc portion of antibodies is relatively well studied in the possible treatment of allergies [154-156]. The challenge in designing such peptides is the large surface of interactions [157-159]. Several studies (reviewed in [160]) have looked at short peptides targeted to different domains of IgG [161, 162] or different Fc receptors such as FcγRI [163, 164] and FcγRIIA [164, 165].

Kinase inhibitors and autoimmune diseases

Divided into 8 major groups, there are 518 kinases encoded in the human genome. Kinases are essential to every aspect of cellular biology and as mentioned previously, one of the critical steps in activation of receptors such as T cell receptor, B cell receptor, Fc receptors and several cytokine receptors is the tyrosine phosphorylation of the receptor themselves and/or their associated adaptor molecules. It is therefore reasonable to speculate that blocking specific kinase activity might block the receptor activation and downstream signaling pathways. This in turn might help reduce inflammatory activity in autoimmune diseases. Thus, tyrosine kinases as well as serine threonine kinases provide a huge avenue for potential therapeutic targets. In support of this, several kinase inhibitors are in clinical trials to treat various autoimmune diseases such as arthritis, inflammatory bowel disease and psoriasis.

One of the first kinases to be targeted was BCR-Abl, a fusion protein resulting from a chromosomal translocation (Philadelphia chromosome) in leukemia patients. The inhibitor of BCR-Abl, called Imatinib, was very successful in early trials of Chronic

Myeloid Leukemia treatment and had few side effects [166]. The effect of Imatinib in targeting other tyrosine kinases also led to the trials of this drug for other malignancies such as gastrointestinal tumors, and hyper-eosinophilic syndrome [167, 168]. Unfortunately, mutations in targeted kinases led to the development of drug resistance, which resulted in searches for new multi-kinase inhibitors. Table 2 lists several FDA-approved kinase inhibitors used in autoimmune diseases [169]:

Agent	Targets for therapeutic activity	Indication/Phase
Tofacitinib	JAK3/JAK1/JAK2	RA/Phase III Psoriasis/Phase II IBD/Phase II
VX-509	JAK3	RA/Phase II
R-348	JAK3	RA/Phase I
Ruxolitinib	JAK1/JAK2	Psoriasis/Phase II
INCB-028050	JAK1/JAK2	RA/Phase II
GLPG-0634	JAK1/JAK2/TYK2	RA/Phase II
AC-430	JAK2	RA/Phase I Lymphoma/Phase I
Lestaurtinib	FLT3/TrkA/JAK2	AML/Phase III Psoriasis/Phase II Pancreatic cancer/Phase II
Fostamatinib	Syk/FLT3/KIT/LCK	RA/Phase III NHL/Phase I/II CLL/Phase I/II
ACU-XSP-001	Syk	Asthma/Phase II
R-343	Syk	Asthma/Phase I
PRT-062607	Syk	RA/Phase I CLL/Phase I NHL/Phase I
Sotrastaurin (AEB071)	PKC	Psoriasis/Phase II IBD/Phase II Solid organ transplantation/Phase II Diffuse Large B-Cell Lymphoma/Phase I
PLX 5622	CSF1R (Fms)	RA/Phase I

Table 2: FDA approved kinases in clinical trial targeted for autoimmune disease therapies. Adapted from Kontzias A et al. (2012).

Contribution of this thesis work

Work in this thesis advances our knowledge about 2 of the multiple players in Fc Receptor pathways – the FcγRI α-chain cytoplasmic domain and serine/threonine residues in the γ-chain ITAM.

Chapter 1 includes data that further advances the idea that the cytoplasmic domain of FcγRI α-chain influences γ-chain mediated signaling in a new system (RBL-2H3). These data establish the idea that Fc receptor α-chain's influence on γ-chain mediated signaling is not cell-type specific (Figure 4, 5, and 6). We also show that different α-chain CY's indeed influence γ-chain mediated signaling differentially (Figure 8). We had previously shown that serine residues in the cytoplasmic domain influence γ-chain signaling [85], but further work needs to be done to define the exact mechanism by which the α-chain CY regulates γ-chain mediated signaling. Targeting specific residues of Fc receptors might provide a more specific alternative to targeting all Fc receptors indiscriminately. As we and others have shown, the Fc receptor α-chain CY's influence only some γ-chain signaling pathways. If each α-chain CY influences specific signaling pathways, or differentially modulate signaling pathways using distinct intracellular molecules, Fc receptor targeted therapies can be better defined based on specific receptor-activated distinct intracellular proteins, leading to development of more effective disease modifying drugs.

Chapter 2 includes data which show that Serine/Threonine residues in the human γ-chain ITAM clearly regulate cellular outcomes differentially. For example, Serine 51 negatively regulates IL-4 production, and our data in conjunction with that of Swann et al., [87] show that Threonine 60 is a positive regulator of γ-chain

mediated signaling. A more detailed study of the functional contribution of each of the residues will provide valuable insight in γ-chain signaling and may identify additional therapeutic targets. The fact that different FcR α-chain CY's differentially regulate γ-chain mediated function, and that γ-chain ITAM serine/threonines also differentially modulate function lead to the compelling hypothesis that the α-chain CY's modulate γ-chain signaling by altering serine/threonine phosphorylation in a manner dependent on the specific α-chain. However, additional studies will be required to test this hypothesis which is beyond the scope of this work.

PKC inhibitors are currently in clinical trials ([170, 171] and Table 2) for treatment of autoimmune diseases like inflammatory bowel disease, psoriasis and Diffuse Large B-Cell Lymphoma. Our data show that the single serine residue (S^{51}) within the human γ-chain can potentially be a target by PKA, several isoforms of PKC, and other as yet unidentified kinases, and one or more phosphatases (still to be identified) are clearly necessary to reverse the phosphorylation events. Perhaps phosphorylation of Serine 51 and other ITAM serine/threonines is involved in regulation of immune-mediated functions which could be modulated by serine/threonine kinase inhibitors or phosphatase inhibitors. While our data suggest redundant roles for PKC's in phosphorylating Serine 51 (Figure 8 A and B), perhaps different kinases might be recruited at different time points, or their activity might be differentially regulated in the presence of different α-chains.

It is interesting to note that Syk kinase, which was previously thought to phosphorylate only tyrosine residues in proteins was recently found to be a dual-specificity kinase with the ability to phosphorylate the serine residue in the Igα ITAM

[172]. The relative similarity of ITAM's across different signaling molecules and the fact that Syk kinase is recruited to the phosphorylated γ-chain ITAM leads to an enticing theory that Syk may also be involved in the phosphorylation of Serine 51. Further studies will be needed to examine whether Syk phosphorylates Serine 51 in response to FcγR stimulation. In clinical trials, the Syk inhibitor, R406, was shown to effectively reduce immune complex mediated inflammation in an arthritis model [173]. A prodrug of R406 – R788 used in a clinical trial for ITP achieved a clinical response in 75% of patients [174]. The effect of the drug seemed to be directed at Syk-dependent phagocytosis by Fc receptors; however, the exact mechanism is not known.

Although details of the mechanism remain unclear, our work has established an RBL-2H3 system for examining the role of ITAM serine/threonine phosphorylation in human γ-chain function and in Fc receptor complex signaling, and using this system we have shown that S^{51} in the ITAM is phosphorylated in stimulated cells, is a negative regulator of γ-chain signaling, and enzymes that modify S^{51} are potential therapeutic targets for modulating γ-chain function. Therefore, this work represents initial steps in a search for signaling molecules that might lead to new effective therapeutics in autoimmune diseases.

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