I, Upasana Parthasarathy, hereby submit this original work as part of the requirements for the degree of Master of Science in Immunology.

It is entitled:
Identifying epitopes of anti-FcaRI monoclonal antibodies on FcaRI ectodomain that trigger the anti-inflammatory ITAMi signaling pathway

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Identifying epitopes of anti-FcαRI monoclonal antibodies on FcαRI ectodomain that trigger the anti-inflammatory ITAMi signaling pathway

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ABSTRACT

Autoimmune disorders are the second leading type of chronic illness in the US, affecting about 50 million individuals. In 2001, the National Institute of Allergy and Infectious Diseases (NIAID) estimated that the annual autoimmune healthcare cost is greater than $100 billion, although this value may be significantly underestimated. Autoimmune diseases such as hypersensitivity reactions are a result of impairment of this FcR regulatory system. Here, we focus on the interaction between Immunoglobulin A (IgA), the most predominant antibody in mucosal sites, and its principal myeloid receptor, FcαRI (CD89). Cross-linking of multiple FcαRI molecules at the cell surface by immune complexed IgA, initiates pro-inflammatory immune responses such as phagocytosis, antigen presentation and antibody-dependent cellular cytotoxicity. These findings led to perceiving FcαRI as a solely activating receptor. In 2005, Pasquier et al. identified that FcαRI, when interacting with monomeric serum IgA, can also drive powerful anti-inflammatory responses through the ITAMs in the FcR γ-chain and thus behave as an inhibitory receptor that controls inflammation. This revealed the dual nature of the ITAM motif, which otherwise is typically considered to be involved in immune cell activation. This inhibitory signaling pathway mediated by FcαRI in association with FcRγ ITAMs was termed the Inhibitory ITAM or ITAMI signaling pathway. Several research groups have shown that some but not all anti-FcαRI monoclonal antibody Fab fragments are capable of triggering the ITAMI pathway via FcαRI. The anti-FcαRI monoclonal antibodies most widely used to study the ITAMI pathway include MIP8a, A3, A59 etc. and these antibodies recognize different extracellular domains in the FcαRI ectodomain.
Hence FcαRI can be considered as a 3 state system: a resting state in which it does not mediate signaling, an activating state in which it triggers pro-inflammatory responses via recruitment of a Syk kinase, and an inhibiting state in which it triggers anti-inflammatory responses via recruitment of SHP-1 phosphatase. Based on this, we hypothesize that anti-FcαRI monoclonal antibodies which monovalently target FcαRI and trigger the ITAMi pathway have their antigenic epitopes clustered in certain regions of the ectodomain, forming hotspots. Identifying key amino acid residues or sequences of residues in these hotspots will enable recognition of optimal regions in FcαRI ectodomain that can be targeted to trigger the ITAMi pathway.

We aim to lay the groundwork to define the characteristics of ligands capable of triggering ITAMi signaling via FcαRI and perform experiments to determine how different FcαRI binding ligands are able to trigger two contrasting pathways solely through the ITAM of the accessory FcRγ receptor. Therefore, the main objective of this thesis is to identify the antigenic epitope of anti-FcαRI monoclonal antibody A59 on FcαRI ectodomain using site-directed mutagenesis and enzyme linked immunosorbent assays.

Understanding the mechanistic basis for ITAMi signaling is a necessary step towards effectively triggering ITAMi responses in immune cells by targeting FcαRI. We believe that this should aid in better defining the characteristics of ligands that are capable of triggering the inhibitory function of FcαRI and characterizing the clustering mode of FcαRI in the three functional states.
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CHAPTER ONE: Introduction

1.1. Human Immunoglobulin A

*Immunoglobulin A (IgA)* is the most abundantly produced immunoglobulin in secretions[1]. It is the most predominant antibody at mucosal surfaces such as respiratory, gastrointestinal, urogenital tracts and forms the first line of host defense against infection at these surfaces. IgA has two heavy and two light chains. The heavy chains have three constant domains each (C_H1, C_H2, C_H3). Two of these constant domains, C_H2 and C_H3 together make up the crystallizable fragment (*Fc*) of the antibody. This Fc region of IgA is known as *Fc-alpha (Fcα).* *Secretory IgA (SIgA)* and *Serum IgA* are the two types of IgA molecules which differ in biochemical, immunological properties and the location of the cells that produce them. Human IgA also occurs in two isotypic forms – IgA1 and IgA2 [1].

*Figure 1 Monomeric IgA1, Adapted from Michael A.Kerr, Biochem. J., Vol. 271, 287, 1999*
1.1.1. Secretory IgA

*Secretory IgA (SIgA)* is produced by plasma cells locally below the mucosal epithelium. It is predominantly dimeric, comprising of two monomeric IgA antibodies linked together by a J (joining) chain[2]. The structure of SIgA has a secretory component interacting with the $C_{H2}$ and $C_{H3}$ domains of both pairs of the heavy chain. SIgA plays a key role in inhibiting bacterial
attachment to mucosal tissues, neutralizing viruses entering the mucosal tissue and neutralizing viruses intracellularly, when transported to mucosal surfaces. SIgA antibodies are mainly responsible for driving immune responses at mucosal surfaces by immune exclusion. It is an FcαRI-independent process that involves binding of SIgA to pathogens and thus hindering their adherence to mucosal epithelia stERICALLY[3].

1.2. Serum IgA

Serum IgA is produced by plasma cells from the bone marrow, lymph node and spleen. It is the second most abundantly produced antibody isotype (serum concentration of 3.5mg/ml) and is considered to be predominantly monomeric IgA₁ (~80%) including ~10% of dimeric IgA and ~10% polymeric IgA [4]. Findings from the past and in the present indicate that serum IgA performs pro-inflammatory as well as anti-inflammatory functions. It is found to trigger pro-inflammatory responses via the up-regulation of cytokine Interleukin-1β (IL-1β) release and down-regulation of anti-inflammatory cytokines IL-10 and IL-12p40 release by Lipopolysaccharide (LPS) activated monocytes[4].

Serum IgA’s potential to trigger anti-inflammatory responses is becoming increasingly significant, and is of major interest in this thesis. Human monomeric serum IgA has been found to play a central role in the regulation of inflammatory responses independent of its antigen binding activity. Release of large amounts of cytokines is considered to be an important pathophysiological effect in most inflammatory diseases. For instance, Tumor necrosis factor-α (TNF-α) and IL-6 are known to be involved in the pathogenesis of inflammatory bowel disease, rheumatoid arthritis etc. Investigations have revealed that serum IgA can inhibit the release of TNF-α and IL-6 from
monocytes activated by *Haemophilus influenza type b* or LPS from *Escherichia Coli (E.Coli.)* [5]. In addition, serum IgA contributes to the regulation of inflammatory response by inducing significant increase of *IL-1 receptor antagonist (IL-1Ra)* in resting and activating monocytes[6].

Serum IgA levels are raised in rheumatic diseases, autoimmune diseases, liver diseases and bacterial infections[1]. These elevated serum IgA levels can be of two forms- either polymeric IgA being a part of IgA immune complexes or monomeric serum IgA. It is this feature that possibly marks the difference between the pro-inflammatory and anti-inflammatory functions. IgA deficiency in humans is often not found to be associated with impaired resistance to infection, but with an increased incidence of autoimmune disease, suggesting that there might be a passive role of serum IgA in regulating inflammatory responses[7]. This is also supported by the fact that probably the low natural frequency of antigen specific serum IgA supports its anti-inflammatory effects and triggering inhibitory functions of its principal receptor.

Systemic inflammatory diseases or certain autoimmune disease triggering factors may sometimes result in uncontrollable, exaggerated and self-amplifying release of inflammatory cytokines manifesting in intravascular thrombosis and lethal septic shock. Thus, it is in such situations that the anti-inflammatory role of serum IgA gains significance and comes into play. It has been established that monomeric serum IgA binds transiently and with lower affinity to its principal receptor. On the other hand, polymeric serum IgA bound to a multivalent antigen binds with very high affinity to the same receptor. Understanding how and under what conditions serum IgA performs these two functions is of utmost importance.
1.3. Human myeloid IgA receptor (FcαRI/CD89)

FcαRI is the principal human myeloid receptor specific for the Fc region of antibody IgA, expressed on myeloid line of cells such as neutrophils, eosinophils, macrophages and dendritic cells[8]. The cluster of differentiation number assigned for FcαRI is CD89. Sequence investigations have revealed that FcαRI belongs to the Ig superfamily, and is closely related to other receptors in the leukocyte receptor cluster including killer inhibitory receptors (KIRs) and LIR family of receptors.

The crystal structure of FcαRI was solved by Herr et al. in 2003[9]. FcαRI is a transmembrane glycoprotein that binds IgA in most its molecular forms (serum IgA and monomeric/dimeric IgA). The FcαRI gene encodes for a hydrophobic leader sequence, two extracellular Ig-like domains oriented approximately at 90°, together known as the ectodomain (a membrane proximal D2 domain and a membrane distal domain D1), a hydrophobic transmembrane segment, and a short cytoplasmic tail[3]. The cytoplasmic tail is devoid of any signaling motifs. It has six N-glycosylation sites- N44, N58, N129, N156, N165 and N177. Due to changes in glycosylation patterns, the receptor size ranges from 55-70kD in monocytes and 70-100kD in neutrophils[10].
The transmembrane domain of FcαRI contains a positively charged arginine residue essential for its association with an aspartate residue in the transmembrane region of the FcR γ-chain/FcRγ, a co-receptor via which FcαRI mediates signaling[3]. FcR γ-chain interacts with a family of receptors belonging to the immunoglobulin superfamily that differ in their binding affinities and signaling pathways they trigger. An activating FcR γ-chain contains an Immunoreceptor Tyrosine-based Activation Motif (ITAM) in its cytoplasmic domain. ITAMs are two short stretches of conserved sequences of amino acids (YxxL-YxxL) via which the FcR γ-chain mediated pro-inflammatory responses such as endocytosis, phagocytosis, cytokine release, ADCC and antigen presentation.

However the binding interaction of FcαRI with FcR γ-chain is different from that of the other receptors. For instance, IgG and IgE Fc receptors lack a positively charged amino acid in their transmembrane domains, which probably results in weaker association with the FcR γ-chain, when compared to the interaction between FcαRI and FcR γ-chain[10].
FcαRI triggers a proinflammatory response when it interacts with multiple IgA molecules bound to a multivalent antigen, i.e. immune complexed IgA. This interaction leads to clustering of several FcαRI molecules at the cell surface. Thus intracellular signaling is initiated by FcαRI via the associated co-receptor FcR γ-chain. Cross linking of the FcαRI-FcR γ-chain complex by a multivalent antigen leads to phosphorylation of the tyrosine residues within the ITAM motifs by protein tyrosine kinases such as Lyn from the Src family of kinases, thus initiating the signaling cascade. The phosphorylated tyrosine residues recruit protein tyrosine kinases such as Syk, resulting in recruitment, phosphorylation and activation of several other downstream proteins such as phosphoinositide-3-kinase (PI3K). This results in the release of inositol triphosphate (IP3) and diacylglycerol (DAG) which trigger calcium release[8]. This signaling pathway triggered by FcαRI-FcR γ-chainITAM complex mediates different intracellular processes such as gene expression by activation of transcription factors, and other specific signaling and functional responses.

1.4. Interaction of IgA with FcαRI

In 1999, Wines et al. identified residues in the first domain of human FcαRI that were involved in the interaction with IgA. The IgA binding site on FcαRI was identified using techniques of chemical modification and site-directed mutagenesis. Chemical modification of recombinant forms of FcαRI with diethylpyrocarbonate (DEPC) suggested a role for histidine residues in the interaction of FcαRI with IgA. In FcRs like FcγRII, FcγRIII, and FcεRI, the FG loop in domain 2 comprises the ligand binding site, and has been established previously as a conserved structural element participating in ligand interaction in most KIR related receptors. In addition, biosensor binding assays of IgA with FcαRI suggested optimal binding to occur at a pH of ~7.5 and loss of binding at higher pH. Since the imidazole side chain of histidine has a pKa of 7, pH profiles
suggested that histidine possibly plays a role in this interaction. Site-directed mutagenesis experiments carried out on this basis led to the identification of Histidine-85 (H85) in the membrane distal domain D1 of FcαRI. Following this, chemical modification of FcαRI with arginyl-specific phenylglyoxal and site-directed mutagenesis led to the identification residues 82-85, RIGH, as key players in this interaction[11].

In 2001, the same group came up with more detailed results, where in they identified additional residues present in the IgA binding site: Tyrosine-35 (Y35), Y81 and Y86 using site-directed mutagenesis and subsequent binding assays to validate their results. Therefore, the IgA binding site on FcαRI was identified as a single face near the N-terminus of the receptor, in the membrane distal D1 domain, distinct from other leucocyte Fc receptors in which the ligand binding site is present in the membrane proximal D2 domain[12].

Figure 5 Crystal Structures of FcαRI with significant residues in the IgA binding sites, Adapted from B Wines et. al., J Immunol, Vol. 162, 2146-2153, 1999
Fcα is similar to the Fc regions of IgG and IgE except that it has differently located interchain disulfide bonds and external rather than internal N-linked carbohydrates. Unlike the 1:1 FcγRIII: IgG and FcεRI: IgE complexes formed, two FcαRI molecules bind to a single IgA molecule, each at one CH2-CH3 domain interface in Fcα dimer (2:1 stoichiometry of binding). Also, FcαRI binds in an upright orientation, with its C-termini near the membrane, whereas FcγRIII and FcεRI bind to their ligands in opposite orientations. This upside-down orientation of IgG and IgE when bound to their Fc receptors is probably because of the high flexibility of the hinge region of IgG and a sharp bend in the Fc region of IgE. However with IgA1, the heavily O-glycosylated hinge region brings about a rigid conformation, thus not allowing it to be flexible enough to adopt the same upside-down orientation when bound to FcαRI [9].
1.5. FcαRI as an Inhibitory Receptor: Dual role of FcRγ ITAM

Until recently, FcαRI was perceived solely as an activating receptor. In 2005, Pasquier et al. identified that FcαRI can also drive anti-inflammatory responses through the ITAMs in the FcR γ-chain and thus behave as an inhibitory receptor that controls inflammation. This revealed the dual nature of the ITAM motif, which otherwise is typically considered to be involved in immune cell activation. This inhibitory signaling pathway mediated by FcαRI in association with FcRγ ITAMs was termed the Inhibitory ITAM or ITAMi signaling pathway[7].

Sustained aggregation of FcαRI by IgA immune complexes promotes cell activation and triggers pro-inflammatory responses such as phagocytosis, respiratory burst, cytokine release and antibody dependent cellular cytotoxicity. However, Pasquier et al. observed that the transient interaction of monovalent ligands, such as monomeric serum IgA with FcαRI leads to inhibition of responses such as IgG-dependent phagocytosis and IgE-mediated degranulation. FcαRI being a moderate affinity receptor and having moderately fast on and off rates while binding to monomeric IgA
suggests that the ITAM_i signaling pathway occurs only when monovalent ligands interact with FcaRI and not immune-complexed IgA. This becomes the first point of distinction between the ITAM mediated pro-inflammatory pathway and the anti-inflammatory ITAM_i pathway triggered by FcaRI[7].

This transient interaction leads to weak/incomplete phosphorylation of FcRγ tyrosine residues by Lyn, a Src kinase. Unlike recruitment of SH2 domain-containing inositol 5'-phosphatase (SHIP) by a tyrosine-phosphorylated Immunoreceptor Tyrosine based Inhibition Motif (ITIM) in other inhibitory receptors, recruitment of Src Homology Protein-1 (SHP-1), a phosphatase, is promoted strongly by the ITAM of FcRγ. SHP-1 mediates long-lasting dephosphorylation and thus inhibition of other signaling pathways[7].

Figure 8A. FcaRI-FcR γ-chain ITAM mediated pro-inflammatory response, Adapted from S.Ben Mkaddem et. al., Autoimmunity Reviews, Vol. 12, 666-669, 2013.
The signaling mechanism underlying the ITAMᵢ signaling pathway involves ITAM-mediated cross-regulatory mechanisms, sequestration of required effectors, and an inhibitory ITAM signaling response that involves the recruitment of SHP-1. Therefore, the signaling mechanism can be broken into two significant steps: (a) dephosphorylation of F-actin by SHP-1 recruited by ITAM, leading to actin depolymerization (b) co-segregation of the inhibitory receptor FcαRI, the targeted activating receptor such as FcγRIII/FcεRI, SHP-1 & other crucial signaling effectors into large polarized intracellular structures called “Inhibisomes”[13].
1.6. FcαRI as an anti-inflammatory agent

Increased levels of serum IgA and secretory IgA are associated with several inflammatory diseases. For instance, IgA nephropathy (IgAN), the most common IgA related disease, is characterized by deposition of polymeric IgA in the kidneys. Other diseases include ankylosing spondylitis, Sjögren's syndrome, alcoholic liver cirrhosis, coeliac disease, inflammatory bowel disease[6].

On the other hand, it has been well established that IgA deficiency is one of the most common immunodeficiencies. Interestingly, IgA deficiency in humans is often not found to be associated with impaired resistance to infection, but with an increased incidence of autoimmune disease, suggesting that there might be a passive role of serum IgA in regulating inflammatory responses[7].

Several disease models have been tested and developed to validate that ITAM induced signaling by FcαRI can inhibit or prevent inflammatory disease development. For instance, in vivo treatment of FcαRI with monomeric IgA has been shown to reduce symptoms in mouse model of asthma[7], and inhibit inflammatory responses in inflammatory renal diseases[13]. Thus, monomeric serum IgA and FcαRI play a central role regulating immune responses in mucosal and systemic compartments in vivo.

Certain anti-FcαRI monoclonal antibodies are found to trigger the same response from FcαRI as monomeric IgA. In models of nonimmune obstructive nephropathy and immune-mediated glomerulonephritis, in vivo treatment of anti- FcαRI Fab fragments reduced inflammation of kidneys by decreasing inflammatory cell infiltration and fibrosis development[14]. These results suggest a promising role for anti-FcαRI monoclonal antibodies to act as general
immunotherapeutic agents to prevent progression of renal, autoimmune and other inflammatory diseases.

The Fab fragments of the anti-FcαRI monoclonal antibodies used widely to study the ITAMᵢ pathway recognize different extracellular domains in the FcαRI ectodomain. The following table provides a summary of the anti-FcαRI monoclonal antibodies, the domain in FcαRI to which they bind and their function. The monoclonal antibody that is being studied and chosen for my thesis is A59.

<table>
<thead>
<tr>
<th>Anti-FcαRI monoclonal antibodies</th>
<th>Binding domain in FcαRI</th>
<th>Blocking/Non-blocking antibody</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP8a</td>
<td>D1[15]</td>
<td>Competes with and blocks monomeric IgA from binding to FcαRI.</td>
<td>Triggers the ITAMᵢ pathway[16].</td>
</tr>
<tr>
<td>A3</td>
<td>Junction between D1-D2[15]</td>
<td>Non-blocking antibody.</td>
<td>Does not trigger the ITAMᵢ pathway[7].</td>
</tr>
<tr>
<td>A59</td>
<td>D2[15]</td>
<td>Non-blocking antibody.</td>
<td>Triggers the ITAMᵢ pathway[7].</td>
</tr>
</tbody>
</table>

*Table 1 Anti-FcαRI Monoclonal Antibodies*
1.7. Epitope Mapping

An epitope, also known as an antigenic determinant, is the surface or region of an antigen/protein that is recognized by an antibody. These epitopes formed by several adjacent amino acid residues are referred to as linear determinants. In contrast, conformational epitopes are formed by residues which are not nearby in sequence, but spatially juxtaposed in the folded protein. Epitope mapping refers to the identification of the molecular structure of an antigen (the epitope or antigenic determinant) that interacts with the paratope (recognition surface) of the antibody[17]. With respect to my thesis, FcαRI is the antigen or protein under consideration, and A59 is the anti-FcαRI monoclonal antibody.
1.8. Hypothesis

The pro-inflammatory role of FcαRI has been well established in the literature over all these years. However, investigations over the past decade have identified FcαRI as a dual functioning receptor. Pasquier *et al.* identified the anti-inflammatory role of FcαRI to be triggered by monovalent IgA or other anti-FcαRI monoclonal antibody Fab fragments. This led to the finding of a new inhibitory ITAM signaling pathway called the ITAMi pathway. FcαRI triggers both these contrasting responses solely through the ITAM of FcRγ.

Hence FcαRI can be considered as a 3 state system: a resting state in which it does not mediate signaling, an activating state in which it triggers pro-inflammatory responses via recruitment of a Syk kinase, and an inhibiting state in which it triggers anti-inflammatory responses via recruitment of SHP-1 phosphatase. The Fab fragments of anti-FcαRI monoclonal antibodies A59, A3 and MIP8a recognize different extracellular domains in the FcαRI ectodomain. MIP8a and A59 Fab fragments trigger the ITAMi pathway. A3 Fab fragment does not trigger the ITAMi pathway. Based on this, we hypothesize that, *anti-FcαRI monoclonal antibodies which monovalently target FcαRI and trigger the ITAMi pathway have their antigenic epitopes clustered in certain regions of the ectodomain, forming hotspots. Identifying key amino acid residues or sequences of residues in these hotspots that are involved in interaction with the monoclonal antibodies will enable recognition of optimal regions in FcαRI ectodomain, to target and trigger the ITAMi pathway.* This will aid in understand how different FcαRI binding ligands are able to trigger two contrasting pathways solely through the ITAM of the accessory FcRγ receptor. Therefore, the objective of my thesis is to identify the antigenic epitope of anti-FcαRI monoclonal antibody A59 on FcαRI ectodomain using epitope mapping approaches.
1.9. Specific Aims

Specific Aim 1:

To construct a wild-type FcαRI ectodomain-Fcɣ (IgG1 Fc-domain) fusion vector and produce sufficient amount of the fusion protein.

**Rationale:** From a biophysical perspective, fusing the FcαRI ectodomain to an Fcɣ will increase the solubility and stability of FcαRI in vitro.

Specific Aim 2:

To perform site-directed mutagenesis of FcαRI ectodomain and express the FcαRI mutants.

**Rationale:** The extracellular domain of FcαRI ectodomain to which the anti-FcαRI monoclonal antibody A59 binds has been established previously. Mutating key residues that are likely to be involved in the receptor-mAb interaction and generating the mutant receptors will aid in identifying the epitopes of A59 on FcαRI.

Specific Aim 3a:

To confirm the ability of FcαRI ectodomain-Fcɣ fusion protein to bind IgA through specific binding assays.

**Rationale:** Analyzing the binding of FcαRI to its principal ligand IgA will aid in assessing the efficiency of (a) construction and transfection of the vector (b) potency of the fusion protein.
Specific Aim 3b:

To examine the ability of the mutant FcαRI ectodomain-Fcγ fusion protein to bind to monoclonal antibody A59 in comparison to the wild type FcαRI ectodomain-Fcγ fusion protein.

**Rationale:** Binding assays will aid in the assessing the functioning of the mutant proteins. This will enable us to understand the contribution of the original residue to the structure or function of the protein and compare it with that of the mutant protein.

Specific Aim 4:

To confirm the absence of any changes in FcαRI ectodomain following its fusion with Fcγ.

**Rationale:** Factor Xa is an endopeptidase that can remove fusion tags from proteins.

Construction of the fusion protein: *FcaRI ectodomain | FacXa cleavage site | Fcγ*

Hence, cleavage with Factor Xa will enable us to separate the FcαRI ectodomain from the parental expression vector.
CHAPTER TWO: Materials and Methods

2.1. Construction of SigpIg-WT FcαRI EC fusion vector

Signal pIgplus (SigpIg), a mammalian expression vector contains a human IgG1 Fc-tail (Fc½) in it that aids in the construction of fusion vectors. It is no longer commercially available. This vector consists of a CMV promoter, and a CD33 leader peptide sequence as the secretory signal, which mediates and enhances secretion of desired fusion protein. The vector has several restriction enzyme cut sites, a FacXa protease cut site that will aid in removing the Fc-tag from fusion proteins and ampicillin/neomycin antibiotic resistance. From a biophysical perspective, fusing the FcαRI ectodomain to an Fcγ means we are increasing the stability of FcαRI and thus facilitating its expression in vitro.

We had a SigpIg-OSCAR fusion vector in our lab already. The objective of cloning was to remove the OSCAR gene from the SigpIg expression vector, and insert the FcαRI ectodomain in its place, and thus construct a SigpIg- wild-type FcαRI ectodomain (SP WT FcαRI EC) fusion vector.

Cloning nomenclature:

Parental vector= SigpIg with OSCAR; Vector=SigpIg without OSCAR; Insert= WT FcαRI EC; Fusion vector= SP WT FcαRI EC.

Polymerase Chain Reaction (PCR) was used to add XhoI and XbaI restriction enzyme cut sites to the WT FcαRI EC. The PCR product, WT FcαRI EC with XhoI and XbaI cut sites, was run on a 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer, and observed at a size of 0.621kb. The gel
pieces were cut and purified using the Qiagen gel purification kit to obtain purified insert. Next, simultaneous double digestion reactions were set up (a) the parental vector with XhoI and XbaI, to cut out OSCAR and (b) insert with XhoI and XbaI, to obtain sticky ends. The digestion products were run on 1% agarose gels in 1X TAE buffer, and observed at a sizes of 6.9kb and 0.621kb respectively. Once the purified vector and insert DNA were obtained using Qiagen gel purification kit, a ligation reaction was set up with T4 DNA ligase (New England Biolabs), to ligate the vector and insert. The ligation product was then transformed using competent DH5α cells, and the transformation mixes were spread on 100mm *Luria broth (LB)* agar ampicillin+ plates and grown overnight at 37°C. Single colonies were picked from these plates, added to 5ml LB+500µl ampicillin miniprep cultures and grown overnight in a 37°C shaker. The DNAs were minipreped and sent for sequencing to Genewiz Inc. Miniprep cultures were used to grow up DNA in 500mL LB+500µL ampicillin maxiprep cultures and produce maxiprep DNA of the fusion vector SP WT FcαRI EC. Double digestion reaction of SP WT FcαRI EC with XhoI and XbaI was set up as a diagnostic to verify size of the fusion vector and cut insert.
2.2. Human Embryonic Kidney 293 T-cells

*Human Embryonic Kidney 293 T-cells (HEK 293T-cells)* stably express the *Simian vacuolating Virus 40 T antigen (SV40 TAg)* that directs viral transcription, replication and episomal amplification of vectors carrying the SV40 origin of replication. In addition, the HEK 293T-cells are semi-adherent, easily transfectable by all kinds of transfections, neomycin-resistant, and double in growth in about 16-24 hours.

2.3. Transient transfection of SP WT FcαRI EC into HEK 293T cells

For transient transfection of large amounts of protein, they required components are HEK 293 T-cells and *Mirus Transit LT-1 (LT-1)*, a polyamine-based lipid reagent. It is a broad spectrum
transfection reagent that has low toxicity and high efficiency of carrying the DNA of interest into the cells.

The transient transfection protocol devised in our lab is an 8-11 day long procedure. The first day involves seeding 100mm sterile collagen coated plates with 2*10^6 cells/plate. The 100mm sterile plates were coated with 2mL collagen/plate and let to sit in the incubator for about 10-15 minutes at room temperature. Then, the collagen was removed from the plates and collected for reuse. The plates were washed with 1X PBS and aspirated to dryness. Once collagen-coated, the plates were seeded with 2*10^6 cells/plate in 10mL complete media (DMEM+10% Fetal Bovine Serum + Penicillin/Streptomycin), and incubated overnight at 37°C, 5% CO₂. On day 2, each 100mm plate was transfected with 4µg of SP WT FcαRI EC, 1mL serum-free media and 160µL of LT-1. The transfection mix was incubated for 15-30 minutes at room temperature before being added to the plate. The transfected plates were incubated overnight at 37°C, 5% CO₂. On days 3 & 4, the cells were observed 24 hours and 48 hours post-transfection.

On day 5, spent cell culture media that contains the secreted fusion protein was collected from the plates and pooled. The spent culture media was spun down at 200Xg for 5 minutes to remove debris. The supernatant was then transferred to a fresh 50mL falcon and stored at 4°C. 0.05% sodium azide was added to the supernatants for long-term storage. 10mL complete media was then added back to the plates and the plates were returned back to the incubator. On days 6 and 7, the cells were observed 4 days and 5 days post-transfection. On day 8, the spent culture media was collected again from the plates and spun down to remove debris. The supernatants were stored at 4°C. Thus, from days 5 and 8, a total of ~250mL of supernatant was collected. This 250mL was considered as the starting volume for calculating final protein concentration after purification.
2.4. Production of WT FcαRI EC- Fcγ fusion protein

For production of fusion protein, *WT FcαRI EC-Fcγ*, the collected supernatants from transfection were run through a set of protein purification steps. The first of this involved dialyzing the supernatants in 20mM Tris at pH 7.4, 300mM Nacl using a dialysis tubing with 12-14kD molecular weight cut off. Dialysis was carried out overnight, with replacement of old with fresh buffer 4 hours after the dialysis was set up. 250mL of the collected supernatant was dialyzed in a total of about 2.5-3 liters of dialyzing buffer.

Protein A affinity column has affinity for Fcγ and thus captures the fusion protein on to the column. First, 20mM Tris at pH 7.4, 300mM Nacl was flushed through the protein A column to rinse before loading the protein. 1mL of the “preload” (dialyzed supernatant before being loaded on to the column) was saved for SDS-PAGE analysis. The dialyzed supernatant was loaded on to the column using a 20mL syringe and a 0.45üM syringe filter. The flow-through from the column was saved for SDS-PAGE analysis. The loaded column was then rinsed with 20mL of the same buffer and the flow-through was saved.

The protein was eluted from the column using elution buffer 0.1M Glycine at a low pH of 2.5. Addition of 200μL 1M Tris buffer at pH 8.0 to the tubes is an important step, as it acts as a neutralizing agent before the protein is eluted into the tubes, thus preventing the protein from unfolding or aggregating due to the sudden change to very low pH. In the first round, protein was eluted with 10mL of 45% elution buffer in five 2mL fractions. In the next round, the protein was eluted with 10mL of 75% elution buffer in five 2mL fractions. 100μL of each of these fractions were saved for SDS-PAGE analysis. Once these fractions were analyzed on SDS-PAGE and their sizes were verified, the fractions that contained the eluted protein were pooled and saved at 4°C.
Following elution, the column was hand stripped using 20mL of 100% elution buffer in order to remove any residual protein in the column. The pooled protein A fractions (5/6 2mL fractions ~ 10-12mL) were then concentrated to ~3-5mL in order to be run over the Superdex 200 HiLoad 16/60 size-exclusion column (S200).

S200 is a fast protein liquid chromatography column used to separate proteins based on size. 20mM Tris at pH 7.4, 300mM NaCl was the running buffer used for this column. The concentrated protein from protein A column was injected into the S200 using a 5mL syringe and the fractions were collected. Based on the peak observed in the output graph, the fractions falling within the peak were pooled and concentrated to 1mL to get a concentration of ~1-2µg/µL of fusion protein.

2.5. Mutations in domain 2 (D2) of FcαRI ectodomain: Binding domain of A59

The extracellular domains of FcαRI ectodomain to which the anti-FcαRI monoclonal antibody (mAb) A59 binds has been established previously. Mutating key residues that are likely to be involved in this receptor-mAb interaction and producing the respective mutant proteins will aid in identifying the epitopes of the mAbs. The residues to be mutated were selected such that charge reversal and differently sized residues replaced the original ones in the wild-type. In this way, we are trying to introduce dramatic changes to the surface of the protein, which might affect binding of A59 to FcαRI but not the structure, folding or nature of the protein.

Solvent accessibility based Protein-Protein Interface iDENtification and Recognition (SPPIDER), a protein interface recognition server, was used as an initial step to predict and select residues in FcαRI domain 2 (D2) which could be at the putative FcαRI-A59 interaction interface.
2.5.1. PyMOL

The location of these residues on the FcαRI ectodomain was visualized using PyMOL, a molecular visualization system that provides high-quality three-dimensional images of proteins. The three-dimensional structure of FcαRI ectodomain was obtained using PyMOL and the residues to be mutated were highlighted.

*Figure 12 Crystal Structure of FcαRI ectodomain generated using PyMOL*
The following residues were chosen to be mutated to other amino residues:

![Diagram showing mutated residues in FcαRI ectodomain]

*Figure 13 Mutated residues in FcαRI ectodomain*

2.6. Site-directed mutagenesis

Perfectly overlapping forward and reverse primers, 25-25 base pairs in length, including 10-15 native bases upstream and downstream of the residue to be mutated, and with a melting temperature \( T_m \) greater than or equal to 78°C, were designed to incorporate the desired changes in FcαRI ectodomain.

The *QuickChange Site-directed mutagenesis kit from Agilent technologies* was used to produce point mutations in domain 2 (D2) of FcαRI ectodomain. The first step was to use the designed primers and the template DNA to synthesize the mutant strands using PCR. The PCR product was digested with restriction enzyme DpnI in order to remove the parental or methylated wild-type
DNA. The DpnI digested PCR products were transformed into competent XL-10 gold cells in prechilled polypropylene round-bottom tube, along with 2µL of beta-mercaptoethanol (βME), and incubated for 30 minutes in ice. The content in the tubes were then subjected to heat-shock at 42°C for about 30-40 seconds, mixed with 1mL Luria broth (LB) and incubated in 37°C shaker for one hour. Two volumes, 20µL and 200µL, of the transformation mix were plated on 100mm LB agar ampicillin+ plates and incubated overnight at 37°C.

Single colonies were picked from the plate and 5mL LB+5µL ampicillin miniprep cultures were grown overnight at 37°C. The miniprep DNA were sent for sequencing (GENEWIZ Inc.). Once the sequences were checked to verify that the desired change had been made in the wild-type sequence, 500mL LB+500µL ampicillin maxiprep cultures were grown overnight at 37°C. The maxiprep DNA were sequenced again to verify that the changes were made and that the desired mutant vector had been produced.

2.7. Producing mutant protein

The mutant vectors were transfected into HEK 293 T-cells in 100mm collagen coated plates. On days 5 & 8, spent cell culture media containing the secreted fusion protein was collected from the plates and pooled. The spent culture media was then spun down at 200Xg for 5 minutes to remove debris. The supernatant was transferred to a fresh tube and stored at 4°C. 0.05% sodium azide was added to the supernatants for long-term storage. 10mL complete media was added back to the plates and the plates were returned back to the incubator.

The supernatants were dialyzed in 20mM Tris at pH 7.4, 300mM NaCl using dialysis tubing with 12-14kD molecular weight cut off. Dialysis was carried out overnight, with replacement of old
with fresh buffer 4 hours after the dialysis was set up. 250mL of the collected supernatant was
dialyzed in a total of about 2.5-3 liters of dialyzing buffer.

The dialyzed supernatants were passed through the protein A affinity column with 20mM Tris at
pH 7.4, 300mM NaCl as the running buffer. The loaded protein was eluted from the column using
elution buffer 0.1M Glycine at a low pH of 2.5. The concentrated protein from protein A column
was injected into the S200 using a 5mL syringe and the fractions were collected. Based on the
peak observed in the output graph, the respective fractions were pooled and concentrated to 1mL
to get a concentration of ~1-2µg/µL of fusion protein.

2.8. Enzyme Linked Immunosorbent Assay

To confirm the ability of WT FcαRI EC-Fcγ to bind to its principal ligand IgA, 200nM (32ug/ml)
human serum IgA1 was bound in triplicate to a 96-well plate for 90 minutes. The wells were
blocked with 200µL TBS Superblock at pH 7.4 containing 0.05% Tween-20 for 30 minutes
followed by several washes with 1X PBST at pH 7.4, containing 0.05% Tween-20. A titration of
different concentrations of WT FcαRI EC-Fcγ, 0-670nM, was added to the wells and incubated
for 60 minutes. Binding of WT FcαRI EC-Fcγ to IgA was detected using a [1:5000] dilution of
HRP-conjugated protein A. After a total of 4 washes with 1X PBST, 100µL of Tetra-Methyl-
Benzidine (TMB), a HRP substrate, was added to the wells to develop and read the plate. 50µL of
2N H₂SO₄ was added to all wells to stop the reactions.

Once the mutant proteins were produced, an ELISA was set up to compare and analyze the binding
of the mutant FcαRI EC-Fcγ proteins to that of the WT FcαRI EC-Fcγ. 1µg/mL (83nM) of the
mutant and WT FcαRI EC-Fcγ were bound in triplicate to a 96-well plate for 90 minutes. The wells
were blocked with 200µL TBS Superblock at pH 7.4 containing 0.05% Tween-20 for 30 minutes followed by several washes with 1X PBST at pH 7.4, containing 0.05% Tween-20. 1µg/mL 7nM of anti-FcαRI monoclonal antibody A59 (BD Biosciences Pharmigen) was added to the wells and incubated for 60 minutes. Binding of mutant or WT FcαRI EC- Fcγ to A59 was detected using a [1:500] dilution of sheep anti-mouse HRP conjugated antibody (GE Healthcare). After a total of 4 washes with 1X PBST, 100µL of Tetra-Methyl-Benzidine (TMB), a HRP substrate, was added to the wells to develop and read the plate. 50µL of 2N H₂SO₄ was added to all wells to stop the reactions. Four controls were used: (a) a non-specific binding control, where in 1µg/mL (6.67nM) of A59 was added to the plates and detected using [1:500] dilution of sheep anti-mouse HRP conjugated antibody (b) a control to detect the amounts of background signal form the detection antibody, where in 1µg/mL (83nM) of the WT FcαRI EC-Fcγ were added to the plates, and detected using [1:500] dilution of sheep anti-mouse HRP conjugated antibody (c) a positive control, where in 1µg/mL (83nM) of the WT FcαRI EC-Fcγ were plated and detected using a [1:5000] dilution of HRP-conjugated Protein A, and a (d) a negative control, where only PBS with 0.05% Tween-20 (PBST) was plated. Then 1µg/mL (6.67nM) of A59 was added to the plates and detected using [1:500] dilution of sheep anti-mouse HRP conjugated antibody.

2.9. Factor Xa protease treatment of fusion protein to remove the Fc-tag

Factor Xa is an endopeptidase that can remove fusion tags from proteins. The construction of the fusion protein is such that the FacXa cleavage site falls in between the FcαRI ectodomain and Fcγ in the fusion vector. 10µg of Factor Xa (purchased from Pierce, Fischer), in 20mM Tris, pH 7.4 300mM NaCl, at a concentration of 0.2µg/µl, was added to 500µg of the wild-type fusion protein
(ratio of 1:50 | Factor Xa: Fusion protein). The reaction was set up shaking at room temperature for a period of 48 hours.
CHAPTER THREE: Epitope mapping to identify antigenic epitope of A59 on FcαRI ecotodomain

3.1. Introduction

Investigation of the ITAMi pathway has suggested that the pathway is triggered by monomeric serum IgA or other monomeric anti-FcαRI monoclonal antibodies. It has been established previously that monomeric serum IgA binds transiently and with lower affinity to its principal receptor. This monomeric interaction is different from that of immune complexed IgA’s interaction with FcαRI.

FcαRI triggers a proinflammatory response when it interacts with immune complexed IgA. This interaction leads to clustering of several FcαRI molecules at the cell surface, and thus activation of intracellular processes such as gene expression, transcription factors, and other specific signaling and functional responses. Cross linking of the FcαRI-FcR γ-chain complex by a multivalent antigen leads to phosphorylation of the tyrosine residues within the ITAM motifs by protein tyrosine kinases such as Lyn from the Src family of kinases, thus initiating the signaling cascade.

In case of monomeric targeting, weak or incomplete phosphorylation of FcRγ tyrosine residues by Lyn, a Src kinase promotes strong recruitment of SHP-1 by the ITAM, and SHP-1 mediates long-lasting dephosphorylation and thus inhibition of other signaling pathways.
This inhibitory signaling pathway mediated by FcαRI in association with FcRγ ITAMs was termed the *Inhibitory ITAM or ITAM*$_i$ signaling pathway.

Several disease models have been tested and developed to validate that ITAM$_i$ induced signaling by FcαRI can inhibit or prevent inflammatory disease development. Anti-inflammatory role of FcαRI has been found to be triggered by certain anti-FcαRI monoclonal antibody Fab fragments[15]. This suggests a promising role for anti-FcαRI monoclonal antibodies to act as general immunotherapeutic agents to prevent progression of renal, autoimmune and other inflammatory diseases.

Based on this, we hypothesize that, anti-FcαRI monoclonal antibodies which monovalently target FcαRI and trigger the ITAM$_i$ pathway have their antigenic epitopes clustered in certain regions of the ectodomain, forming hotspots. Identifying key amino acid residues or sequences of residues in these hotspots that are involved in interaction with the monoclonal antibodies will enable recognition of optimal regions in FcαRI ectodomain, to target and trigger the ITAM$_i$ pathway. Therefore, the objective of my thesis is to identify antigenic epitope of anti-FcαRI monoclonal antibody A59 on FcαRI ectodomain using site-directed mutagenesis of FcαRI ectodomain.

### 3.2. Results

#### 3.2.1. Construction of Sigplg-WT FcαRI EC fusion vector

In order to increase the solubility and stability of FcαRI *in vitro*, the FcαRI ectodomain construct was inserted into a human IgG1 Fc-tail (Fcγ) containing mammalian expression vector, Sigplg. *Polymerase Chain Reaction (PCR)* was used to add XhoI and XbaI restriction enzyme cut sites to
the WT FcαRI EC. The PCR product, WT FcαRI EC with XhoI and XbaI cut sites, was run on a 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer, and observed at a size of 0.621kb. The gel pieces were cut and purified using the Qiagen gel purification kit to obtain purified insert. Next, simultaneous double digestion reactions were set up (a) the parental vector with XhoI and XbaI, to cut out OSCAR and (b) insert with XhoI and XbaI, to obtain sticky ends. The digestion products were run on 1% agarose gels in 1X TAE buffer, and observed at sizes of 6.9kb and 0.621kb respectively. Once the purified vector and insert DNA were obtained using Qiagen gel purification kit, a ligation reaction was set up with T4 DNA ligase (New England Biolabs), to ligate the vector and insert. The ligation product was then transformed using competent DH5α cells, and the transformation mixes were spread on 100mm Luria broth (LB) agar ampicillin+ plates and grown overnight at 37°C. Single colonies were picked from these plates, added to 5ml LB+500µl ampicillin miniprep cultures and grown overnight in a 37°C shaker. The DNA was minipreped, verified by sequencing and maxipreped to produce the fusion vector - SP WT FcαRI EC. The vector map of constructed fusion vector is shown in Figure 14.
3.2.2. Transient transfection of SP WT FcαRI EC into HEK 293T cells

The constructed fusion vector was transiently transfected into HEK 293-Tcells. As a part of an 8 day long transfection procedure, 100mm sterile collagen coated plated were seeded with $2\times10^6$ cells/plate. Each plate was transfected with 1mL serum-free media, 4µg of the fusion vector DNA and 160µL of transfection reagent LT-1, on day 2. On days 5 & 8, ~10mL/plate of spent culture media was collected and pooled. The plates were fed with 10mL fresh complete media and returned back to the incubator. The spent culture media was spun down at 200Xg for 5 minutes to remove debris. The supernatant was then transferred to a fresh 50mL falcon and stored at 4°C. At the end of each transfection, ~250mL of spent culture media containing the secreted fusion protein was collected.

**This 250mL is called the “starting volume”, before going into dialysis and further protein purification.**
3.2.3. Production of WT FcαRI EC- Fcy fusion protein

3.2.3.1. Dialysis and Protein A affinity column

The starting volume from transfection was run through three protein purification steps. The first step involved dialyzing the supernatants in 20mM Tris at pH 7.4, 300mM Nacl using a dialysis tubing with 12-14kD molecular weight cut off. Dialysis was carried out overnight, with replacement of old with fresh buffer 4 hours after the dialysis was set up. 250mL of the collected supernatant was dialyzed in a total of about 2.5-3 liters of dialyzing buffer.

The dialyzed supernatants were then loaded onto a Protein A affinity column. 20mM Tris at pH 7.4, 300mM Nacl (Loading Buffer) was flushed through column to rinse it before loading the protein. The dialyzed supernatant was then loaded onto the column in batches, using a 20mL syringe and a 0.45µM syringe filter. After the entire 250mL was loaded onto the column, the
column was rinsed using 20mL of the loading buffer. The protein was eluted from the column in two rounds using 0.1M Glycine, pH 2.5 (Elution Buffer). In the first round, protein was eluted with 10mL of 45% elution buffer in five 2mL fractions. In the next round, the protein was eluted with 10mL of 75% elution buffer in five 2mL fractions. Following elution, the column was hand stripped using 20mL of 100% elution buffer in order to remove any residual protein in the column.

The fractions collected from the column at every stage were run on an SDS-PAGE gel, under non-reducing conditions (Figure 16). 15µL of each of the fractions were mixed with 5µL of non-reducing Laemmli protein sample buffer for SDS-PAGE (without β-mercaptoethanol). The fusion protein consisting of FcαRI ectodomain construct, a linker and Fcy is at a size of ~60kD under reducing conditions. Under non-reducing conditions, the fusion protein is seen to come off at ~120kD (between the 100kD and 150kD markers) in the gel.
Figure 16 Fractions collected when WT FcaRI EC- Fcγ fusion protein run through Protein A affinity column: SDS-PAGE gel under non-reducing conditions.

**Lane 1:** Dual colored protein standard; **Lane 2:** Protein eluted with 45% elution buffer*, fraction 1; **Lane 3:** Protein eluted with 45% elution buffer, fraction 2; **Lane 4:** Protein eluted with 45% elution buffer, fraction 3; **Lane 5:** Protein eluted with 45% elution buffer, fraction 4; **Lane 6:** Protein eluted with 45% elution buffer, fraction 5; **Lane 7:** Protein eluted with 75% elution buffer, fraction 6; **Lane 8:** Protein eluted with 75% elution buffer, fraction 7; **Lane 9:** Protein eluted with 75% elution buffer, fraction 8; **Lane 10:** Protein eluted with 75% elution buffer, fraction 9; **Lane 11:** Protein eluted with 75% elution buffer, fraction 10; **Lane 12:** Positive control: WT FcaRI EC- Fcγ fusion protein 1ug.

*Elution buffer: 0.1M Glycine, pH 2.5
In Figure 16, Lanes 3, 4, 5 and 6 represent eluted fractions from the protein A column that contain the fusion protein. These specific fractions were pooled (four/five 2mL fractions ~ 8/10mL) and concentrated to ~3-5mL.

3.2.3.2. Superdex 200 size exclusion column

The Superdex 200 HiLoad 16/60 size exclusion column was attached to the FPLC and the inlet to pump A was immersed in one liter of 20mM Tris at pH 7.4, 300mM Nacl (Running buffer). Pump A was washed with the running buffer and following the pump wash, 20mL of the running buffer was injected into the injection valve using a syringe. The S200 column was equilibrated in the running buffer before the protein was loaded on to the column. After equilibration, the following settings were made: Flow=1.5mL/min; Gradient=0%B; High alarm pressure=0.30MPa; Fraction size=4mL. The concentrated protein from protein A column (~3-5mL) was injected into the injection valve using a syringe, and the run was started.

WT FcαRI EC-Fcγ peak typically comprised fractions B12/B11-B7/B6 (depending on the size of fractions collected-2/4mL) (Figure 17). Once the run was completed and the readout was obtained, the fractions enclosed within the protein peak in the readout were run on an SDS-PAGE gel under reducing and non-reducing conditions (Figures 18&19). The size of the fractions were confirmed after SDS-PAGE analysis- the fusion protein comes off at ~60kD under reducing conditions and ~120kD under non-reducing conditions. Following this, the fractions that contained the protein (B11, 10, 9, 8 and 7 in Figure 17) were pooled and concentrated to~1mL (1-1.5µg/µL).
Figure 17 WT FcaRI EC-Fcy run on Superdex 200 size exclusion column - Readout from column
Figure 18 Fractions collected when WT FcαRI EC-Fcy fusion protein was run through S200 size exclusion column: SDS-PAGE gel under non-reducing conditions

Lane 1: Dual colored protein standard; Lane 2: Blank; Lane 3: Protein eluted from S200, fraction B12; Lane 4: Protein eluted from S200, fraction B11; Lane 5: Protein eluted from S200, fraction B10; Lane 6: Protein eluted from S200, fraction B9; Lane 7: Protein eluted from S200, fraction B8; Lane 8: Protein eluted from S200, fraction B7; Lane 9: Protein eluted from S200, fraction B6; Lanes 10, 11, 12: Blank.
Figure 19 Fractions collected when WT FcαRI EC-Fcy fusion protein was run through S200 size exclusion column: SDS-PAGE gel under reducing conditions.

Lane 1: Dual colored protein standard; Lane 2: Blank; Lane 3: Protein eluted from S200, fraction B12; Lane 4: Protein eluted from S200, fraction B11; Lane 5: Protein eluted from S200, fraction B10; Lane 6: Protein eluted from S200, fraction B9; Lane 7: Protein eluted from S200, fraction B8; Lane 8: Protein eluted from S200, fraction B7; Lane 9: Protein eluted from S200, fraction B6; Lanes 10, 11, 12: Blank.
3.2.4. Site-directed mutagenesis: Mutations in domain 2 (D2) of FcαRI ectodomain: Binding domain of A59

Perfectly overlapping forward and reverse primers, 25-25 base pairs in length, including 10-15 native bases upstream and downstream of the residue to be mutated, and with a melting temperature ($T_m$) greater than or equal to 78°C, were designed to incorporate the desired changes in FcαRI ectodomain.

The **QuikChange Site-directed mutagenesis kit from Agilent technologies** was used to produce point mutations in domain 2 (D2) of FcαRI ectodomain. The first step was to use the designed primers, and the template DNA to synthesize the mutant strands using PCR. The PCR product was digested with restriction enzyme DpnI in order to remove the parental or methylated wild-type DNA. The DpnI digested PCR products were then transformed into competent XL-10 gold cells in prechilled polypropylene round-bottom tube, along with 2µL of beta-mercaptoethanol (βME), and incubated for 30 minutes in ice. The content in the tubes were then subjected to heat-shock at 42°C for about 30-40seconds, mixed with 1mL Luria broth (LB) and incubated in 37°C shaker for one hour. Two volumes, 20µL and 200µL, of the transformation mix were plated on 100mm LB agar ampicillin$^+$ plates and incubated overnight at 37°C.

Single colonies were picked from the plate and 5mL LB+5µL ampicillin miniprep cultures were grown overnight at 37°C. The miniprep DNA were sent for sequencing (GENEWIZ Inc.). Once the sequences were checked to verify that the desired change had been made in the wild-type sequence, 500mL LB+500µL ampicillin maxiprep cultures were grown overnight at 37°C. The maxiprep DNA were sequenced again to verify that the changes were made and that the desired mutant vector had been produced.
3.2.5. Production of mutant protein

The mutant vectors were transfected into HEK 293 T-cells in 100mm collagen coated plates. On days 5 & 8, spent cell culture media containing the secreted fusion protein was collected from the plates and pooled. The spent culture media was then spun down at 200Xg for 5 minutes to remove debris. The supernatant was transferred to a fresh tube and stored at 4°C. 10mL complete media was added back to the plates and the plates were returned back to the incubator.

The supernatants were dialyzed in 20mM Tris at pH 7.4, 300mM NaCl using dialysis tubing with 12-14kD molecular weight cut off. Dialysis was carried out overnight, with replacement of old with fresh buffer 4 hours after the dialysis was set up. 250mL of the collected supernatant was dialyzed in a total of about 2.5-3 liters of dialyzing buffer.
The dialyzed supernatants were passed through the protein A affinity column with 20mM Tris at pH 7.4, 300mM NaCl as the running buffer. The protein was eluted from the column in two rounds using 0.1M Glycine, pH 2.5 (Elution Buffer). In the first round, protein was eluted with 10mL of 45% elution buffer in five 2mL fractions. In the next round, the protein was eluted with 10mL of 75% elution buffer in five 2mL fractions. Following elution, the column was hand stripped using 20mL of 100% elution buffer in order to remove any residual protein in the column.

The eluted fractions were run on an SDS-PAGE gel, under non-reducing conditions (Figure 21). 15µL of each of the fractions were mixed with 5µL of non-reducing Laemmli protein sample buffer for SDS-PAGE (without β-mercaptoethanol). The mutant protein is at a size of ~60kD under reducing conditions. ~120kD (between the 100kD and 150kD markers) under non-reducing conditions.

**Figure 21 (A)-(G): Fractions collected when the different mutant FcαRI EC- Fcy fusion proteins were run through Protein A affinity column: SDS-PAGE gel under non-reducing conditions**

Lane 1: Dual colored protein standard; Lane 2: Protein eluted with 45% elution buffer*, fraction 1; Lane 3: Protein eluted with 45% elution buffer, fraction 2; Lane 4: Protein eluted with 45% elution buffer, fraction 3; Lane 5: Protein eluted with 45% elution buffer, fraction 4; Lane 6: Protein eluted with 45% elution buffer, fraction 5; Lane 7: Protein eluted with 75% elution buffer, fraction 6; Lane 8: Protein eluted with 75% elution buffer, fraction 7; Lane 9: Protein eluted with 75% elution buffer, fraction 8; Lane 10: Protein eluted with 75% elution buffer, fraction 9; Lane 11: Protein eluted with 75% elution buffer, fraction 10; Lane 12: Positive control: WT FcαRI EC- Fcy fusion protein 1ug.
In Figure 21, for each of the mutant proteins, the lanes with prominent bands at ~120kD represent the eluted fractions from protein A column that contain the fusion protein. These specific fractions were pooled (~four 2mL fractions=~8mL) and concentrated to ~3-5mL.

The concentrated protein from protein A column (~3-5mL) was then loaded onto an equilibrated Superdex 200 HiLoad 16/60 column and the protein purification run was started.

Mutant FcαRI EC-Fcγ peaks typically enclosed fractions B12/B11-B7/B6 (depending on the size of fractions collected-2/4mL) (Figures 22-28). Once the runs were completed and the readouts were obtained, the fractions enclosed within the protein peak in the readout were run on an SDS-PAGE gel under reducing and non-reducing conditions. The size of the fractions were confirmed after SDS-PAGE analysis- the fusion protein comes off at ~60kD under reducing conditions and ~120kD under non-reducing conditions. Following this, the fractions that contained the protein were pooled and concentrated to~1mL (1-1.5µg/µL). An average protein yield of ~2.6mg for a starting volume of 250mL was obtained for most of the mutants. E119K had a strikingly low yield, almost three fold lesser than the average yield of the other mutants. We suspect that this could be due to misfolded mutant protein.
Figure 22 S108E FcαRI EC-Fcy run on Superdex 200 size exclusion column – Readout from column
Figure 23 D110K FcaRI EC-Fcγ run on Superdex 200 size exclusion column – Readout from column
Figure 24 E119K FcaRI EC-Fcy run on Superdex 200 size exclusion column – Readout from column
Figure 25 E140K FcαRI EC-Fcy run on Superdex 200 size exclusion column – Readout from column
Figure 26 H129E FcαRI EC-Fcy run on Superdex 200 size exclusion column – Readout from column
Figure 27 H153E FcaRI EC-Fcy run on Superdex 200 size exclusion column – Readout from column
Figure 28 F185A FcαRI EC-Fcy run on Superdex 200 size exclusion column – Readout from column
The starting volume of culture media for the wild-type and each of the mutants was 250mL, except for E140K. Due to an accidental spill in lab, about 200mL of culture media of E140K was lost. Once the O.D. values for binding were determined after protein purification, each of the proteins were concentrated to a final volume of 2mL. The extinction coefficient for each of the proteins was determined using the ProtParam tool in ExPASy Bioinformatics portal[18]. The O.D. values were divided by the extinction coefficient values to determine the final concentrations of the wild-type and mutant proteins.

3.2.6. Enzyme Linked Immunosorbent Assay

3.2.6.1. To confirm the ability of the fusion protein WT FcαRI EC- Fcy to bind its principal receptor IgA, an ELISA was set up with the following template. Human serum IgA1 was bound in triplicate to a 96-well plate for 90 minutes. The wells were blocked with 200µL TBS Superblock at pH 7.4 containing 0.05% Tween-20 for 30 minutes followed by several washes with 1X PBST at pH 7.4,
containing 0.05% Tween-20. A titration of different concentrations of WT FcαRI EC-Fcγ, 0-670nM, was added to the wells and incubated for 60 minutes. Binding of WT FcαRI EC-Fcγ to IgA was detected using a [1:5000] dilution of HRP-conjugated protein A. After a total of 4 washes with 1X PBST, 100µL of Tetra-Methyl-Benzidine (TMB), a HRP substrate, was added to the wells to develop and read the plate. 50µL of 2N H₂SO₄ was added to all wells to stop the reactions.

**WTSPFcaRI EC[nM] vs Binding (OD values)**

![Graph showing binding of WT FcαRI EC-Fcγ to human serum IgA](image)

*Figure 29 Binding of WT FcαRI EC-Fcγ to human serum IgA*

This is a dose response curve of the wild-type fusion protein with its principal ligand IgA. An increase in binding was observed for increasing concentrations of the fusion protein eventually leading to saturation of binding. **Non-specific binding control**: No IgA1 was bound to the plate. (1) 0nM (2) 167nM and (3) 500nM of the fusion protein were added to the plate and detected using HRP conjugated IgA. **Positive control**: The plate was coated with different concentrations of the
fusion protein and HRP-conjugated Protein A was then added to the plate. *Background signal detection:* IgA1 was bound to the plate. HRP conjugated protein A was added to the plate.

3.2.6.2. **To compare and analyze the binding of WT FcαRI EC-Fcγ versus the mutant FcαRI EC-Fcγ proteins to anti-FcαRI monoclonal antibody A59,** an ELISA with the following template was set up: 1µg/mL (83.3nM) of the mutant and WT FcαRI EC-Fcγ were bound in triplicate to a 96-well plate for 90 minutes. The wells were blocked with 200µL TBS Superblock at pH 7.4 containing 0.05% Tween-20 for 30 minutes followed by several washes with 1X PBST at pH 7.4, containing 0.05% Tween-20. 1µg/mL (6.67nM) of anti-FcαRI monoclonal antibody A59 (BD Biosciences Pharmigen) was added to the wells and incubated for 60 minutes. Binding of mutant or WT FcαRI EC-Fcγ to A59 was detected using a [1:500] dilution of sheep anti-mouse HRP conjugated antibody (GE Healthcare). After a total of 4 washes with 1X PBST, 100µL of *Tetra-Methyl-Benzidine (TMB),* a HRP substrate, was added to the wells to develop and read the plate. 50µL of 2N H₂SO₄ was added to all wells to stop the reactions.

**Controls:** (a) Non-specific binding control: 1µg/mL (6.67nM) of A59 was added to the plates and detected using [1:500] dilution of sheep anti-mouse HRP conjugated antibody (b) Control to detect the amounts of background signal: 1µg/mL (83nM) of the WT FcαRI EC-Fcγ was added to the plates, and detected using [1:500] dilution of sheep anti-mouse HRP conjugated antibody (c) Positive control: 1µg/mL (83nM) of the WT FcαRI EC-Fcγ was plated and detected using a [1:5000] dilution of HRP-conjugated Protein A, and (d) Negative control: PBS with 0.05% Tween-20 (*PBST*) was plated. Then 1µg/mL (6.67nM) of A59 was added to the plates and detected using [1:500] dilution of sheep anti-mouse HRP conjugated antibody.
Figure 30 Raw data showing binding of WT FcαRI EC-Fcγ to A59 versus binding of mutant FcαRI EC-Fcγ to A59

Figure 31 Raw data showing average binding of WT FcαRI EC-Fcγ versus mutant FcαRI EC-Fcγ to A59 (n=3)
The above results are not dose response curves, but represent the amount of binding of the wild-type versus mutant proteins at specifically chosen concentrations of A59. Many monoclonal antibodies recognize their antigen with affinity at a $K_d$ range of about 1-5nM. This is the functional range of binding. Therefore 6.67nM and 2nM of A59 were chosen for the sake of the above experiments. In Figure 30, the graph represents the raw data from three trials of the experiment.

The average binding values and standard deviation values were calculated from these three trials for wild-type and mutant at 6.67nM of A59 and the respective average values were plotted in Figure 31. Error bars were used to indicate one standard deviation of uncertainty, to give an idea of how far the reported values might be from the true value of binding.

Overall, the results suggested that there was considerable variability in the binding of the mutant proteins to A59, and E140K specifically showed diminished binding consistently in all the trails, when compared to the wild-type and other mutants.

3.3. Discussion

In the last decade, the anti-inflammatory role of FcαRI mediated by the ITAMs of FcR $\gamma$-chain on the same cell has been demonstrated by several groups. This inhibitory ITAM signaling mechanism involves recruitment of phosphatase SHP-1 to the phosphorylated ITAM sequence, instead of Syk kinase as in the activating ITAM pathway. This novel inhibitory pathway is known as ITAMi (i.e., ITAM inhibitory) signaling. Monteiro and his group first demonstrated the ITAMi pathway in FcαRI, the IgA-specific receptor and showed that monomeric IgA as well as some but not all anti-FcαRI monoclonal antibodies were capable of triggering the ITAMi pathway in FcαRI.
Based on these past studies, we hypothesize that anti-FcαRI monoclonal antibodies which monovalently target FcαRI and trigger the ITAMi pathway have their antigenic epitopes clustered in certain regions of the ectodomain, forming hotspots.

Experiment 3.2.6.2 was carried out to compare and analyze the binding of WT FcαRI EC-Fcγ protein versus the mutant FcαRI EC-Fcγ proteins to A59.

83.3nM of the fusion protein (wild-type and mutants) was bound to a 96-well plate. 6.67nM of A59 was added to the plate in triplicate and binding of A59 to the fusion protein was detected using [1:500] dilution of sheep anti-mouse HRP conjugated antibody. This experiment was repeated three times to see verify repeatability of the obtained binding values. The average binding value of the three trials was calculated for wild-type and mutant proteins, and the respective values were plotted (Figure 31).

We obtained an unusually low yield of protein for E119K, only a third as high as the ideal cases was obtained for the same amount of starting volume. In addition, a sizeable aggregate peak (~24mL) was also observed in this case (Figure 24). Similarly, even though the protein yield was comparable to wild-type, a considerable amount of aggregate was observed in H129E as well, which was strikingly different from other such mutants such as S108E, D110K etc. (Figure 26). Decreased yield of two of the mutant FcαRI EC-Fcγ proteins was observed when compared to the wild-type. Due to an accidental spill in the lab, most of the E119K protein was lost. Therefore, a lesser yield of the protein was observed (Figure 25). However, the ratio of starting volume to protein yield/ml of culture media was similar to the wild-type. About 200ml of the culture media (starting volume) was lost. However, for about 50ml of starting volume, we still got a yield of 0.52mg. With all these considerations in mind, E140K still showed lesser yield when compared to the wild-type and all the other mutants, consistently through all three trials.
H153E on the other hand did show lesser yield when compared to the wild-type in two out of the three trials, but we suspect that there are chances of this being attributed to experimental artefacts. Therefore repetition of experiments (increase in “n” number of trials) would be required to comment further on the binding of this mutant.

E140K lies on the right-edge of domain 2 whereas H153E lies on the left-edge of domain 2 (Figure 20).

We believe that these results lead us in a direction that will help decide and focus on certain regions of domain 2 while trying to identify the epitopes of A59. Based on the possibility that E140K might be a part of the epitope, the next ideal step would be to mutate a string of residues close to it and analyze the behavior of those mutant proteins.
CHAPTER FOUR: Conclusions and Future Directions

4.1 Significance

Autoimmune disorders are the second leading type of chronic illness in the US, affecting about 50 million individuals[19]. In 2001, the National Institute of Allergy and Infectious Diseases (NIAID) estimated that the annual autoimmune healthcare cost is greater than $100 billion, although this value may be significantly underestimated[20]. Similarly, allergies affect about 50 million Americans annually with a cost over $14 billion[21]. The receptors for Fc region of immunoglobulins, called Fc Receptors (FcRs) play key roles in activation and inhibition of immune system responses. Many human autoimmune diseases, such as allergies/hypersensitivity reactions are a result of impairment of this FcR regulatory system[22]. The most prominent activating immune functions of FcRs that have been well established, include FcγR (IgG receptor) mediated phagocytosis in macrophages, FcεRI (IgE receptor) mediated degranulation in mast cells, and proliferation of B-cells. The FcRs are stably associated with FcR γ-chain/ FcRγ, and their activating functions are triggered when clustering of FcR-FcRγ complexes occur.

In this thesis, we focus on the interaction between Immunoglobulin A (IgA), the second most predominant antibody in serum, and its principal myeloid receptor, FcαRI (CD89). Findings until 2005 led to perceiving FcαRI as a solely activating receptor. In 2005, Pasquier et al. identified that FcαRI can also drive powerful anti-inflammatory responses through the ITAMs in the FcR γ-chain
and thus behave as an inhibitory receptor that controls inflammation. This revealed the dual nature of the ITAM motif, which otherwise is typically considered to be involved in immune cell activation. This inhibitory signaling pathway mediated by FcαRI in association with FcRγ ITAMs was termed the Inhibitory ITAM or ITAMI signaling pathway[7].

Until now research groups in this field have focused only on the downstream signaling pathways involved in ITAMI and cellular localization of FcαRI in inhibisomes. However, there has been no mechanistic experiments to determine how different FcαRI binding ligands are able to trigger two contrasting pathways solely through the ITAM of the accessory FcRγ receptor. Important questions such as at which specific sites do these inhibitory ligands bind on FcαRI and what the oligomerization states of FcαRI might be are yet to be addressed.

Therefore, the main objective of this thesis is to identify the antigenic epitopes of anti-FcαRI monoclonal antibody A59 on FcαRI ectodomain using site-directed mutagenesis and enzyme linked immunosorbent assays.

4.2 Future Directions

The main aim of this thesis was to perform epitope mapping in order to identify key residues in domain 2 of FcαRI ectodomain involved in the interaction of A59 with FcαRI. Site-directed mutagenesis and ELISA’s helped us identify residue E140K that might be a part of the epitope. The most ideal future directions of this project would be to perform those experiments which will aid in confirming whether these residues really are a part of the epitope. The protein production yield for E119K was strikingly low compared to the other mutants and the wild-type. For such mutants, the protein purification runs can be repeated two or three more times, to observe the trend
of the protein being produced. This will help in determining whether the protein is misfolded or identify other reasons causing this low yield.

The next step would be to test the binding of all mutants to human serum IgA. The binding of WT FcαRI EC (wild-type fusion vector) to its principal ligand IgA has been shown (Figure 29). A similar ELISA can be set up to test the binding of mutants to IgA. We would expect to see normal binding of the mutants to IgA, since IgA binds to domain 1 but the mutations have been made in domain 2 of FcαRI ectodomain. Following this, the binding of the mutants to other anti-FcαRI monoclonal antibodies, like MIP8a and A3 can be tested. MIP8a binds to domain 1 of FcαRI ectodomain and triggers the ITAMi pathway whereas A3 binds at the junction of domain 1 and domain 2 and does not trigger the ITAMi pathway. Results from these experiments will help in determining if mutations in domain 2 disrupt/affect only binding of A59 to FcαRI ectodomain or not. On the other hand, if results from such experiments suggest that multiple ligands do not bind to one or more of the mutants, then that would indicate that the mutants are misfolded.

In addition, more sophisticated and informative techniques such as Surface Plasmon Resonance (SPR), and tryptic digest and Mass Spectrometry (MS) analysis can be used to map epitopes of these antibodies.

SPR can be considered as a better and more efficient technique when compared to ELISA, based on the required end products of the experiments. SPR is a more precise and sensitive technique than ELISA, since it involves an automated method of detecting binding. When compared with ELISA, it can be used to study the kinetics of binding, measure binding at lower concentrations, and look at much more complicated binding models apart from simple 1:1 binding interactions.
With respect to this project, SPR can be used to study the binding interaction between free analyte molecules in solution (WT-FcαRI EC fusion protein) and probe molecules which are immobilized on a sensor chip (monoclonal antibody A59). The same experiment can be repeated for the mutant proteins in order to compare and analyze their binding to A59 versus that of the wild-type.

With respect to tryptic digest and MS analysis, the technique of epitope excision can be employed to immobilize the antibody (A59) on an affinity column. The antigen, in this case being the fusion protein, can be bound to the immobilized antibody and then digested with suitable proteases such as trypsin, lysine etc. Following the protease digest, the unbound peptides can be washed, and bound peptides can be analyzed using mass spectrometry[24]. Another technique involving mass spectrometry would be to use cross-linkers to chemically modify the antigenic peptides, and use the MS spectrum to distinguish between the cross-linker modified antigenic peptides and the unmodified antigenic peptides (that constitute the epitope)[25].

Eventually, when the precise epitope of A59 is mapped, the same experiments and project can be extended to mapping epitopes of other common anti-FcαRI monoclonal antibodies like MIP8a and A3. Since MIP8a triggers the ITAMi pathway and A3 does not (not been established in the literature as of yet), along with the results obtained for A59, it will be interesting to compare the epitope sites for these three antibodies. *Figures 32&33* show some suggested mutations in the binding domains of MIP8a and A3 for future analysis.

Determining the epitopes of MIP8a, A59, A3 and several other anti-FcαRI monoclonal antibodies will provide the opportunity to target and trigger the ITAMi pathway. The inhibition responses mediated by ITAMi pathway can be analyzed using functional assays such as degranulation assays.
We consider FcαRI as a 3 state system: a resting state in which it does not mediate signaling, an activating state in which it triggers pro-inflammatory responses via recruitment of a Syk kinase, and an inhibiting state in which it triggers anti-inflammatory responses via recruitment of SHP-1 phosphatase. Therefore, the long-term goal of this project would be to determine epitopes and analyze signaling responses of various existing neutralizing, activating and inhibiting ligands for FcαRI and also create a panel of antibodies for testing responses. This will be the step towards determining the precise localization of epitopes of different ligands and the oligomerization state of FcαRI during ITAMi signaling.
Figure 32 Suggested Mutations in Domain 1 (Binding domain of MIP8A)

Figure 33 Suggested Mutations in Domain 1 - Domain 2 junction (Binding region of A3)
The abovementioned experiments and analyses should pave way for achieving the overarching goal of this project, which is to better define the characteristics of ligands that are capable of triggering the inhibitory function of FcαRI and to characterize the clustering mode of FcαRI in the three functional states.

The long-term goal would be to develop significant therapeutics which are targeted towards the epitopes which trigger the ITAMi pathway, and hence be able to control the progression of autoimmune and inflammatory diseases.
References


