



Delivery of inhibitory signalling via co-crosslinking of
FcεRI and FcγRIIB with engineered micro-surfaces as
a new therapeutic concept

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Abstract

Mast cell and basophil mediators play a major role in the development of immunoglobulin E (IgE)-dependent allergic disorders. One of these mediators is histamine, which is released by activation of inflammatory cells through cross-linking of the high affinity IgE receptor (FcεRI). The current immunotherapies are accompanied by major drawbacks, such as an increase the risk of anaphylaxis and systemic reaction, difficulties in allergen extract standardisation of allergen-specific immunotherapy (SIT), and a small but worrying increase in the incidence of cancer with omalizumab. It is known that co-aggregation of immunoglobulin G (IgG) low-affinity receptor (FcγRIIB) with FcεRI can lead to inhibition of mast cell degranulation, thus inhibiting production of pharmacological mediators. The potential underlying this principle has been highlighted by many approaches published in the literature.

In this study, we designed a novel immunotherapy vector that takes advantage of the most recent advances in drug delivery systems using microparticles. This micro-carrier will carry the Fc parts of both IgG and IgE in defined stoichiometric ratios to be directed towards the allergic inflammatory cells (mast cells and basophils). Fcγ receptors have different binding affinities for IgG1, either with high affinity in the case of FcγRI, or low as with FcγRIIB. We engineered mutant IgG-Fc fragments with increased binding affinity for FcγRIIB. These recombinant Fc-genes were successfully transfected, expressed and purified from the cell supernatant of a high yield HEK293-6E cell line mammalian expression system. To obtain a site-specific conjugation between the recombinant Fc proteins and microparticles, a novel 15 amino acid peptide tag known as Avi-Tag was used. Both particle size and Fc density have an effect on the level of the immune response. On the reporter cell lines derived from the parental RBL-2H3 cell lines (RS-ATL8 and NFAT-DsRed), a 0.5µm particle coated

with IgE Fc significantly affected the activation response compared to other particles sizes (1,2 and 6 μm). Cell activation was also affected by the density of IgE Fc, and a lower dilution ratio of added IgE Fc to particles (1:50) showed the highest activation value. Unexpectedly, the preliminary data for the release of histamine from purified human peripheral blood basophils treated with different Fc-particle conjugate formulations did not show any effect for the suggested therapy.

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List of Abbreviations

AOX1:	Alcohol oxidase promoter
BCR:	B cell receptor
Bet v:	Betula verrucosa antigen
bFGF:	Basic fibroblast growth factor
BiP:	Heavy chain binding protein
BSA:	Bovine serum albumin
Btk:	Bruton's tyrosine kinase
C3a:	Complement 3a
C3aR:	Complement 3a receptor
CCL:	Chemokines ligand
CD:	Cluster of differentiations
Ce:	Epsilon chain
Cg:	Gamma chain
CH:	Constant heavy chain
CL:	Constant light chain
CMV:	Cytomegalovirus
CRAC:	Calcium release-activated channels
Cys:	Cysteine
DCs:	Dendritic cells
Der f:	Dermatophagoides farinae
Der P:	Dermatophagoides pteronyssinus antigen
DMEM:	Dulbecco's Modified Eagle's Medium
DOK1:	Docking protein 1
DPBS:	Dulbecco's Phosphate-Buffered Saline

DTT: Dithiothreitol

EBNA1: Epstein-Barr virus nuclear antigen 1

EBV: Epstein-Barr virus

EF1: Elongation factor 1

enh MLP: Adenovirus major late promoter enhancer

ER: Endoplasmic reticulum

ERK: Extracellular-signal-regulated kinase

Fab: Antigen binding fragment

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

Fc: Crystallisable fragment

FcεR: Fc epsilon (IgE) receptor

FcγR: Fc gamma (IgG) receptor

Fel d: Felis silvestris catus antigen

FOXP3: Forkhead box P3

G-CSF: Granulocyte-colony stimulating factor

Gab2: Grb2-associated binding like protein 2

GE2: IgG-IgE fusion protein

GM-CSF: Granulocyte macrophage -colony stimulating factor

GSH: Reduced glutathione

GSSG: Oxidized glutathione

HDC: Histidine decarboxylase

hEF1- HTLV: Hybrid of elongation factor 1α and human T-cell leukemia virus

HEK-293: Human embryonic kidney cells-293

hIgE: Human immunoglobulin E

hIgG: Human immunoglobulin G

HRP: Horseradish peroxidase

IFN- γ : Interferon gamma

IgA: Immunoglobulin A

IgD: Immunoglobulin D

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IgM: Immunoglobulin M

iHDAC: Histone deacetylase inhibitor

IL: Interleukin

IMAC: Immobilised metal affinity chromatography

InS (1,3,4,5)P₄: Inositol (1,3,4,5)-tetrakisphosphate

IP3: Inositol 1,4,5-triphosphate

ITAM: Immunoreceptor tyrosine-based activation motif

ITIM: Immunoreceptor tyrosine-based inhibition motif

LB medium: Lysogeny broth medium

LIF: Leukemia inhibitory factor

LT: Leukotriene

MAPK: Mitogen-Activated Protein Kinase

MC-CPA: Mast cells-carboxypeptidase

MGY: Minimal Glycerol media

MM: Minimal Methanol media

MP: Microparticles

MTA: Material transfer agreement

NFAT: Nuclear factor of activated T-cells

NGF b: Nerve growth factor

NR: Non-reducing

NRB: Non-reducing buffer

OriP: Origin of replication

PAF: Platelet activating factor

PAGE: Polyacrylamide gel electrophoresis

PEI: Polyethyleneimine

PGD₂: Prostaglandin D₂

Phl p: Phleum pratense antigens

PIK3: Phosphatidyl- inositol 3-kinase enzyme

PLC: Phospholipase C

PtdIns: Phosphatidylinositol

PVDF: Polyvinylidene difluoride

R: Reduced

RASGAP: RAS GTPase activating protein

RB: Reducing buffer

RBL: Rat basophilic leukemia

SCF: Stem cell factors

SD: Standard deviation

SDS: Sodium dodecyl sulfate

SH2: Src homology domain 2

SHIP: *Src* homology 2 (SH2) domain-containing inositol 5-phosphatase

SIT: Allergen-specific immunotherapy

SLE: Systemic lupus erythematosus

SLP76: Leukocyte-specific phosphoprotein of 76 kDa

βGlo pAn: Beta-globin 3'UTR and polyadenylation sequence

SV40 pAn: Simian Virus 40 late polyadenylation signal

SYK: Spleen tyrosine kinase

TBS-T: Tris buffered saline with tween 20

TEV: Tobacco etch virus

TGFβ: Transforming growth factor beta

Th: T helper cells

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

v/v: Volume per volume

VEGF: Vascular endothelial growth factor

VL: Variable light chain

w/v: Weight per volume

WT: Wild type

YPD: Yeast Extract Peptone Dextrose Medium

YPDS: Yeast Extract Peptone Dextrose Medium with sorbitol

Chapter 1. General introduction

1.1 Allergy: Background

The term 'allergy' is defined as an alteration in immune response to foreign substances followed by certain immunologic reactions^{1,2}. These foreign substances are called allergens. Regardless of age, gender or genetic background, allergy can happen to anyone. A number of factors, including genetic and environmental, have been identified as triggers for the development of allergy and asthma. Although it is known that asthma is associated with certain genetic backgrounds^{3,4}, environmental factors such as indoor and outdoor air pollution, diet, tobacco use, occupational exposure and respiratory infection also play an important role in the pathogenesis of asthma and allergy⁵.

Allergies are very common in children and range from a mild symptomatic disease which requires only a topical treatment, to a severe life-threatening or debilitating condition that needs constant treatment and counselling². In some studies, a validated questionnaire tool is used to measure the effect of allergies on the quality of life; such studies conclude that allergies have a major negative effect on both adults and children. Allergies not only have an adverse effect on the quality of life for children, but also for their families and carers⁶⁻⁹.

Several epidemiological studies on the prevalence of the allergic diseases have been performed in different parts of the world¹⁰⁻¹². It is reported that the prevalence of asthma and allergies in USA and Europe is increasing and is accompanied by a rise in some allergic diseases such as atopic eczema and rhinoconjunctivitis^{13,14}. In the UK, there was a rise in admission rates to hospitals due to certain forms of systemic allergies from January 1990 to April 2003. Anaphylaxis and food allergies have the

highest increase in admission rates, as they rose from 6 to 41 per million and 5 to 28 per million respectively during that period^{15,16}.

1.1.1 Diseases related to the activation of the allergic cells

Although the allergic disorders are not similar in term of the associated symptoms, locations and the severity scale, they share some common features at the cellular and molecular level. For example, the allergic reaction that developed in case of asthma, allergic rhinitis and atopic dermatitis is mediated by the crosslinking of immunoglobulin E on the surface of mast cells¹⁷. Moreover, the inflammatory mediators (histamine, lipid mediators and interleukins) which released from the activated cells are also common in most of the allergic disorders. Here we describe some common examples of allergic manifestations.

- **Asthma** is a chronic inflammation of the airways, associated with recurrent blocking of airflow, constant airway hypersensitivity and airway reshaping¹⁸. The aetiology of asthma is complicated and interconnected between genetic factors and environmental influencers. The hypothesis that T helper type 2 (Th2) cells are to a large extent implicated in asthma comes from findings from an original paper explaining the functional effect of CD4 T helper cells combined with studies that presented the findings that Th2 cells were found in airways of asthmatic people^{19,20}. These results, alongside the connection between Th2 cytokines such as IL-4 and IL-13 and allergic diseases, helped in the development of this hypothesis.
- **Rhinitis** is an inflammatory disease of the mucosal membrane of the nose and is identified by one of three nasal symptoms: itching, sneezing and congestion. It is often associated with symptoms of the eye, ear and throat²¹. Adults are often faced with the complications of chronic rhinitis such as a

sleep disturbance and a limitation of activity. Children may experience behavioural disorders, lack of concentration, irritability and a loss of interest in social and educational activities²². In 15% to 38% of patients with allergic rhinitis, the disease gradually progresses from the nasal mucosa and descends down the respiratory tract ending up in allergic asthma – part of the so-called ‘allergic march’²³.

- **Atopic dermatitis** is a chronic inflammation of the skin linked by a change in the morphology of skin lesions and tissue remodelling. Dendritic cells (DCs) and keratinocytes play a crucial regulatory role in skin pathology of atopic dermatitis. Pruritus and sleep disorder are key signs of atopic dermatitis²⁴.
- **Anaphylaxis** is ‘a severe, life-threatening, generalised or systemic hypersensitivity reaction’¹⁷. It is identified by the development of sudden and often fatal difficulties in circulation, airways or breathing, or a combination of the three. Generally, it is associated with changes in the skin and mucus²⁵. Immunologic anaphylaxis involves the activation of mast cells and basophils by IgE or is as a result of complex- or complement-mediated reactions. Non-immunologic anaphylaxis occurs when the mast cells and basophils are activated through non-immunologic mechanism such as non-steroidal anti-inflammatory drugs, exercise and cold exposure²⁶.
- **Urticaria** is a heterogeneous family of disorders distinguished by the rapid development of pruritic and swollen skin lesions or angioedema^{27,28}. Urticaria is generally classified as acute or chronic depending on whether the symptoms persist for less or more than 6 weeks. Physical urticaria represents a presence of external influencing stimuli that induce lesions²⁷. Activation of histamine receptors in mast cells can lead to vascular changes in urticarial

lesions such as an increase in the permeability and dilatation of the cutaneous blood vessels. In many cases, the cause is not known – this is termed chronic idiopathic urticaria²⁹.

1.2 Hypersensitivity

There is a widely accepted classification of hypersensitivity developed by Gell and Coombs³⁰ in which hypersensitivity is subdivided into four categories. The first three types of hypersensitivity are recognized as an antibody mediated response (IgE in type I and IgG in types II and III) while Type IV is mediated by T cells (helper or cytotoxic T cells). Although the hypersensitivity classification is based on the underlying mechanism and the period of the hypersensitivity reaction, there are some similarities between the four types. The common characteristics are: 1) the immune response in all types is adaptive and regulated by both T and B cells; 2) the inflammatory reactions occur after the re-exposure to the allergen; 3) due to the adaptive immune response, sensitization to the allergen can persist for a long time in the absence of the subsequent exposure.

1.2.1 Type I hypersensitivity

Type I hypersensitivity reaction is the most common type. It is a mostly IgE-mediated reaction which involves an immediate response after exposure to the allergen. Diseases or manifestations such as hay fever, anaphylaxis and allergic asthma are typical cases of type I hypersensitivity. In this category, tissue mast cells and blood basophils are stimulated through the binding between IgE and allergen binding site on the surface of these cells. This binding will cause degranulation of these cells and will release active preformed or *de novo* synthesised pharmacological mediators such as histamine, platelet activating factor (PAF), prostaglandins, and leukotrienes³⁰.

Some mediators of activated mast cells such as lipid mediators and vasoactive amines induce a rapid increase in the permeability of the vessels and the contraction of the smooth muscle which occurs within minutes of antigen exposure. In late phase reaction, the cytokines and chemokines are involved in this reaction which occurs over several hours³¹.

1.2.2 Type II hypersensitivity

In type II hypersensitivity, also known as cytotoxic or antibody-dependent hypersensitivity, IgG and IgM are directed toward antigens on the surface of host cells. This binding produces immune complexes and complement activation that leads to cell lysis. The proliferation of B cells with the production of cytokines and antibodies will trigger the cell death. Autoimmune diseases, transplantation and drug reaction can be linked with type II hypersensitivity^{31,32}.

1.2.3 Type III hypersensitivity

Type III hypersensitivity is also caused by IgG and IgM antibodies. Unlike a type II reaction, in type III hypersensitivity, the soluble antigens are not bound with host cells but with antibodies in the bloodstream, which then cause an inflammatory reaction. When the level of antigen-antibody complexes is increased, they can precipitate in joints and different tissues such as the skin, eyes, and kidney, resulting in complement activation and inflammatory reactions³³. This reaction can also be associated with some systemic diseases such as systemic lupus erythematosus (SLE) and serum sickness³⁴.

1.2.4 Type IV hypersensitivity

Type IV hypersensitivity is also defined as a cell-mediated or delayed hypersensitivity. It usually takes two or more days to develop and is the second most common type of

hypersensitivity response. Type IV reactions occur when T cells are overstimulated and release cytokines to activate monocytes and macrophages leading to inflammation and cell death³⁵. Contact dermatitis is an example of type IV reaction in which lymphocyte production can develop local inflammation such as a rash, which identifies this skin disorder³⁶.

1.3 IgE mediated allergy: cells and molecules components

As stated before, IgE-mediated allergy (type I hypersensitivity) is the most common type of allergy, and the allergic response is dependent on the presence of four critical components: the allergen, the corresponding specific (a.k.a. cognate) IgE, the high affinity IgE receptor and the allergic effector cells (mast cells or basophils).

1.3.1 Allergen

Allergen is defined as any protein or substance that is able to cause allergic reaction, mostly by activating the IgE-mediated response. It can be found in certain foods, tree or grass pollen, moulds latex, and animal sources such as dander dust mite fecal pellets. There are a variety of ways in which these allergens can enter the body, including ingestion, inhalation, injection or by skin contact². It is not fully understood why some but not all substances can cause an inflammatory reaction, but there are some shared features in these allergens as they are all glycoproteins, several allergens have low molecular weight and are highly soluble in body fluid³⁷. These allergens can be considered as major allergens if they can produce an allergic response in a majority of patients. Der p I and Der p II from house dust mite, Fel d I from the cat, Bet v I from pollen of the birch tree and Phl p 1 and Phl p 5 from timothy grass are examples of major allergens³⁸. There is also a relationship between the type of allergic disorders and the type of allergen; asthma can be induced from house dust mites and cat allergens, while hay fever is caused by allergens derived from grass or tree pollen³⁹.

1.3.2 Immunoglobulin E (IgE)

IgE is an immunoglobulin with a molecular weight of ≈ 190 kDa which is thought to play a significant role in the host defence against parasitic infection. Compared to other immunoglobulins isotypes, IgE cannot cross the placenta and does not show complement activation by the classical pathway⁴⁰. IgE is regarded as the lowest serum antibody with a concentration of 50-200 ng/ml compared to 10 mg/ml of IgG. This lower concentration represents 0.004% of total circulating immunoglobulins. Moreover, IgE has the shortest half-life in serum of approximately ≈ 1 to 2 days in human and ≈ 5 -12 hours in mice⁴¹. However, the half-life of cell-bound IgE is much longer. People with atopic disease, which is generally developed due to individuals genetic susceptibility, such as asthma, hay fever, eczema, parasitic (e.g. helminthic) diseases or Hyper IgE Syndrome (HIES)²¹, have a higher level of IgE in the circulation (10 times the normal level).

Like the monomer of other human immunoglobulins, IgE consists of two identical heavy chains (H) and two identical light chains (L) with variable (V) and constant (C) regions joined together by disulphide bridges. The heavy chain of IgE is composed of four epsilon (ϵ) constant domains (C ϵ 1-4) with one variable region. The pair of variable regions of the light and heavy chains, which are responsible for the binding with the antigen, and the adjacent constant chain is known as the Fab region. the Fc region is consisting of the remaining constant domains which contain the binding site for the IgE receptor (Fc ϵ R) see Figure (1-1)⁴². The synthesis of IgE from B cells and plasma cells is made up of different combinations of (VDJ) regions and this process is influenced by activated T cells which produce IL-4 or IL-13 and CD40 ligand that enhance the transcription of the germ line C ϵ that leads to isotype switching to IgE^{21,43}.

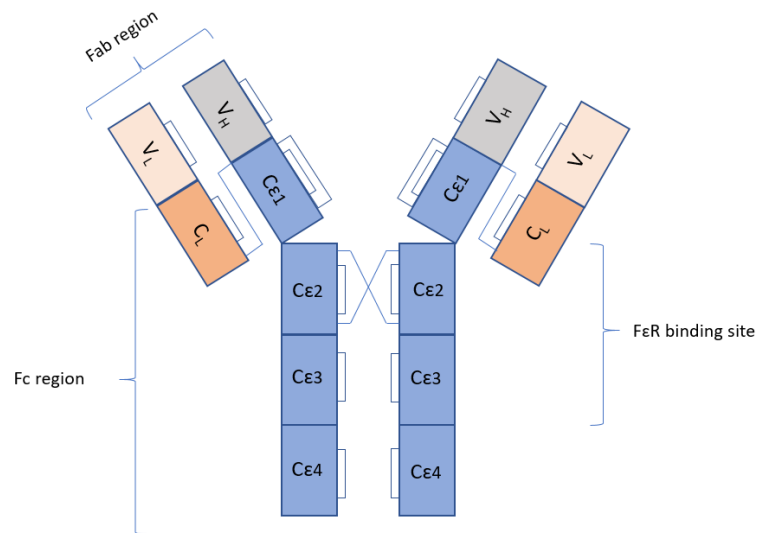


Figure 1-1 : Diagrammatic structure of an IgE molecule.

1.3.3 IgE high affinity receptor (FcεRI)

1.3.3.1 Structure and function

FcεRI, also known as the high-affinity IgE receptor, is one of the two known receptors for the Fc domain of IgE. The interaction between FcεRI and IgE is characterised by a slow dissociation rate ($k_{-1} < 10^{-5} \text{ s}^{-1}$) and accounts for the high affinity binding (see Section 1.3.3.2). The structure of FcεRI differs between humans and mice. In mice, it is a tetrameric receptor complex ($\alpha\beta\gamma_2$), whereas in humans FcεRI is expressed as $\alpha\beta\gamma_2$ tetrameric complex on mast cells and basophils or as $\alpha\gamma_2$ trimeric complex in antigen-presenting cells (see Figure 1-2). The two extracellular domains of the α chain are responsible for the binding of IgE. The β chain, which has four transmembrane domains that separate the amino and carboxy terminal tails both facing the cytoplasm, acts as an inducer for the maturation of FcεRI and as an amplifier for the transduction of signals. The two disulphide-connected γ chains contain a transmembrane domain and cytoplasmic tail, and are important for signal

transduction. The β - and γ - chains are critical in propagating the downstream signal by tyrosine phosphorylation of their immunoreceptor tyrosine-based activation motif (ITAM)^{44–46}. Activation of mast cells and basophils through crosslinking of IgE-Fc ϵ RI with polyvalent antigen will lead to degranulation of effector cells and the production of inflammatory mediators and vasodilators⁴⁷.

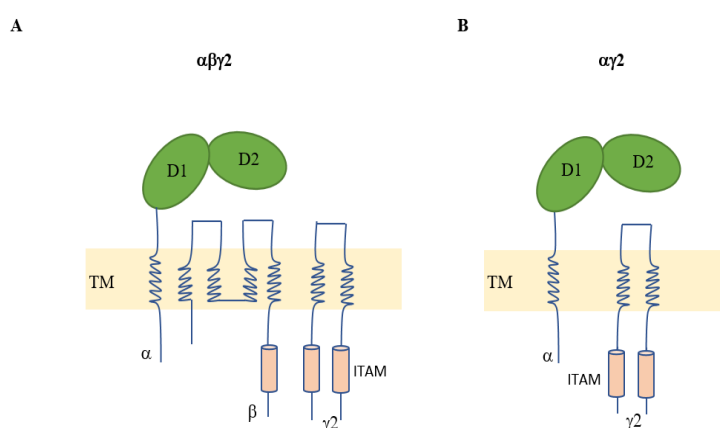


Figure 1-2 : The schematic structure of Fc ϵ RI.

(A) represents the tetrameric structure of Fc ϵ RI while (B) represents the trimeric structure.

1.3.3.2 IgE- Fc ϵ RI binding

The structural binding between the Fc domain of IgE and the α chain of Fc ϵ RI is a vital early step of the allergic reaction, and has been explored in countless studies^{48–50}. The stoichiometry of the binding is 1:1 and it is considered high affinity ($K_d = 10^{-9} - 10^{-10}$ M). The analysis of the X-ray crystal structure has shown that the interaction of IgE with its receptor involves C ϵ 3 domains and the C ϵ 2–C ϵ 3 linker residue of IgE with no involvement of C ϵ 4 domain. While, Fc ϵ RI has two different binding sites for IgE; the first is around Y131 at the C-C' region of the D2 domain of the receptor with one of the C ϵ 3 domain, and the second involves the interaction between the cluster of four

tryptophan residues at the receptor D1-D2 interface with P426 of the second C ϵ 3 domain (see Figure 1-3)⁴⁸⁻⁵⁰.

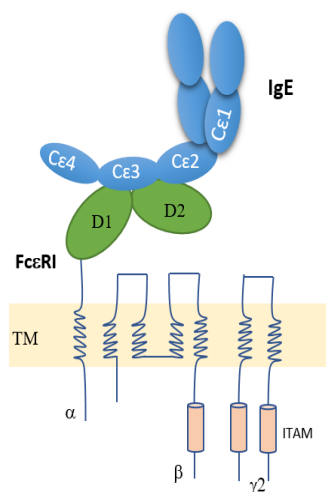


Figure 1-3 : Schematic diagram representing the binding complex of IgE molecule and high affinity IgE receptor (Fc ϵ RI).

1.3.3.3 The role of IgE in Regulating Fc ϵ RI expression

The expression of high affinity Fc ϵ RI on the surface of mast cells and basophils is normally regulated by the presence of receptor subunits ($\alpha\beta\gamma$ chains) and the quantity of IgE⁵¹. The initial insight on the significant role of IgE on regulating the expression of Fc ϵ RI was by Malveaux *et al*⁵² who observed a direct relationship between the level of serum IgE and basophil-bound IgE. The expression of Fc ϵ RI was also shown to be increased by monomeric IgE^{53,54}. Later, other groups of researchers who worked on IgE-deficient mice also found that incubation of IgE *in vitro* or injection of IgE *in vivo* could upregulate the low Fc ϵ RI expression⁵⁵. Many reports noticed a similar effect of IgE on the expression level of Fc ϵ RI on different allergic- and antigen-presenting cells (mast cells, basophils and dendritic cells(DCs)), which led to enhanced function⁵⁶⁻⁵⁹. Using anti-IgE antibody during the treatment of atopic diseases will reduce the level

of both serum IgE and FcεRI expression on basophils⁵⁶. The reason for the ability of IgE to upregulate the expression of FcεRI is due to the stability of the receptors on the cell surface by its ligand. This stability results in prevention of receptor internalisation and subsequent degradation while preserving the underlying synthesis; this will permit the receptors to be accumulated on the cell surface⁵¹.

1.3.4 Cells of allergic reactions: mast cells and basophils

Mast cells and basophils are regarded as critical effector cells in the allergic response. Both express tetrameric high affinity IgE receptors (FcεRI) on their surface and contain many granules loaded with a considerable amount (2-6 pg/cell) of preformed histamine^{60,61}. The release of histamine and other chemical mediators of inflammation from these cells is an important early step in the complicated sequences of cellular and molecular events which promote the allergic reaction^{62,63}.

1.3.4.1 Mast cells

Origin and development

Human mast cells originate from CD34⁺ hematopoietic progenitor cells in bone marrow. They circulate in the bloodstream as immature cells, and their maturation is completed in the connective tissues or on mucosal surfaces under the effect of stem cell factor (SCF) which binds to the c-kit receptor. Some cytokines such as interleukin 4 (IL-4), IL-5, and interferon gamma (IFN-γ), have also an effect on the maturation of mast cells, and SCF stimulates the adhesion, migration and proliferation of mast cells⁶⁴. Increased numbers of mast cells are associated with the presence of an immediate hypersensitivity response⁶⁵. A wide range of membrane receptors is expressed by mast cells; for example, high affinity IgE receptor, complement receptors, histamine receptors (H₁, H₂ and H₄) and toll-like receptors (TLR).

Activation and function

Mast cells can be activated through crosslinking of FcεRI or via complement receptors or TLR stimulation (see Figure 1-4)^{65,66}. During activation, mast cells release different types of mediators such as preformed mediators (histamine, proteoglycans and neutral proteases), or *de novo* produced substances, such as lipid mediators (leukotriene C₄ (LTC₄), platelet activating factor (PAF), prostaglandin D₂ (PGD₂)), cytokines, chemokines and growth factors. Mast cells also activate macrophages and epithelial cells to produce lymphocyte and eosinophil chemoattractants, and via the production of IL-4 they initiate the development of Th2 cells⁶⁷. Stimulation of mast cells through IgE-dependent mechanisms can also promote consequences of events leading to immediate hypersensitivity reaction and late phase reaction⁶⁸.

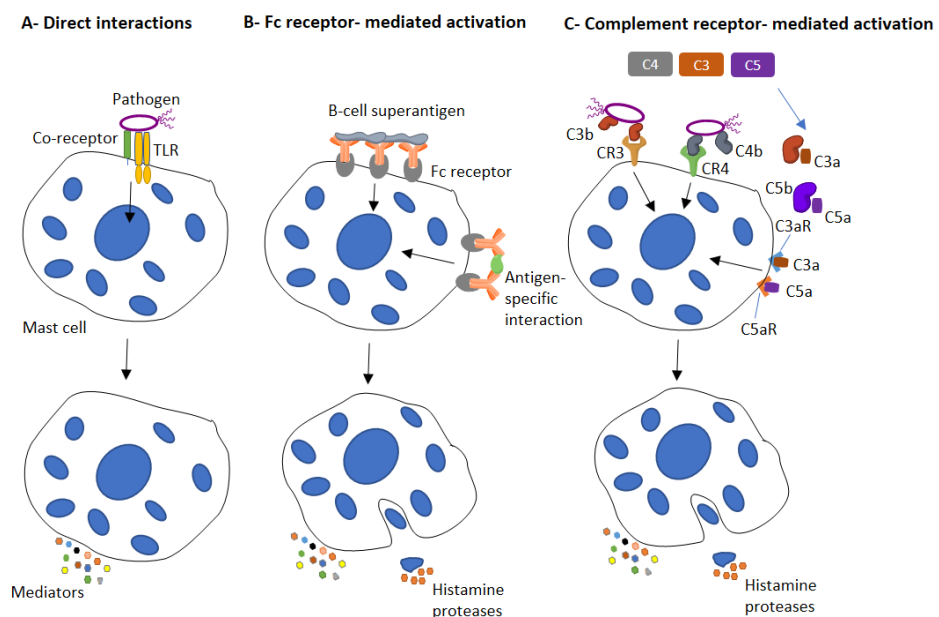


Figure 1-4: Different types of receptors are involved in the activation of mast cells.

1.3.4.2 Basophils

Origin and development

Basophils are granulocytes that account for less than 1% of all white blood cells. They also originate from CD34⁺ hematopoietic stem cells and complete their proliferation and maturation in the bone marrow, before circulating as mature granulocytes in the periphery. IL-3 is the predominant cytokine responsible for their development and differentiation, acting via the CD123 receptor which is highly expressed on this cell type⁶⁹. Although basophils and mast cells have many features in common such as expression of the high-affinity IgE receptor (FcεRI), histamine release and Th2 cytokines expression, there is a wide agreement that basophils are found in different cell lineages to mast cells⁷⁰. Thus, the description as 'circulating mast cells' frequently found in older scientific articles and immunology textbooks is obsolete and misleading, and modern experimental work indicates that their function is complementary and non-redundant.

Activation and function

Like mast cell activation, basophils can be activated through cross-linking of a functional FcεRI receptor ($\alpha\beta\gamma_2$), leading to granule exocytosis and mediator release⁴⁴. Basophils express complement receptors such as C3aR and C5aR and can be also strongly activated via C3a and C5a. Stimulation of any of these receptors will induce the synthesis of eicosanoids such as LTC₄, gene expression of IL-4 and IL-13 and histamine release^{71–75}. Unlike mast cells, PGD₂ and IL-5 are not released by basophils. The discovery of 2D7 and BB-1, two monoclonal antibodies specific for unknown antigen and basogranulin, respectively, has led to detection of basophils by Immunohistochemistry in tissues during the late phase reaction; it is now documented that basophils recruit into the tissue such as inflamed lung⁷⁶, skin⁷⁷, or

nose⁷⁸. The detection was confirmed by measuring the concentration of mast cells and basophil-specific biochemical mediators, the histamine (released from both), PGD₂ (from mast cells only) and LTC₄ (from basophils and mast cells)^{79–81}.

In summary, the overall outcome of the activation of these cells is: the release of stored mediators (e.g. histamine, heparin, chondroitin sulphate) and the de novo synthesis of cytokines and arachidonic acid metabolites (lipid mediators).

1.4 Signalling event of IgE cell activation

FcεRI cross-linking through IgE by a multivalent allergen originates a sequence of biological actions beginning with Lyn tyrosine kinase (member of Src family kinases) that has phosphorylation action on the ITAM motifs of both β- and γ- chains of FcεRI^{82,83}. After the phosphorylation of ITAM motifs, the cytoplasmic signals are propagated by the activation of Src homology domain 2 (SH2) containing enzymes such as the protein kinases Lyn and Syk. Other proteins including SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa (SLP76), phospholipase C (PLC) γ1, γ2 and Vav are then phosphorylated by the stimulated Syk⁸⁴. According to Parravicini *et al.*, aggregation of FcεRI will lead to Fyn activation which results in phosphorylation of the adapter protein Grb2-associated binder-like protein 2 (Gab2). Subsequently, Gab2 will activate phosphatidylinositol 3-kinase (PIK3) via the binding with p85 subunit to generate phosphatidylinositol-3,4,5 triphosphate (IP₃) which in turn recruit some of pleckstrin homology (PH) domains such as Btk and PLC-γ to the membrane. The phosphorylation of PLC-γ will mediate the hydrolysis of PIP₂, producing the calcium releasing-second messengers IP₃ and 1,2-diacylglycerol and activate protein kinase C (PKC)⁸⁵. Eventually, the binding between IP₃ and some specific receptors on the surface of the endoplasmic reticulum leads to Ca²⁺ depletion,

and a short transient increase of cytosolic calcium, which in turn results in a large influx of extracellular calcium through the activation of store-operated Ca^{2+} current (CRAC)⁸⁶. Other studies have also shown that the early signal events of FcεRI will lead to stimulation of ERK-associated MAPKs (Vav, Raf1, MEK) and GTPases (Rac, Ras, Rho). These processes will activate MAPK pathways, which in turn lead to granule exocytosis and production of leukotrienes and cytokines^{87,88} (Figure 1-5).

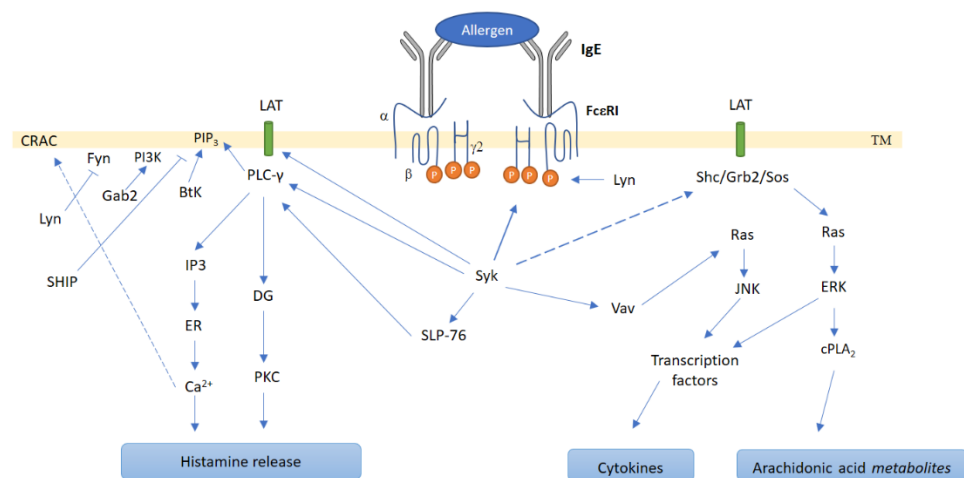


Figure 1-5: The signal pathway of IgE receptor (FcεRI) crosslinking.

The first event after the cross-linking of FcεRI is activation of different tyrosine kinases such as Lyn. Following the activation of Lyn, the β- and γ-subunit of immunoreceptor tyrosine-based activation motifs are phosphorylated. Lyn also stimulates Syk and leads to signal propagation. Once Syk is activated, PLC/ calcium pathway, the small GTPase pathway, the MAPKs pathway and the phosphatidylinositol 3-kinase (PI3K) pathway are different ways for the signal transduction. Eventually, all of these steps will lead to an increase the release of intracellular calcium, triggering cell degranulation and cytokine production.

1.5 Inflammatory mediators

Mast cells and basophils encompass functional mediators that have several potential actions in inflammation and tissue remodelling. In granules in the cell's cytoplasm, some of these mediators are deposited preformed, while others are formed *de novo* upon suitable cell activation. Cytokines, recently identified as inflammatory mediators, can also be produced either as preformed (TNF α in mast cells and perhaps small amounts of IL-4 in basophils)⁷³, or synthesized mediators of activated cells^{89,90} (Table 1-1).

Table 1-1 : major mediators known to be released from mast cells and basophils adapted from Gri et al⁹¹.

Class	Mediators	Physiological effects
Preformed		
Biogenic amine	Histamine 5-hydroxytryptamine	Vasodilatation effect Leukocyte regulation, pain, vasoconstriction
Proteoglycan	Heparin, heparin sulphate Chondroitin sulphate	Angiogenesis, coagulation Tissue remodeling
Proteases	Tryptase Chymase MC-CPA/ Carboxypeptidase A Cathepsin B,C,D,E,G,L	Inflammation, pain, tissue damage, PAR activation Inflammation, pain, tissue damage Enzyme degradation Pathogen killing, tissue remodeling
Lysosomal enzymes	β - hexosaminidase, β - glucuronidase	ECM remodeling
Cytokines	TNF α (mast cells), IL-4 (basophils)	Inflammation, leukocyte proliferation and activation immunoregulation
Others	Endothelin Kinins Granzyme B	Sepsis Inflammation, pain, vasodilatation Anti-inflammatory effects
Newly sensitised		
Lipid-derived	LTB ₄ [*] , LTC ₄ , PGD ₂ [*] , PAF	Inflammation, leukocyte recruitment, endothelial adhesion, smooth muscle cells contraction, vascular permeability
Cytokines	IL-1 α & β , IL-3, IL-4, IL-5 [*] , IL-6, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL14, IL-15, IL-16, IL-17, TNF α	Inflammation, leukocyte proliferation and activation immunoregulation

Chemokines	CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL16, CCL17, CCL19, CCL20, CXCL2, CXCL3, CXCL4, CXCL5, CXCL8, CXCL10, CX3CL	Leukocyte chemotaxis
Growth factors	TGF β , SCF, G-CSF, M-CSF, GM-CSF, VEGF, NGF β , LIF, bFGF	Growth of different cell types

*Indicate the mediators that are not produced from basophils.

1.5.1 Preformed mediators

1.5.1.1 Histamine

Histamine is considered an important mediator and is involved in many physiological and pathological responses such as neurotransmission and several other brain functions, secretion of hormones from the pituitary gland, and inflammatory conditions. The synthesis of histamine starts from histidine by the effect of histidine decarboxylase (HDC) within the Golgi zone⁹². When histamine is stored in cell granules it forms a complex with proteoglycans. In each basophil cell, there are about 2-4 pg of histamine, while in the lung mast cells, there are 3–6 pg^{93,94}. The level of histamine is regulated by diamine oxidase and histamine N-methyltransferase^{95,96}. In modified mast cell-deficient and congenic normal mice, studies show that mast cells are responsible for all the histamine deposited in normal tissue. However, mast cells do not account for histamine released from the central nervous system and glandular stomach tissues⁹⁷.

1.5.1.2 Neutral proteases

Neutral proteases are the main protein components that are secreted from the secretory granules of mast cells. Tryptase is regarded as the major protease of human mast cells and it is produced in α and β forms. The secretion of α -Tryptase is constitutive, and in mastocytosis, which is a condition associated with elevation of mast cells numbers, the level of α -Tryptase is also increased. On the other hand, β -Tryptase is secreted during exocytosis and represents a marker for activation of mast cells. Furthermore, the levels of tryptase are also raised in the serum of anaphylactic patients and in nasal discharges of patients experiencing allergic rhinitis. Tryptase has many roles *in vitro* that might be critical in the mechanism of asthmatic inflammation including blockage of fibrinogenesis and fibrinogen inactivation, positive regulatory effect on synthesis of IL-8 and (Intercellular Adhesion Molecule 1) ICAM-1 expression by epithelial cells of bronchi^{98,99}. Chymase, cathepsin-G, and carboxypeptidase A are also other serine protease that are found in the granules of some human mast cell. Based on the composition of neutral proteases in their secretory granules, human mast cells are classified as MC_T and MC_{TC} types. The MC_T cells, which are mainly distributed in the alveolar region of the lung and in the mucosa of small intestine, contain a measurable level of tryptase only. While the second type (MC_{TC}) contain both tryptase and chymase and they predominate in the skin and sub-mucosa of the small intestine⁶⁶.

1.5.2 Lipid mediators

Prostaglandin D₂ (PGD₂), and leukotriene C₄ (LTC₄) represent significant lipid mediators of mast cells^{100,101}. Both these products have bronchial constriction effect with short half-lives in plasma. The leukotriene D₄ (LTD₄) and leukotriene E₄ (LTE₄), which are the metabolic products of LTC₄, also have an impact on bronchial

constriction¹⁰². Prostaglandin D₂ is generated from arachidonic acid under the effect of cyclooxygenase enzyme (COX-1 and COX-2), while leukotriene is produced by the enzymatic action of lipoxygenase¹⁰³. Moreover, in allergen-challenged patients, there is an elevated level of PGD₂, its metabolite 9 α and 11 β -prostaglandin F₂ in the secretion of bronchi and in the urine of sensitised people to aspirin¹⁰⁴. Regarding production of arachidonic acid, human mast cells are different. For example, after stimulation, lung mast cells release leukotriene C₄ 10 times more than skin mast cells¹⁰⁵. Platelet activating factor (PAF) is another product of activated lung mast cells and basophils and is also released from other cells including macrophages, neutrophils, eosinophils, platelets and endothelial cells. PAF is a bioactive and potent inflammatory mediator and it is involved in several inflammatory disorders^{106,107}. PAF is inactivated by PAF acetyl hydrolase and deficiency of this enzyme is connected with development of asthma in some people¹⁰⁸.

1.5.3 Cytokines

As mentioned earlier, cytokines can either be stored in mast cells in granules or synthesized over a period of time after crosslinking of IgE^{89,90}. Evidence from several studies show that basophils are the main source of IL-4 and IL-13 after allergen challenge of peripheral blood cells⁷³⁻⁷⁵. Moreover, mast cell express a variety of multifunctional cytokines including IL-4, IL-5, IL-13 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) that are involved in the progression of asthma^{89,109}. Tumor necrosis factor alpha (TNF- α) derived from mouse mast cells plays an important role in the recruitment of neutrophils in inflammation of peritoneum and septic shock^{110,111}. From the nasal biopsy specimens of people with allergic rhinitis, it has been found that IL-4 and IL-6 have immunoreactivity with anti IL-4 and IL-6 antibodies and are localised mainly to mast cells, while IL-5 is located in mast cells

and eosinophils¹¹². Similar results were shown of elevated levels of cytokines such as IL-4 and IL-10 in the nasal fluid of children with seasonal allergic rhinitis¹¹³. Some cytokines such as IL-8, macrophage inflammatory protein-1 α and macrophage chemoattractant proteins act predominantly as chemoattractant substances for macrophages and circulating white blood cells¹¹⁴. However, they also stimulate the histamine release from mast cells, basophils and have the ability to activate eosinophils^{115,116}.

1.6 Immunoglobulin G (IgG)

IgG is the predominant protein among other immunoglobins types. This class of antibody represents 10-20% of plasma proteins and accounts for about 75% of total antibodies in the blood serum¹¹⁷. It is an important antibody in the secondary immune response to infection and able to cross the placenta to be an abundant antibody in the fetal blood with postpartum protection¹¹⁸. Additionally, IgG can promote the phagocytic effect, complement system activation and toxin neutralisation through the binding with Fc γ receptors on the surface of phagocytes (macrophage and DCs), or Natural killer (NK) cells¹¹⁷. This immunoglobulin can be classified into four subtypes (IgG1, IgG2, IgG3 and IgG4), termed in order of their relative amounts in the bloodstream¹¹⁷.

1.6.1 The structure of IgG

IgG structure is similar to other immunoglobulin isotypes in terms of having two light chains referred to as Kappa (K) or Lambda (λ) and two identical heavy chains with approximate sizes of 25 and 50 kDa, respectively, connected by inter-domain disulphide bridge. One variable region (VH), three constants regions (C γ 1-3) and a hinge region linking C γ 1 and C γ 2 are found in each heavy chain, while the light chain is made of one variable region (VL) and constant region (CL). The two variable domains of light and heavy chain (antigen binding site) with the next constant domain is called the F_{ab} region. The lower part of IgG, which comprises the hinge region with the constant fragments (C γ 2-C γ 3) is called F_c region (see Figure 1-6)¹¹⁹⁻¹²¹.

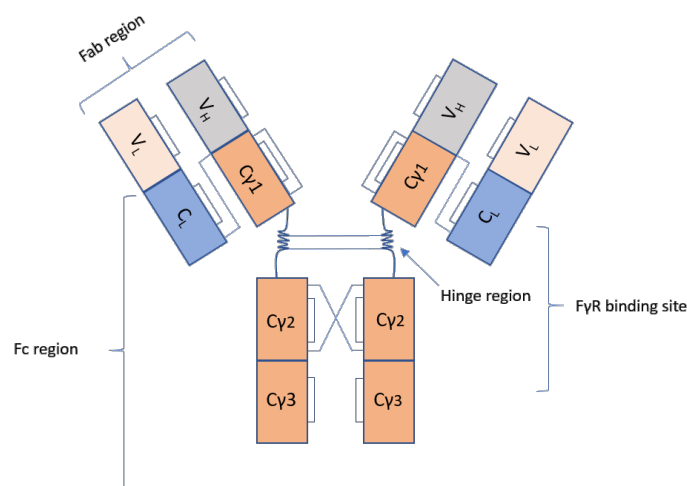


Figure 1-6 : Schematic representation of IgG structure.

The four IgG isotypes display a high similarity in overall structure and have over 90% homology in amino acid sequences. Each subtype of IgG is based on the variations of amino acid sequence in both hinge region and C γ 2 chain, and on the number of inter-heavy chain disulphide bonds¹²². These small variations have a critical impact on the binding profile to Fc γ receptor and C1q as the tertiary structural will be influenced

and having an effect on the properties of each isotype (see table 1-2)¹²²⁻¹²⁴. Regarding the antigen binding region, there is a variability in the amino acids residues between all IgG subtypes in their N- terminal domains of variable regions (heavy and light chains) as well as differences in the ratio of Kappa and Lambda light chains. these are considered as unique feature of each IgG subclass¹²⁵.

Table 1-2 : General properties of IgG subclasses.

Properties	IgG1	IgG2	IgG3	IgG4
Molecular weight (KDa)	146	146	170	146
Number of Amino acids (hinge region)	15	12	62	12
Number of inter-domain <i>disulphide</i> bonds	2	4	11	2
Relative abundance (%)	60	32	4	4

From Vidarsson *et al*¹²².

1.7 IgG Fc Receptors

FcRys are membrane proteins present on the surface of some immune cells. They bind selectively to the Fc region of IgG. In terms of their function, they are classified as activating receptors (FcγRI, FcγRIIA, FcγRIIIA And FcγRIIIB) or inhibitory receptors (FcγRIIB)¹²⁶. The group of activating receptors shows different binding affinities toward different subtypes of IgG molecule in humans. The relatively high affinity binding is present in FcγRI and low to medium binding affinity in FcγRIIA, FcγRIIIA and FcγRIIIB. The inhibitory receptor (FcγRIIB) is considered as a low affinity binding receptor to its ligand IgG¹²⁷. In activating type receptors, the effector cells are activated through an intracellular ITAM motif present on the cytoplasmic tail of the receptors. In receptors bearing an immunoreceptor tyrosine-based inhibitory motif (ITIM), such as FcγRIIB, the ITAM-mediated cellular activation will be inhibited. The

net effect of the positive and negative signalling of this activation will be mediating of the degranulation, mediator release in the inflammatory response, phagocytosis, antibody-dependent cytotoxicity (ADCC) and cytokine release or inhibition of these responses. The ITAM-mediated cellular activation will be inhibited. Because this receptor is central to the aim of this project, we will discuss in detail the inhibitory FcγRIIB receptor on the inflammatory cells^{47,128}.

1.7.1 Inhibitory FcγRIIB receptor

Structure and function

As stated above, FcγRIIB is an inhibitory low affinity receptor ($K_d > 10^{-7}$ M) for the Fc portion of IgG. It is expressed by several inflammatory cells such as mast cells, basophils, macrophages and neutrophils⁴⁷. There are three isoforms of FcγRIIB that have been shown to exist in humans and mice. Two of these, FcγRIIB1 and FcγRIIB2, are constructed from two extracellular Ig-like domains for binding to the IgG-Fc region, one trans-membrane domain and one cytoplasmic domain linked with (ITIM) motif. FcγRIIB3 is the soluble isoform of all these receptors and it is characterised by lack of the sequence from the transmembrane and the first cytoplasmic regions¹²⁶.

There are three inhibitory roles mediated by FcγRIIB; two of them are ITIM-dependent, such as the inhibition of cellular proliferation and calcium mobilisation, while one is ITIM-independent such as apoptosis. FcγRIIB also organises negatively innate and adaptive immune reactions by inhibiting the activating signal transductions by co-aggregation with a B cell receptor (BCR) or by activating Fc receptors. It has also been determined that FcγRIIB inhibits the activation of basophils, mast cells T cells and B cells; in FcγRIIB- deficient mice studies, the significance of inhibitory signalling of FcγRIIB in controlling immune reaction was confirmed, as gene targeted mice showed autoimmunity, anaphylactic reaction and elevated production of

antibodies¹²⁶.

IgG- FcγRIIB binding

Generally, the direct contact between FcγRII and IgG includes the second domain of FcγRII receptors. Some amino acid residues of the second Ig-like domain are involved in ligand interaction, such as 109-116, 130-135 and 154-161. It is also believed that D1-D2 interface of FcγRIIB is involved in IgG interaction sites, where the first domain will be essential for stabilisation of the binding, while the second domain will bind with the Fc portion of IgG. The results of mutagenesis experiments indicate that the lower region residues 234 to 237 of IgG have a crucial role in the binding with FcγRII and with FcγRI^{129,130}. It has been found from the crystal structure of the complex of hIgG1 with the extracellular domain of Fcγ receptors that the D2 domain of the Fcγ receptor is embedded between CH2 domains of IgG Fc portion (Figure 1-7). Due to the low affinity binding between IgG1-Fc region and FcγRIIB receptor, many studies have also attempted to increase the binding affinity. They have demonstrated that substitution of specific amino acids to the sequence of IgG-Fc region will result in a higher binding affinity¹³¹⁻¹³³.

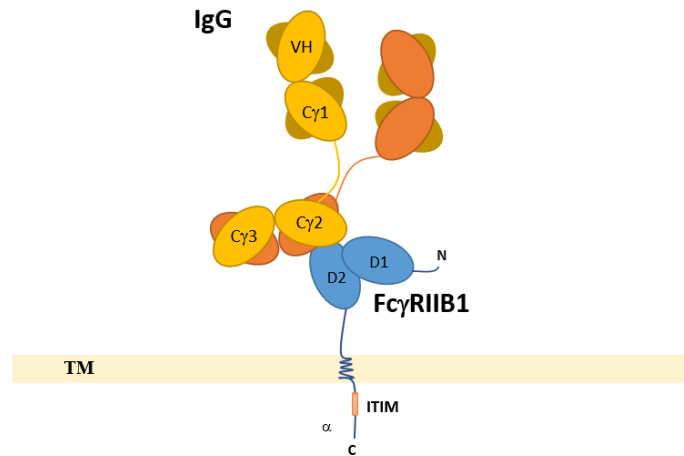


Figure 1-7: Schematic diagram of IgG-FcγRIIB interaction.

1.7.2 Co-aggregation of FcεRI and FcγRIIB

Co-aggregation of ITIM-containing receptors such as FcγRIIB with ITAM-containing receptors such as FcεRI and BCR will help in regulating and reducing the potentially dangerous activity of many immune cells, including mast cells and basophils. This results in a blockage of a variety of downstream biological activities of the cells that contain these receptors¹²⁶. The initial inhibitory signal event starts with tyrosine phosphorylation of ITIM in the cytoplasmic tail of FcγRIIB via Lyn kinase¹³⁴. This will lead to recruitment of the *src* homology 2 (SH2) domain-containing inositol 5-phosphatase (SHIP) 1, which has a dephosphorylating enzymatic action to decrease the level of phosphatidylinositol-1,3,5-trisphosphates. This eventually reduces the inflow of extracellular calcium into mast cells and basophils and inhibits the activating signals induced by FcεRI^{135,136}. Recent findings have offered insight into the mechanisms through which SHIP facilitates the inhibitory signal of FcγRIIB in mast cells. When co-engaging with FcεRI, FcγRIIB can inhibit calcium mobilisation that is induced by FcεRI but could not affect both the tyrosine phosphorylation of β- and γ-chains in FcεRI, and activation of Syk^{134,137}. The removal of the 5-phosphate from

PtdIns(3,4,5)P₃ (PIP₃) and Ins(1,3,4,5)P₄ is also catalysed by the enzymatic activity of SHIP¹³⁷, and recent studies indicate that SHIP has a direct role in the cleavage of PtdIns(3,4,5)P₃, which in turn leads to the prevention of the recruitment and activation of Btk and PLCγ1 and the signal events needed for FcεRI-mediated degranulation of mast cells¹³⁶ (Figure1-8).

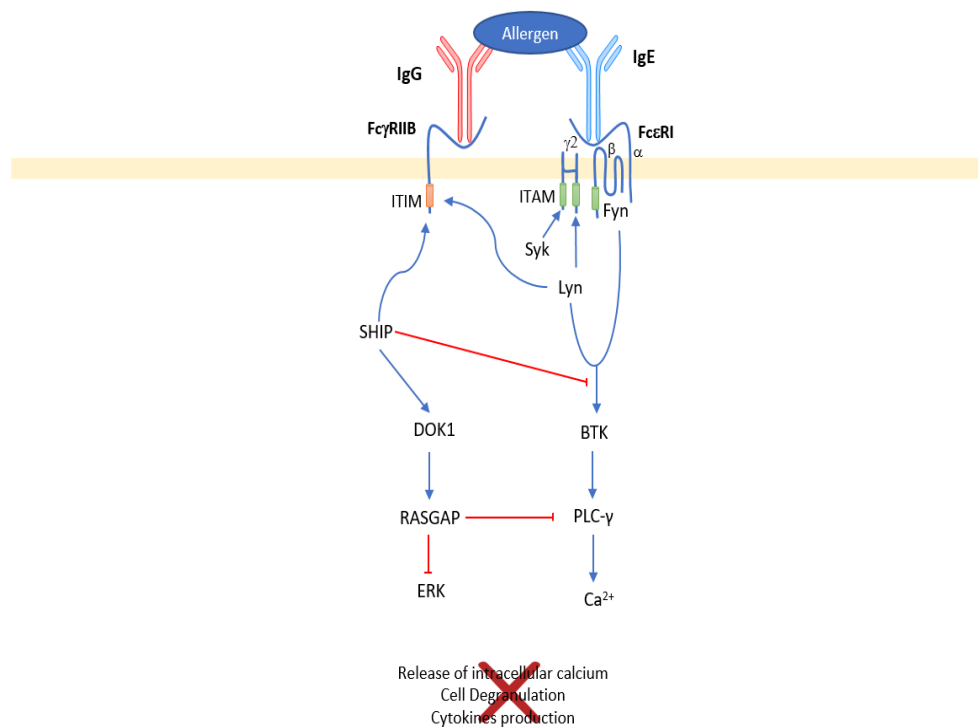


Figure 1-8 :Schematic diagram of inhibition of FcεRI signal transduction by FcγRIIB.

The first inhibitory signal event begins with tyrosine phosphorylation of ITIM in the cytoplasmic tail of FcγRIIb via Lyn kinase. The next step is recruitment of the src homology 2 (SH2) domain-containing inositol 5-phosphatase (SHIP) 1, which has an enzymatic action to decrease the level of phosphatidylinositol-1,3,5-trisphosphates. This ultimately reduces the inflow of extracellular calcium into mast cells and inhibits the activating signals induced by FcεRI.

1.8 Current approaches taking advantage of FcγRIIB-mediated inhibitory activity

The concept of FcγRIIB-mediated negative regulation is of great interest to many groups of academic and industrial researchers who seek to develop a new technique to treat IgE-mediated allergies. It was highlighted first by Daeron *et al.* on human basophils, seeing this as a potential target of immunotherapy¹³⁸. This concept was then applied further into models related to the biological system^{139–142}.

The first strategy was developed by using a human IgG-IgE fusion protein (GE2) composed of Fc regions IgE and IgG with linker (γHinge-CH₂-3-15aa linker-CH₂-4). This fusion protein has been shown to inhibit histamine release from human basophils and lung tissue fragments and reduce the activity of the tissue to the stimulation by allergen^{140–142}. Other therapeutic platforms were developed by designing a fusion protein with more focus on the enhancing of the FcγRIIB affinity. From these trials, one recent paper by Cemerski and colleagues has shown that using dual-targeting way with 100-fold increase in FcγRIIB affinity can inhibit the cell activation in more specific and effective way compared to the native IgG Fc region¹⁴³.

Two other immune-based therapies were established by fusing the human IgG Fc region and major allergens to form chimeric fusion protein (Fcγ- Fel d I) and (Fcγ-Der f2). The approach was to target the antigen-specific mediated IgE allergy. In the first platform (Fcγ- Fel d I), it was observed that the mediator release was inhibited in dose-dependent response in the basophils of donors allergic to cat Fel d I¹⁴². In the second therapeutic agent (Fcγ-Der f2), the expression levels of several inflammatory mediators were low after applying the treatment on the collected bronchoalveolar lavage fluid (BALF) from allergic mice. The level of anti-Der f2 antibodies (IgE and IgG-sub classes) from the blood sample was also low¹⁴⁴. Both recombinant fusion IgG

major allergens supporting these strategies were found to be functional as allergen-specific immunotherapies. Another approach is called 2G10 was designed to be a chimeric human IgG1 anti-idiotypic which has ability to bind to IgE via its Fab region while Fc region will deliver the inhibitory signalling through the binding with FcγRIIB receptor. This strategy shows a dose dependent inhibition of cells transfected with Fcγ receptors and purified basophils when these cells were activated with anti-IgE or Der p I allergen¹⁴⁵.

1.9 Current immunotherapies and their limitations

Several potential therapeutic approaches have been proposed to inhibit the activation of inflammatory cells and prevent the release of their mediators.

One of these is allergen-specific immunotherapy (SIT), which is used for the treatment of allergic rhinitis. It is dependent on regular subcutaneous injections (SCIT) or a sublingual tablet (SLI) of extract containing the allergen. This can reduce responsiveness to a specific allergen by initiating the synthesis of blocking of IgG4 antibodies and afford relief from symptoms associated with the allergy. SIT also produces a rise in the levels of both allergen-specific IgG4 and IgA antibodies, and a decline in the level of allergen-specific IgE antibodies¹⁴⁶. SIT can stimulate Treg cells (CD4⁺ CD25⁺ FOXP3⁺) that synthesize two cytokines (IL-10 and TGFβ) to a high level. These two cytokines are involved in the attenuation of T_H2- cells responses¹⁴⁷. Recently, one paper shows that the mechanism behind the inhibitory action of the allergen immunotherapy is belong to the production of IgG antibodies which stimulate the inhibitory function of FcγRIIB receptor and suggesting also that co-aggregation of FcγRIIA is required for the activation of the inhibitory signalling of FcγRIIB¹⁴⁸.

Omalizumab, a humanised IgG1 monoclonal antibody, has been also used to inhibit the inflammatory response of severe allergic asthma by the prevention of IgE binding to FcεRI. The inhibitory action of omalizumab depends on the binding to IgE with a higher affinity than the binding of IgE molecule to its high affinity receptor, and this leads to a decline in the level of the circulating IgE and inhibits its binding with FcεRI¹⁴⁹. However, omalizumab does not suppress the activating signals of mast cells that IgE molecules are already being cross-linked with a high affinity FcεRI^{150,151}. There are also others approaches of immune inhibitors that have been designed to inhibit kinases that are involved in the development and activation of mast cells and basophils; for instance Syk, Btk and c-kit^{152,153}. Some monoclonal antibodies against Th2 cytokines IL-4, IL5 and IL-13 and their receptors are also in the progress stage as a treatment option to control allergic inflammation¹⁴⁶.

Although the current immunotherapies have been shown to be an effective treatment for patients with allergies, many potential problems are associated with their use. Administration of allergen immunotherapy by subcutaneous injection is accompanied with problems in patient compliance, due to issues such as repeated swelling at the site of injection¹⁵⁴ and the year-long duration of treatment. Many studies have also reported an increase in the risk of anaphylactic shock and systemic reactions¹⁵⁵. SIT needs at least 3 years to produce a sufficient immunological action, and it is not effective with all types of allergy¹⁵⁶. Difficulties around the standardisation of allergen extracts are still an issue among manufacturers, and probably lead to a variation in the estimation of allergen extract potency¹⁵⁷. Regarding the use of omalizumab, some concerns have been raised about its safety in terms of the relationship between omalizumab and the increase of incidences of cancer, as IgE appears to be associated with protection against cancer in some studies^{158–161}. Some adverse effects have been

also identified following its use, such as local reactions (erythema, swelling and bruising) around the site of injections, fatigue, headache and nausea^{162,163}. Because of the previous disadvantages of conventional immunotherapy, the design of potent and selective immunotherapy with tolerable side effects is a necessity.

1.10 Microparticles as drug delivery platform

A microparticulate drug carrier is one of the possible methods that affords a sustained and controlled release of a therapeutic agent, and overcomes the difficulties of the traditional ways of administration. Developing vehicles in micron and submicron sizes to deliver new biologic medicines such as protein and nucleic acid has attracted huge interest recently. It is a viable option which leads to good patient compliance and a decrease in side effects¹⁶⁴.

The microparticles are small solid particles or liquid droplets surrounded by a polymeric membrane made from natural or synthetic material. They have a diameter range of 0.1 μm to 1000 μm with different degrees of thickness and permeability¹⁶⁵. Albumin microsphere was the first delivering agent and was proposed by Kramer in 1974, followed by using haemoglobin as a biodegradable vehicle for medicine administration¹⁶⁶. Microparticles are classified in two subcategories based on the method of preparation; microcapsule and microsphere. In a microcapsule, the drug is confined in the core and enclosed with a polymeric coat, while in a microsphere, the drug is dispersed uniformly on the matrix¹⁶⁷.

1.10.1 Microsphere for drug targeting

When the therapeutic agent is administrated orally or by injection, it will be distributed throughout the body by the effect of several intrinsic properties of the drug. Thus, it could interact at a site which is not required, causing undesirable site

effects. In such a case, it will be of great benefit if the drug could be directed to the specific site to enhance the therapeutic value with low toxicity profile. The modified microsphere is one such technique that has the potential to provide this effect¹⁶⁸. Many formulations of surface-modified microspheres with ligands which have been explored, and show a site specificity by binding to a selective receptor on the target cell which could be used to target, for example, cancerous cells, inflammatory cells or to increase the drug uptake into the circulation by enhancing the microsphere adhesion to the intestinal epithelium^{169–172}. One example of this formulation seeks to mimic the rolling adhesion behaviour of leukocytes to deliver the anti-inflammatory drugs to the site of inflammation. The targeted delivery system was a polylactic-co-glycolic acid (PLGA) microsphere modified with Sialyl Lewis^x (sLe^x)-biotin. This biotinylated ligand is directed to selectins on the surface of endothelial cells¹⁶⁹.

1.10.2 Potential advantages of using microparticles in immunology

Over the years, there has been increasing attention to using microparticles as a drug vehicle, either to enhance the immune response or to reduce autoimmune reaction.

In terms of vaccination against viral or bacterial infection, microparticles have been used for activation of cellular immunity by providing an adjuvant action through the development of antigen delivery to the immune cells. Microparticles have a role in enhancing the antigens' availability to the immune cells by acting as a depot to release them in a controlled way^{173,174}. They could also provide antigen integrity by protecting from degradation, especially in oral formulations¹⁷⁵. The type of immune response can also be modulated when microparticles are used alone or with other immune-activating agents¹⁷⁶. Numerous studies have shown that polymeric microparticles coated with antigens can offer an effective and sustained immune response against

some pathogenic bacteria such as *Pseudomonas aeruginosa*, *Bordetella pertussis* or *Vibrio cholerae*^{177–179}.

In hypo-sensitisation therapy, it is an essential to introduce new allergen vehicles to make the immunisation system simple, and to reduce the dose frequency to improve efficacy. Microparticles have been developed as new vectors to deliver the allergen. One of the efforts to develop this was investigating the integrity and activity of *Dermatophagoides pteronyssinus* (D.Pt) allergen entrapped in microparticles as the hyposensitising agent. The outcomes of the study show that following the microencapsulation, the integrity of Der p I (D.Pt allergen) was not affected, and the binding activity to IgG was not changed¹⁸⁰. Another trial of microparticle formulation was applied to improve the efficacy of the sublingual immunotherapy in a murine model of asthma. The uptake of allergen by dendritic cells was enhanced by a mucoadhesive chitosan microparticle which in turn increased the priming of specific T-cells in the cervical lymph nodes, and therefore reduced pulmonary inflammation¹⁸¹.

In an attempt to reduce organ rejection during the transplantation, a system based on microparticles was employed to enhance the immunological tolerance by stimulation of dendritic cell receptors via immunoglobulin-like transcripts (ILTs). Two ligands were coated on the surface of microparticles (HLA-G1 peptide and monoclonal antibody) targeted the inhibitory ILT4 receptor and CD11c marker respectively. Targeting these receptors will help to modulate the DC to have a tolerogenic role. This dual coated microparticle system could be useful in regulating specific immune reactions in transplantation and autoimmune diseases¹⁸².

1.11 Project aim and objectives

Due to the major drawbacks of conventional immunotherapy in the treatment of allergies, development of a selective and potent immunotherapy with acceptable adverse reactions is required.

The aim of this project is to create a therapeutic technique that specifically inhibits the activation of mast cells and basophils via co-aggregation of FcεRI and FcγRIIB, while avoiding the stimulating effects that are mediated by FcεRI crosslinking. This approach takes advantage of polymeric micro-surfaces which enable a defined stoichiometry of both ligands (IgE and IgG) to simultaneously engage their receptors (FcεRI and FcγRIIB).

This aim will be achieved through the following specific objectives:

- Creation of an IgG Fc variant with improved binding affinity to FcγRIIB. This step is essential to obtain a better stoichiometric ratio on the surface of microparticles.
- Incorporation of two tags (Avi and His) at the C-terminal of IgG Fc variants and IgE Fc constructs. This step allows for the purification of the proteins and to be biotinylated at a specific site (monobiotinylation).
- Recombinant expression of IgG and IgE Fc fragments from eukaryotic systems and purification using Immobilized metal ion affinity chromatography (IMAC). This is an essential part in the design of the proposed drug delivery system.
- Conjugation of biotinylated human IgG Fc variants and IgE Fc regions with streptavidin-coated microparticles. This step will be achieved by using

enzymatic biotinylation (BirA enzyme) to allow sequence-specific (mono)-biotinylation on Avi-tagged proteins.

- Testing the microparticles coated with IgE Fc region on the RBL cell lines.
The purpose of this step is to optimise the conditions (particle size, concentrations and Fc ligand density on the particle surface).
- Testing the proposed drug delivery system on purified human basophils.
This final step is to analyse the functionality of the delivery system and to see if the release of the cells' mediator (histamine) could be controlled.

Chapter 2. General Materials and Methods

This chapter provides the general material and methods that were used in this work. More details will be found in the experimental procedure sections of each subchapter. All purchased materials in this section are from Sigma Aldrich, UK unless otherwise stated.

2.1 Materials

2.1.1 Cells

Adherent HEK-293 cell line was purchased from ATCC® (cat no. 1573). RS-ATL8 luciferase reporter cells were obtained through a material transfer agreement (MTA) with Ryosuke Nakamura (National Institute of Health Science, Tokyo, Japan). NFATp-DsRed-Express2 fluorescence reporter cells were created in-house. Two strains of *Escherichia coli* were used for bacterial culture, XL10-Gold from Stratagene, UK and DH5α cells from Thermo Fisher Scientific, UK. For yeast expression, *P. pastoris* X-33 was kindly gifted by Dr Marcos Alcocer, University of Nottingham, UK.

2.1.2 Microparticles

Streptavidin conjugated microparticles, 0.5, 1.2 and 6 μm with stock particle concentration of 1.25 % w/v were purchased from Polysciences, Inc., Warrington, PA, USA.

2.1.3 Reagents, culture media and buffer solutions

2.1.3.1 Mammalian culture

Minimal essential medium (MEM) or Dulbecco's Modified Eagle Medium (DMEM) were used for adherent cell culture. FreeStyle F17 Expression Medium (Thermo Fisher Scientific, UK) was used for suspension cell culture. L-glutamine, heat-inactivated foetal bovine serum (FBS), penicillin-streptomycin and kolliphor P-188 were

supplemented to the media. Trypsin-EDTA and Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} or Mg^{2+} were used for cell maintenance.

2.1.3.2 Bacterial culture

For the bacterial cells growth and preparation of the competent cells, the following media and buffer solutions were used:

- **Lysogeny broth (LB) medium and agar:** 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract and NaCl in 1L distilled water. For LB agar, 1.5 % of bacterial agar is added to the LB medium. Next, all medium and agar are autoclaved to be sterilised and stored at room temperature and at 4 °C, respectively.
- **Inoue buffer:** In 1 L distilled water, 55 mM of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 15 mM of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 250 mM of KCl and 10 mM HEPES were added, and the buffer was sterilised using a 0.22 μm syringe filter and divided into 50mL falcon tubes and stored at 4°C.
- **Super optimal broth (SOB) medium:** 2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 in 1 L distilled water. This medium was autoclaved and stored 4°C.

2.1.3.3 Yeast culture

For the growth of *Pichia pastoris* cells and the preparation of the competent cells, the following media and buffer solutions were used:

- **YPD (Yeast Extract Peptone Dextrose) medium:** in 1 L final volume, 1 % (w/v) yeast extract and 2 % (w/v) peptone were dissolved in 950 mL distilled water and autoclaved. 2 % (w/v) filter-sterilised glucose was added to the medium and stored at room temperature.
- **YPBS (Yeast Extract Peptone Dextrose medium with Sorbitol) agar:** 18 %

(w/v) sorbitol and 2 % (w/v) agar were added to YPD medium before the autoclaving step, then 2 % (w/v) filter-sterilised glucose was added to the medium and stored at 4°C.

- **BMGY (Buffered Glycerol-complex Medium) & BMMY (Buffered Methanol-complex Medium):** in 1 L final volume, 0.5 % (w/v) yeast extract and 1 % (w/v) peptone, 100mM potassium phosphate buffer pH6, 1.34 % (w/v) yeast nitrogen base (YNB) and 0.4 µg/mL biotin were added and the media autoclaved. Next, 1 % or 0.5 % (v/v) of filter-sterilised glycerol or methanol were added to the BMGY and BMMY media, respectively.

2.1.3.4 Protein screening

For the analysis of proteins with SDS-PAGE gel and western blot, the following buffers were used:

- **10X SDS-PAGE running buffer:** 248 mM Trizma, 1.92 M glycine and 1 % (w/v) SDS were dissolved in 1 L final volume of distilled water to prepare 10X stock solution. The buffer was then diluted to 1X for running the gel.
- **2X Non-Reducing Sample Buffer (NRSB):** For 100 mL, 125 mM Trizma pH7, 20 % (v/v) glycerol, 4 % (w/v) SDS and 0.1 % (w/v) bromophenol blue were dissolved and the buffer kept at 4°C.
- **2X Reducing Sample Buffer (RSB):** prepared by adding 5% β-mercaptoethanol to 100mL of non-reducing sample buffer.

2.2 Methods

2.2.1 PCR and agarose gel electrophoresis

For all PCR work, RNase and DNase pyrogen-free materials were used, and these include sterile filter tips with strip PCR tube (STARLAB, UK) and ultra-pure water (Invitrogen, UK). All primers used in this project were designed using SnapGene software. These primers were purified according to the purpose of the PCR reaction. For cloning and sequencing application, the primers were desalted. For site-directed mutagenesis, they were purified with HPLC or PAGE. They were prepared with nuclease-free water to a concentration of 100 μM (see Tables 2-1 and 2-2 for amplification and cloning primers lists) and the final concentration of primers in the PCR reaction was 0.5 μM . All cycling was performed on a PTC-200 (MJ Research) Peltier gradient thermal cycler. For the thermal cycling conditions of PCR, see sections 3.2.1.2 and 4.2.1. Following the PCR reaction, agarose gel electrophoresis was used to analyse the PCR product. The gel was prepared by dissolving appropriate amounts of agarose powder in 0.5X Tris-borate EDTA (TBE) buffer (Thermofisher Scientific, UK) then the solution was microwaved until all the agarose powder was dissolved (usually 7-10 minutes). Following the heating step, the solution was cooled to 50-60 $^{\circ}\text{C}$ to and mixed with ethidium bromide at a final concentration of 0.5 $\mu\text{g}/\text{mL}$. Next, the solution was poured into the gel tray with a well comb and cooled at room temperature until the gel was made up. Different percentages of agarose gel (0.8 to 2 %) were prepared based on the size of the PCR products. The samples were loaded into the gel in parallel with a DNA ladder (1 kb or 100 bp, New England Biolabs, UK) and run at 100 volts for one hour. Finally, the gel was visualised using the Gene Genius Gel Imaging System (Syngene, UK).

Table 2-1: Amplification primers.

Primer Name	5'-3' Sequence
IgG Fc S267E/H268F FOR	GTGGTGGACGTG GAGTTC GAAGACCCTGAG
IgG Fc S267E/H268F REV	CACGCATGTGACCTCAGGGGTCC
IgG Fc S267E FOR	GTGGACGTG GAG CACGAAGACCCTGAG
IgG Fc S267E REV	CACCACGCATGTGACCTCAGGGGTCC
IgG Fc G236A FOR	CTGAACTCCTG CCCG GACCGTCAGTC
IgG Fc G236A REV	GTGCTGGGCACGGTGGGCATG
IgG Fc I332E FOR	CTCCCAGCCCC GAG GAGAAAACCATC
IgG Fc I332E REV	GGCTTTGTTGGAGACCTTGCACTTGACTCCTTGC
IgG Fc S324T FOR	CAAGTGCAAGGTC ACCA ACAAAGCCCTC
IgG Fc S324T REV	TACTCCTTGCCATTCAGCCAGTCCTGGTG
IgG Fc L328F FOR	CAACAAAGCC TTCC CAGCCCCATC
IgG Fc L328F REV	GAGACCTTGCACTTGACTCCTTGCCATTCAGC
IgG Fc S239D FOR	CCTGGGGGGACCG GAC GTCTTCCTCTTC
IgG Fc S239D REV	AGTTCAGGTGCTGGGCACGGTGGGCATG
IgG Fc Avi-His FOR	GAATGGCATGAACATCATCATCATCATCATTGAGTGCTAGCTGGCCAGA CATGATAAGATACATTG
IgG Fc Avi-His REV	GATCTTTTGAGCTTCAAAAATATCATTGAGTCCTTTACCCGGAGACAGGG AGAGGCTCTTC
IgE-Fc Avi-His FOR	GAATGGCATGAACATCATCATCATCATCATTGACGTACTCCTGCCTCCCTC TAGACCTAGCTG
IgE Fc Avi-His REV	GATCTTTTGAGCTTCAAAAATATCATTGAGTCCTTTACCGGGATTACAGA CACCGCTCGCTG
IgG Fc- Pichia FOR	TATTAAGCTGCAGACAAAACCTCACACATGCCACCG
IgG Fc- Pichia REV	TATTAATGCGGCCGCTTTACCCGGAGACAGGGAGAGGC
IgE Fc- Pichia FOR	TATTAAGCTGCAGCTAGCACAGAGCCCATCCGT
IgE Fc- Pichia REV	TATTAATGCGGCCGCTTTACCGGGATTACAGACACCGCT
IgE Ce2-Ce4 FOR	GTCTGCTCCAGGGACTTCACCCGCCACCGTGAAGATCTTACAGTC
IgE Ce2-Ce4 REV	CGAATTCGTGACAAGTGCAAGACTTAGTGCAATGCAAGACAGGAGTTGC
IgEK Avi-His FOR	TGGCACGAGGGCGGCTCTCACCACCATCATCACCATTGATGTACAGCTAGC TGCCAGACATGATAAGATAC
IgEK Avi-His REV	CTCGATCTTCTGAGCCTCGAAGATGTCGTTGAGGCCAGCAGCGGCTTTACCG GGATTTACAGACACCGCTCGCTG
IgG Fc- pTT5 FOR	TCAGCAGATATCGGACAAAACCTCACACATGCCAC
IgG Fc- pTT5 REV	TTAATAGCGGCCGCTCAATGATGATGATGATGATG
IgE Fc ^{Phe229} - pTT5 FOR	TATAATGATATCGTTACCCCGCCACCGTGAAGATC
IgE Fc ^{Phe229} - pTT5 REV	GCAGCAGGATCCTCAATGATGATGATGATGATG
IgE Fc- pYD5 FOR	CTACTAGGATCCGTCTGCTCCAGGGACTTCACC
IgE Fc- pYD5 REV	TAGTAGGATATCATGATGATGATGATGATGGTCGACG

Table 2-2: List of colony PCR and sequencing primers.

Construct	Primer Name	5'-3' Sequence
Backbone		
pPICZ alpha B	IgG Fc- Pichia FOR	TATTAAGCTGCAGACAAAACACACATGCCACCG
pPICZ alpha B	IgG Fc- Pichia REV	TATTAATGCGGCCGCTTTACCGGAGACAGGGAGAGGC
pPICZ alpha B	IgE Fc- Pichia FOR	TATTAAGCTGCAGCTAGCACAGAGCCCATCCGT
pPICZ alpha B	IgE Fc- Pichia REV	TATTAATCGCGGCCGCTTTACCGGATTACAGACACCGCT
pPICZ alpha B	Seq-Primer-Pichia FOR	AGAAGGGGATTTCGATGTTGC
pPICZ alpha B	Seq-Primer-Pichia REV	GCAAATGGCATTCTGACATCC
pFUSE vectors	Seq-Primer pFUSE FOR	CATGTACAGGATGCAACTCC
pFUSE vectors	Seq-Primer pFUSE REV	CACTGCATTCTAGTTGTGG
pVITRO1-dv-IgEK	Seq-primer IgEK FOR	CATGCCTGAGGACATCTCG
pTT5	pTT5 FOR	GATATTCACCTGGCCCGATCTG
pTT5	pTT5 REV	GGTTCAGTTGGCAAGTTGTACCA
pYD5	pYD5 FOR	GCTCTGCACAACCACTACACG
pYD5	pYD5 REV	GTCCTCCGAGTGAGAGACAC

2.2.2 Preparation of bacterial competent cells by the Inoue method

Escherichia coli XL10-Gold or DH5 α were inoculated in 10mL SOB medium to grow overnight at 37°C with a shaking speed of 250 rpm. The next day, 125 mL of fresh SOB medium was inoculated in a 1 L flask with 5 ml of overnight culture and incubated at 25°C until OD₆₀₀ reached 0.55. The cells were then placed on ice and divided into to 4 centrifuge tubes (each contains 30mL) and centrifuged at 2,500 g for 10 minutes at 4°C. The cell pellets from two centrifuge tubes were resuspended in 20mL Inoue buffer to obtain 40mL of cell-containing buffer in two centrifuge tubes. Next, the same centrifuge step was used and the cell pellets of each tube were resuspended in 5mL Inoue buffer then pooled together to obtain 10 mL in total. 750 μ L of DMSO was added and mixed gently. Finally, the cells were incubated for 10 minutes at 4°C and the Eppendorf tubes were used to prepare 100 μ L aliquots before being frozen in liquid nitrogen and kept at -80°C until use.

2.2.3 Preparation of *P. pastoris* for electroporation

P. pastoris X-33 was grown in 5 mL of YPD medium at 30 °C overnight. The following morning, 500 mL of fresh YPD medium was inoculated in a 2 L flask with 0.25 mL of overnight culture and incubated overnight at 30 °C until OD₆₀₀ reached 1.3-1.5. Cells were pelleted by centrifugation at 1,500 g for 5 minutes at 4°C, and the pellet re-suspended in 500 mL of ice-cold sterile water. The centrifuge step was repeated a further three times, with the pellet re-suspended in 250 mL ice-cold sterile water, followed by 20 mL of ice-cold 1 M sorbitol, finishing with 1 mL of ice-cold 1 M sorbitol, resulting in a final volume of approximately 1.5 mL. Cells were kept on ice until use.

2.3 Mammalian cell culture

All cell culture work was undertaken in a class II microbiological safety cabinet under sterile conditions.

2.3.1 Cell maintenance and freezing

2.3.1.1 Adherent cells

In a cell culture incubator maintained with 5 % CO₂ and at 37°C, RBL cells (RS-ATL8 and NFAT-DsRed) and adherent HEK 293 cells were cultured in sterilised 75 cm² flasks with vented caps, with 15 mL of MEM and DMEM media , respectively. These media were supplemented with 10 % of FBS, 1% penicillin-streptomycin and 1 % L-glutamine. After every 2-3 days, the medium was changed when the cell confluency reached 80-100 % using a cell splitting ratio of 1/10. This process is performed by discarding the old medium and washing the cells with 10mL of DPBS without Ca²⁺ or Mg²⁺, and then 2-3mL of trypsin-EDTA is added to the flask and the cells incubated for 10-15 minutes. Next, the cells are harvested in falcon tubes and completed to 10mL with fresh medium to be centrifuged at 450 – 600 g for 5 minutes. The cell pellet is then diluted

to 10 mL of fresh medium and 1 mL of this cell containing medium is transferred to the flask with 9 mL of fresh medium to prepare the 1/10 splitting proportion. Finally, the selective antibiotics were added to the corresponding cultures to maintain the expression of the transfected genes.

To prepare stocks of the cells, the harvested cells after the splitting step were resuspended in the freezing medium (10 % of DMSO in MEM or DMEM medium) at concentration of $5-10 \times 10^6$ cells/mL and aliquots in cryogenic storage vials (Thermo Fisher Scientific, UK). Before transferring these vials to liquid nitrogen storage, a Mr. Frosty Freezing Container (Nalgene, USA) was used to store the cells at -80°C overnight.

2.3.1.2 Suspension cells

In sterile conditions at 37°C in 5 % CO_2 , HEK293-6E cells were thawed and added to a disposable 125 mL Corning Erlenmeyer flask containing 16mL of FreeStyle F17 Expression Medium supplemented with 2 % L-glutamine and 0.1 % kolliphor P-188 without G418 on an orbital shaker plate which started at 120 rpm. Once the cells reached 1×10^6 cells/mL, they were diluted with the same amount of fresh F17 medium, and then 25 $\mu\text{g/mL}$ G418 (Invitrogen, USA) was added as soon as the cells established a doubling time ($\approx 20-24$ hours). The cells were maintained by diluting them every three days to a cell density of 0.25×10^6 .

To freeze HEK293-6E cells, a freezing mixture (10 % of DMSO in F17 medium) was added to the harvested cells to obtain a density of $5-50 \times 10^6$ cells/mL, and aliquots were prepared in cryogenic storage vials to be stored in a Mr. Frosty Freezing Container at -80°C for overnight, and on the following day they were transferred to liquid nitrogen storage.

2.3.2 Preparation of transfection reagent (Polyethyleneimine, PEI)

In a 1 L glass beaker on a stirring plate, Linear PEI with MW≈25,000 (Polysciences, Inc., USA) was prepared by adding 500mg to sterilised water (final concentration 1mg/mL), followed by dropwise addition of HCl to adjust the solution to pH < 2.0. Then after a few minutes, more HCl was added to readjust the pH to < 2.0. It was stirred for 2-3 hours at maintained pH < 2.0 until the PEI had fully dissolved. Once this step was achieved, NaOH was added to return the pH to 7.0. Finally, the solution was filtered through 0.22 µm membrane to sterilise, and aliquots were stored at -20°C.

Chapter 3. Design, expression, and purification of recombinant mutant IgG Fc variants and IgE Fc

3.1 Introduction

The first objective of this work was to design mutant hIgG Fc variants and hIgE Fc expression constructs for the production of proteins with high binding affinity to effector cell Fc receptors, and the expression of these mutants as ligands on the surface of the delivery vehicle. These Fc recombinant proteins are designed to aid in controlling the harmful effects of activated immune cells (mast cells and basophils) by co-aggregation of the inhibitory receptor (FcγRIIB) with the activating receptors (FcεRI)^{140,141}. However, due to the differences in receptor binding affinity between the hIgE and hIgG Fc regions ($K_d > 10^{-9}$ M and $> 10^{-7}$ M respectively),⁴⁷ the hIgG Fc variants require enhanced binding affinity for a more balanced distribution ratio of hIgG to hIgE regions on the surface of micro particles. Once achieved, this higher binding affinity will permit the engagement of inhibitory signalling at a lower concentration than the native IgG (Figure 3.1).

Generally, the Fc region mediates IgG binding to all effector molecules, including Fcγ receptors and complement factor C1q; modification of this region can cause significant alteration of binding affinity to effector receptors^{183,184}. Moreover, engineering of the Fc region of human IgG to create Fc variants with improved binding affinity for the Fcγ receptor (FcγRIIB) has been reported by several studies^{131,185–187}. This enhanced activity is achieved by changes in the amino acid sequence which are obtained from either random mutational analysis or rational computational analysis.¹⁸³ The site selected for amino acid substitution in the IgG Fc region is primarily in the Cγ2 domain, which plays a crucial role in binding FcγRIIB^{188,189}.

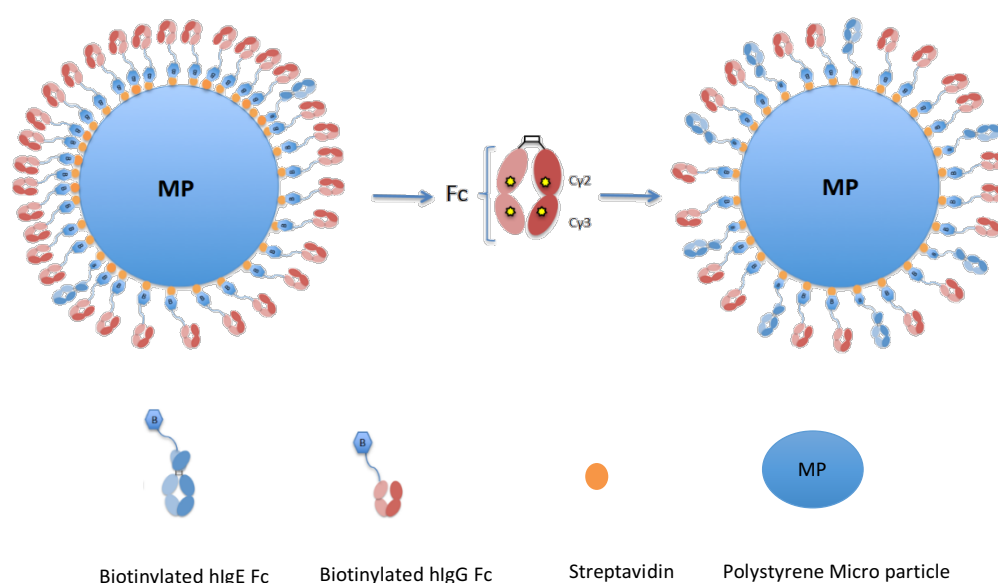


Figure 3-1: schematic diagram of streptavidin coated micro particles conjugated with Fc regions of IgG and IgE.

(A) The stoichiometric ratio between wild type human IgE and IgG Fc (hIgE Fc and hIgG Fc) on the micro particle surface. (B) An enhanced stoichiometric ratio between wild type hIgE and an engineered hIgG Fc with enhanced binding affinity.

In this chapter, different strategies were developed to optimise the expression and purification of hIgG Fc variants and hIgE Fc recombinant proteins (Figure 3.2). In the case of IgG Fc fragments (the CH2 and CH3 domains), we aimed to design a series of mutant IgG Fc fragments with enhanced FcγRIIB affinity, and express, purify, and conjugate these to micro particles. A 6xHis-tag and an AviTag were inserted at the C-terminus to aid in purification and particle conjugation respectively. 6xHis and AviTags were also inserted at the C-terminal of the IgE Fc (CH1-CH4). Two modifications were made in IgE Fc expression trials: expression with a Kappa constant chain, and deletion of the first domain of IgE constant region.

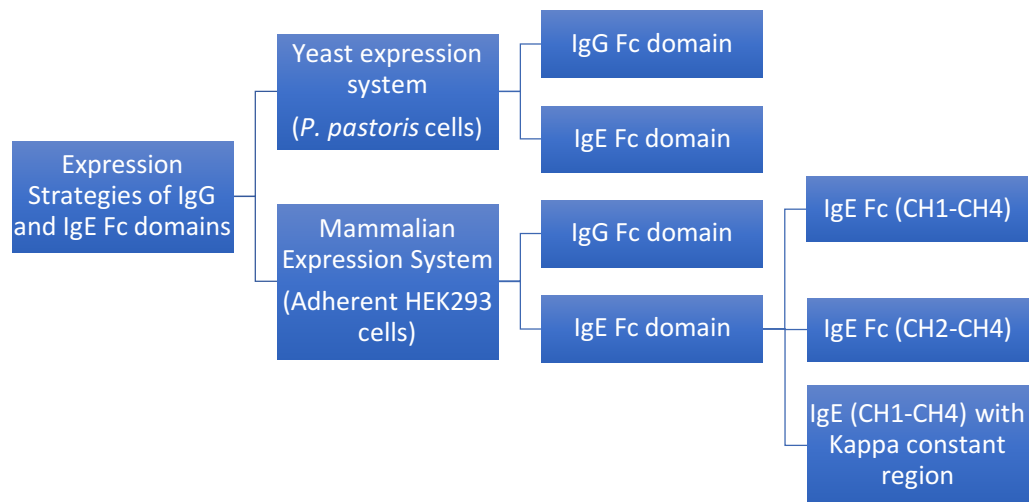


Figure 3-2: Schematic diagram of the expression strategies of IgG and IgE Fc proteins.

3.2 Experimental procedures

3.2.1 Generation of IgG Fc variants and IgE Fc constructs

All IgG and IgE expression vectors are summarised below in Table 3.1.

Table 3-1: Summary of IgG Fc and IgE Fc derivative constructs.

Construct name	Mutation positions	Vector backbone	Fc variant ID
pFUSE-EFT+EA-6xHis/Avi	S267E/ H268F/ S324T/ I332E/ G236A	pFUSE-hIgG1-Fc2	EFT+EA
pFUSE-EF-6xHis/Avi	S267E/L328F	pFUSE-hIgG1-Fc2	EF
pFUSE-DE-6xHis/Avi	S239D/ S267E	pFUSE-hIgG1-Fc2	DE
pFUSE-hIgG1-Fc-6xHis/Avi	-	pFUSE-hIgG1-Fc2	WT-hIgG Fc
pFUSE-CHlg-hE-6xHis/Avi	-	pFUSE-CHlg-hE	WT-hlgE Fc
pFUSE-CHlg-hE(2-4)-6xHis/Avi	-	pFUSE-CHlg-hE	WT-hlgE(2-4)
pVITRO1-dv-IgEK-6xHis/Avi	-	pVITRO1-dv-IgEK	DV-IgEK _{Avi}
pPICZ alpha B-EFT+EA-6xHis/Avi	S267E/ H268F/ S324T/ I332E/ G236A	pPICZ alpha B	EFT+EA
pPICZ alpha B-EF-6xHis/Avi	S267E/L328F	pPICZ alpha B	EF
pPICZ alpha B-DE-6xHis/Avi	S239D/ S267E	pPICZ alpha B	DE
pPICZ alpha B-hIgG1-Fc-6xHis/Avi	-	pPICZ alpha B	WT-hlgG Fc
pPICZ alpha B-CHlg-hE-6xHis/Avi	-	pPICZ alpha B	WT-hlgE Fc

3.2.1.1 Site-Directed Mutagenesis

Site directed mutagenesis was used to create the Fc variants of IgG. The whole plasmid was amplified using back-to-back primers, one of which contained the desired mutation. Three constructs with different mutations were generated, one with quintuple mutations and two with double mutations (Table 3-1). Amino acid substitution was done one at a time to enable confirmation of the presence of each mutation. Site directed mutagenesis was also used to add affinity tags (6xHis and AviTags) to the C-terminus of the sequence of the IgG Fc variants and IgE Fc domains, and to delete the first domain of IgE Fc (Figure 3-3).

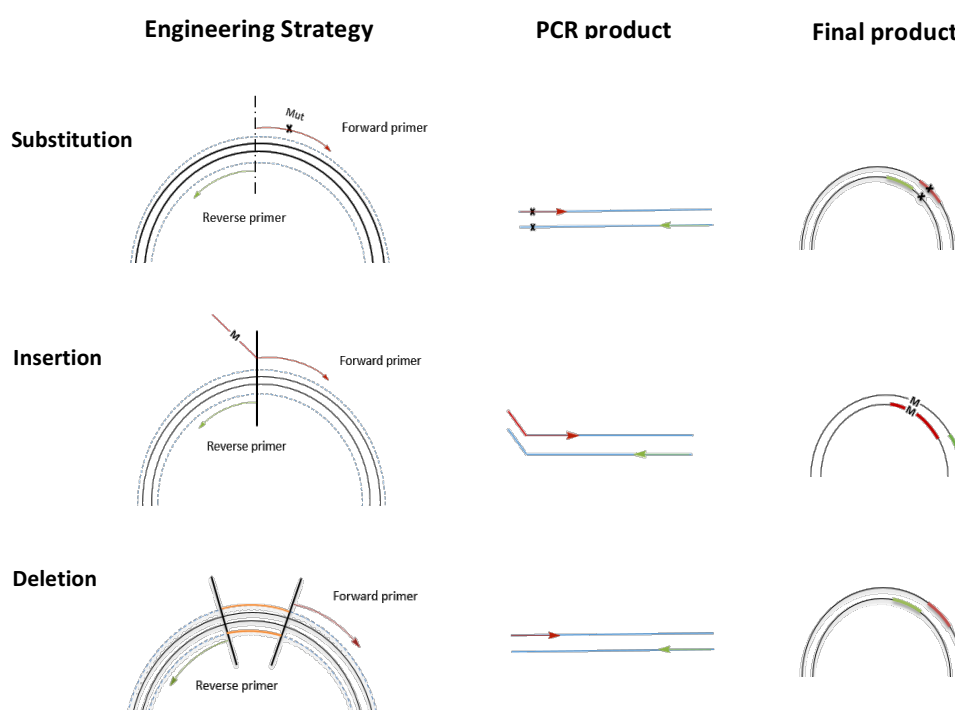


Figure 3-3: Schematic diagram showing site directed mutagenesis strategies.

3.2.1.2 Polymerase chain reaction amplification

Phusion High Fidelity DNA polymerase (New England Biolabs, USA) was used in site directed mutagenesis to amplify the whole plasmid containing genes for IgG and IgE Fc fragments, and to amplify the same genes to be cloned into *P. pastoris* expression vectors. PCR consisted of a 3-step thermal cycling protocol according to the manufacturer's instructions using the primers listed in Table 2-1. The cycling condition was set up as follows: initial denaturation at 98°C for 30 seconds, followed by 25 cycles of: denaturation at 95°C for 10 seconds, annealing at 72°C for 30 seconds, and extension at 72°C for 30 seconds/kb, with a final at 72°C, 10-minute extension step.

3.2.1.3 Cloning

Gene products generated by site directed mutagenesis were ligated directly into the desired vector using a Quick ligation kit (New England Biolabs, USA). 5 µL of PCR product was added to 5 µL of 2x Quick Ligation buffer and 0.5 µL of T4 DNA ligase. The reaction was mixed thoroughly and incubated at room temperature for 5 minutes, before being chilled on ice and stored at -20°C. For cloning into a *P. pastoris* vector, the amplified PCR products of IgG and IgE Fc fragments and the pPICZ alpha B vector were digested with *Pst*I and *Not*I restriction enzymes under conditions specified by the manufacturer. Digested products and vector were purified by a Wizard® SV Gel and PCR Clean-Up System (Promega, UK) and mixed in a 3:1 molar ratio (insert to vector) before being ligated with the aforementioned Quick ligation kit according to the manufacturer's protocol.

The ligation mixtures of both amplification strategies were used to transform XL10 gold ultra-competent cells for plasmid propagation using the heat shock method. The competent cell and plasmid mixtures were incubated on ice for 30 minutes before

being placed at 42°C for 45 seconds, this was followed by 2 minutes incubation on ice. After the heat shock procedure, cells were grown in LB medium for one hour at 37 °C before being plated onto LB agar containing 50 µg/mL zeocin (InvivoGen, USA) for selection. Plates were then incubated overnight at 37 °C. To determine the success of the mutagenesis, random colonies were picked and cultured overnight at 37 °C in LB media supplemented with 50 µg /mL zeocin. The following morning, plasmids were extracted using the Wizard® Plus SV Minipreps DNA Purification System (Promega, UK). In *Pichia* cloning, separate colonies were selected for colony PCR screening using JumpStart Taq DNA polymerase (Sigma Aldrich, UK) with primers shown in Table 2-2 and an annealing temperature of 58°C. Agarose gel electrophoresis of the PCR products was used to confirm gene insertion. Positive colonies were picked and grown in LB medium supplemented with 50 µg/mL zeocin and the plasmids purified. All vectors were sent to Source BioScience LifeSciences for DNA sequencing, along with appropriate sequencing primers (Table 2-2).

3.2.2 Expression in *P. pastoris* cells

3.2.2.1 Transformation using electroporation method

pPICZ alpha B derivative constructs were linearised by digestion with *PmeI* (New England Biolabs, USA) and concentrated to 1 µg/µL before being mixed with electrocompetent *Pichia* cells. 10 µg of linearized DNA was mixed with 80 µL of cells in an ice-cold 0.2 cm electroporation cuvette and incubated for 5 minutes on ice. The cells were pulsed by a Micro Pulser™ electroporation device (Bio-Rad, UK) according to the manufacturer's instructions for yeast. Following the incubation step, 1 mL of ice-cold 1 M sorbitol was immediately added to the cuvette and the content of the cuvette were collected and transferred to a sterile 15 mL tube and incubated without shaking at 30°C for 1.5 hours. Following incubation, 20 and 200 µL of the

electroporation mixtures were spread on YPD media with sorbitol (YPDS) agar plates containing 100 µg/mL of zeocin and incubated for 3 days at 30°C, with covering the plates to prevent light degradation of zeocin.

3.2.2.2 Genomic DNA isolation of transformed *Pichia* cells for PCR screening

From *Pichia* cell cultures grown overnight at 30°C in YPD media, 1.5 mL was transferred to a microcentrifuge tube and the cells pelleted by centrifugation at 20,000 g for 5 minutes. 200 µL of Harju lysis buffer (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added. Tubes were immersed in liquid nitrogen for 2 minutes, followed by incubation at 95°C for 1 minute; this step was repeated twice. The tubes were mixed by vortex for 30 seconds, and 200 µL of chloroform was added followed by a further 2 minutes mixing. A 3 minute, 20,000 x g centrifugation step was carried, resulting in two distinct layers; the upper layer was transferred to a new microcentrifuge tube containing 500 µL cold ethanol, followed by gentle mixing. DNA was precipitated by incubation at -20°C for 10 minutes, followed by a centrifugation step of 20,000 g for 5 minutes. Supernatants were removed and pellets were washed with 500 µL of 70 % ethanol, followed by another centrifugation step and the supernatant removed. Pellets were dried by incubation at 60°C for 5 minutes and re-suspended in 50 µL of water.

3.2.2.3 Protein expression screening

Eight to ten colonies were randomly picked and inoculated separately in 200 µL fresh YPD medium supplemented with 100 µg/ mL zeocin and grown overnight with shaking at 30°C. The following morning, 10 µL of each culture was grown in 1 mL of Minimal Glycerol media (MGY) with 100 µg/mL zeocin in 24-well plates (Corning Life Science, USA) overnight with shaking at 30°C. Cells were harvested by centrifugation at 3000 x g for 5 minutes at room temperature and washed in Minimal Methanol medium (MM)

to remove any glycerol residue. The centrifugation step was repeated after decanting the supernatant and the cells re-suspended in MM medium for induction of protein expression. Methanol was added every 24 hours to final concentration of 1 % to maintain expression. Cells were pelleted by centrifuged at 1,300 x g for 5 minutes at room temperature and the supernatant collected to be analysed by dot-blotting to screen for expression of IgG and IgE Fc proteins.

3.2.3 Protein expression in adherent HEK-293 cells

3.2.3.1 Stable Expression

Human Embryonic Kidney 293 (HEK-293) cells were cultured in sterile conditions at 37°C with 5 % CO₂ in a humidified incubator and transfected with 0.25 µg of pFUSE derivatives vectors using X-tremeGENE9 DNA transfection reagent (Roche Diagnostics, Germany) in 24-well plates at 70 to 80 % confluency. A ratio of 6:1 (v/w) of transfection reagent/DNA was used to prepare the transfection mixture in Opti-MEM Reduced Serum Medium (Thermo Scientific, UK) according to the manufacturers recommended conditions. After 24 hours, pDsRed-Express N1 (Clontech, USA), which is a constitutively expressed red fluorescent protein, was used as reporter to evaluate the transfection efficiency, and the transfected HEK-293 cells were visualised by fluorescence microscopy on an EVOS fluorescence microscope (Advanced Microscopy Group, now part of Thermo Scientific, UK). The next day, the medium was changed and cells were selected in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % FBS, 1 % penicillin-streptomycin, 1 % L-glutamine (Sigma Aldrich, UK) and 300 µg/ mL zeocin. Medium was changed every 2-3 days. When the cells reached confluency, they were detached from the plates using trypsin-EDTA (Sigma Aldrich, UK) and transferred to 6 well-plates, T25 flasks and T75 flasks (Corning Life Science, UK). The supernatants were collected for analysis of the secreted proteins

using SDS-PAGE, stained with both instant blue staining and Western blotting as described in section 3.2.4.

3.2.3.2 Transient Expression

In a T75 flask, HEK-293 cells were grown in DMEM with 10 % FBS, 1 % penicillin-streptomycin and 1 % L-glutamine with a medium change every 2-3 days. At 70-80 % confluency the cells were washed with Dulbecco's Phosphate-Buffered Saline without calcium and magnesium (DPBS) (Sigma Aldrich, UK) and detached by trypsinisation for transfer into two T225 flasks. Each of these flasks was sub-cultured to confluency with the same maintenance procedure and again split two ways, this time making 4 T225 flasks. The process was repeated once more, resulting in 8 T225 flasks of cells. Transfection was conducted using the GeneJuice transfection reagent (Merck Millipore, USA) at a ratio of 3:1 (GeneJuice to pFUSE derivatives vectors) in Opti-MEM medium when the cells reached 70-80% confluency. Transfection mixture was prepared by adding Opti-MEM media to 960 μ L of GeneJuice transfection reagent up to a final volume of 320 mL, followed by mixing and incubation for 5 minutes. DNA vector was added to a final concentration of 1 μ g/mL and incubated for 15 minutes at room temperature. The transfection mix was divided equally between the 8 T225 flasks and cultures maintained at 37°C with 5 % CO₂. The next day, media were replaced with fresh Opti-MEM media supplemented with 3.75 mM valproic acid and incubated for further 6 days. Following this, the supernatant was harvested by centrifugation at 2,000 g for 15 minutes at 4°C. Several cOmplete®, Mini, EDTA-free I protease inhibitor cocktail tablets (Roche, UK) were added to the supernatant for storage at 4°C in preparation for the batch purification step as described in section 3.2.5.

3.2.4 Analysis of secreted IgG and IgE Fc proteins

3.2.4.1 SDS-PAGE and Coomassie staining

The supernatants of the *Pichia* and HEK-293 expression cultures were mixed with an equal amount of 2x reducing and non-reducing sample buffers (RSB & NSRB) before heating at 100°C for 5 minutes. 15 to 20 µL of samples were loaded into gels (4–20 % Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad, UK) and run in SDS-PAGE buffer in Mini-PROTEAN™ Tetra Cell (Bio-Rad, UK) for protein separation. Instant blue staining (Expedeon, UK) was used for protein detection and the gel was incubated for 45 minutes at room temperature with shaking before being washed with deionised water.

3.2.4.2 Western and Dot blotting

After SDS-PAGE separation, proteins were transferred to 0.2 µm nitrocellulose membrane using a semi-dry transfer method (Trans-Blot® Turbo™ Transfer System, Bio-Rad, UK). For dot blotting, a Microfiltration unit (Thermo Scientific, USA) was loaded with 400 µl of supernatant from *Pichia* cultures to coat a preactivated PVDF membrane with methanol (Bio-Rad, UK). Next, both membranes (nitrocellulose and PVDF) were blocked with 5 % skimmed milk in a Tris buffered saline buffer with 0.01 % Tween 20 (TBS-T) for 1 hour at room temperature with shaking. After blocking, the membrane was incubated with anti-Human IgG Fc or anti-Human IgE Fc peroxidase-conjugated antibodies (Sigma Aldrich, UK) at dilutions of 1:4,000 and 1:10,000 respectively and incubated overnight at 4°C. The following day, membranes were washed four times with TBS-T. Chemiluminescence reagents (250 mM luminol, 90 mM coumaric acid and 30 % H₂O₂ dissolved in 100 mM Tris-Cl (pH 8.0)) were added for protein detection and membranes were visualised using a Fuji LAS 4000 imager. For detection of c-Myc tagged proteins, the membrane was incubated overnight after

blocking with a mouse anti-c-Myc:Biotin antibody (Bio-Rad, UK) at dilution of 1:5,000. Unbound primary antibody was removed by washing four times with TBST, and followed by incubation for one hour with a Streptavidin-Horseradish Peroxidase (HRP) conjugated secondary antibody (Bio-Rad, UK) diluted 1:5,000. 6xHis-tagged proteins were detected using a mouse anti-His primary antibody and a goat anti mouse IgG HRP conjugated secondary antibody (Sigma Aldrich, UK) following the same protocol as c-Myc tag detection. Both antibodies were diluted 1:3,000.

3.2.5 Protein purification using immobilised metal affinity chromatography (IMAC)

3.2.5.1 Column purification

Purification of His-tagged IgG Fc proteins from stable transfection cultures was carried out using gravity column chromatography. Filtrated supernatants were loaded onto a PD-column (Bio-Rad, UK) containing nickel resin (cOmplete His-Tag Purification Resin, Roche, UK) using an EP-1 Econo Pump (Bio-Rad, UK) with a flow rate of 2 mL/min. The column was washed (20 mM Tris-Cl, 300 mM NaCl, pH 7.6) at the same flow rate before elution with an increasing concentration of elution buffer (20 mM Tris-Cl, 300 mM NaCl, 500 mM imidazole, pH 7.6). Eluted fractions were evaluated using SDS-PAGE gel and instant blue staining.

3.2.5.2 Magnetic bead purification

A Dynabeads® His-Tag Isolation & Pulldown (Thermo Scientific, UK) kit was used to partially purify proteins from samples containing IgG Fc proteins from stable and transient expression. Dynabeads were prepared by vortex mixing of the vial, followed by transfer of the beads to a microcentrifuge tube and removal of the supernatant using an EasySep™ Magnet (STEMCELL Technologies, Canada). Filtered supernatant was mixed with an equal amount of binding/washing buffer (100 mM sodium phosphate, 600 mM NaCl, 0.02 % Tween 20, pH 8.0) and added to the beads. The

suspension was incubated for 5 minutes on a roller at room temperature, and placed in the EasySep™ Magnet in a standard FACS tube for 2 minutes to remove the supernatant. Beads were washed 3 to 4 times with 300 µL of the washing buffer, using the EasySep™ Magnet to remove supernatant. Protein was eluted by mixing the beads with 100 µL of elution buffer (100 mM sodium phosphate, 600 mM NaCl, 0.01 % Tween 20, 300 mM imidazole, pH 8.0) and incubation for 5 minutes. The supernatant was collected for evaluation of protein purity using SDS-PAGE and instant blue staining.

3.3 Results

3.3.1 Designing and cloning of IgG Fc variants and IgE Fc into expression vectors

The choice of Fc variants to utilise was based on published studies. Three IgG Fc variants which have been described to increase the binding affinity to FcγRIIB receptor were selected: one with a quintuple mutation, EFT+EA, and two with double mutations, EF and DE (Table 3-2).

Table 3-2: Mutations introduced into hIgG1 and increased binding affinity for FcγRIIB.

Sample ID	Mutations	Fold increase in FcγRIIB binding*
EFT+EA	S267E/ H268F/ S324T/ I332E/ G236A	120
EF	S267E/L328F	100 430
DE	S239D/ S267E	150

* These fold increase is according to published studies¹³¹⁻¹³³.

Sited directed mutagenesis was used to incorporate mutations into the vector, amplifying the whole IgG Fc (pFUSE-hIgG1-Fc2, 4194 bp) vector in the process. Analysis by gel electrophoresis (Figure 3-4), shows the three constructs that were generated, one with EFT+EA mutations (Figure 3-4A, lane 2; and lane 1 of 3-4B, 3-4C, and 3-4D). The second construct with EF mutations is present (Figure 3-4A, lane 1; 3-4B, lane 2) and the third construct with DE mutations was also successfully cloned (Figure 3-4A, lane 1; 3-4B, lane 3).

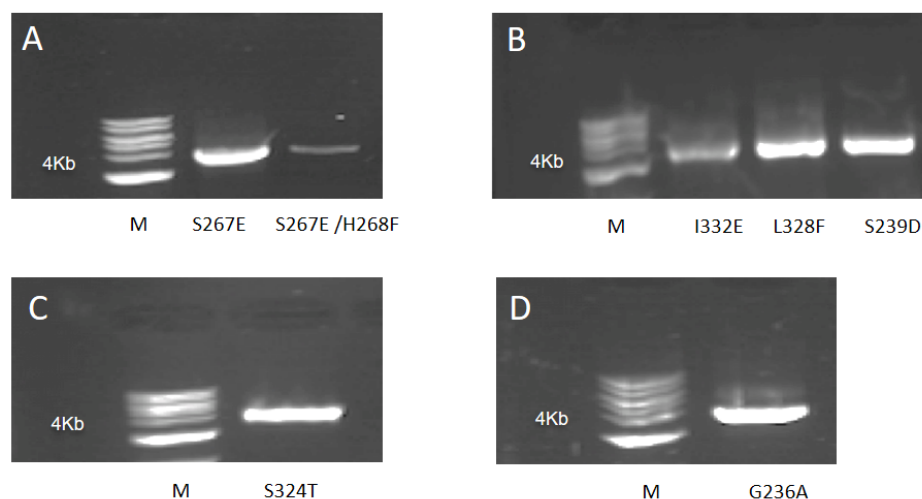


Figure 3-4: Gel electrophoresis separation of IgG-Fc vector after site directed mutagenesis method.

IgG-Fc vector (4194 bp) mutant vector constructs were separated in a 1.2 % agarose gel with 1 kb ladder. (A) Lane 1: vector containing mutation S267E; lane 2: vector containing both S267E/H268F mutations. (B) Lane 1: vector with I332E mutation; lane 2: vector with L328F mutation; lane 3: vector with S239D mutation. (C) Vector with S324T mutation. (D) Vector with G236A mutation.

Amplified constructs were subjected to PCR clean up, ligated and transformed into *E. coli* XL-10 gold competent cells for plasmid propagation. Random colonies were picked and grown in LB media before extraction and purification of the vectors. To confirm the presence of the mutations in the sequence of the generated IgG Fc genes, DNA sequencing was conducted and the results aligned to the wild type. Mutagenesis was successful in all three cases: the EFT+EA Fc variant (Figure 3-5), the EF double mutation Fc variant (Figure 3-6), and the DE double mutant Fc variant (Figure 3-7).

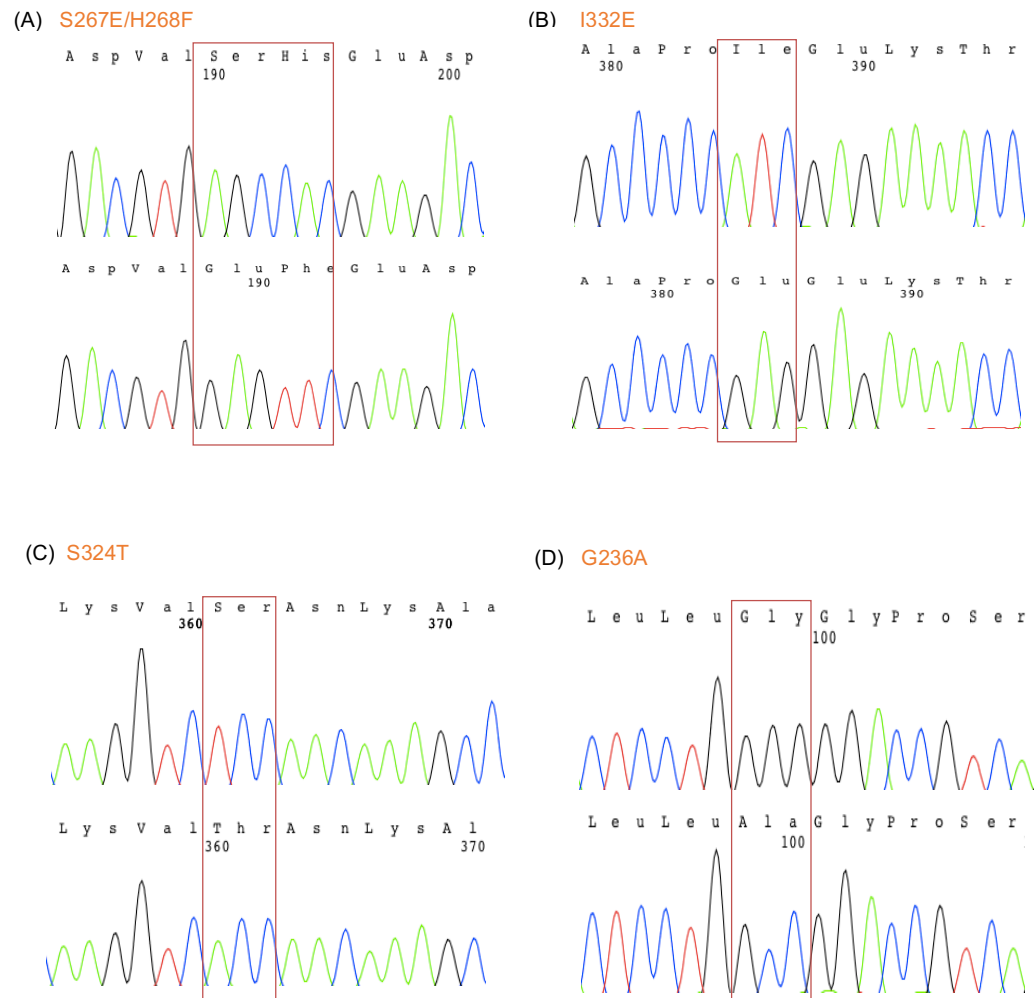
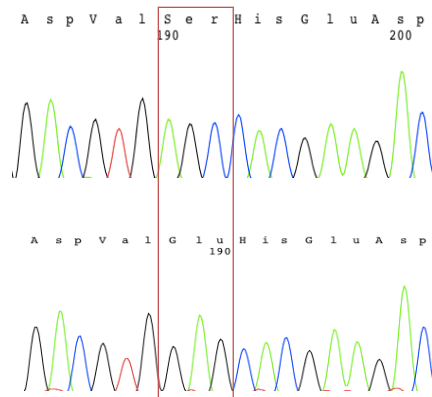


Figure 3-5: DNA sequencing results showing the first Fc variant with EFT+EA mutations.

(A) The S267E/H268F mutation, (B) the I332E mutation, (C) the S324T mutation, (D) the G236A mutation. The top sequence represents the wild type while the bottom sequence is mutant.

(A) S267E



(B) L328F

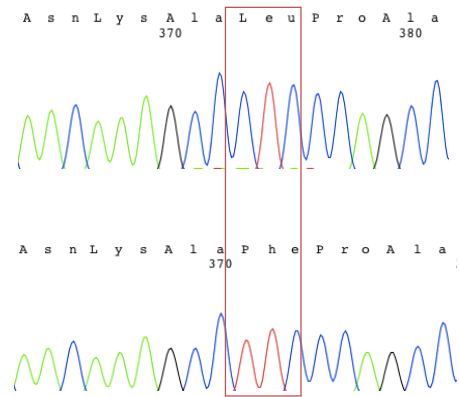
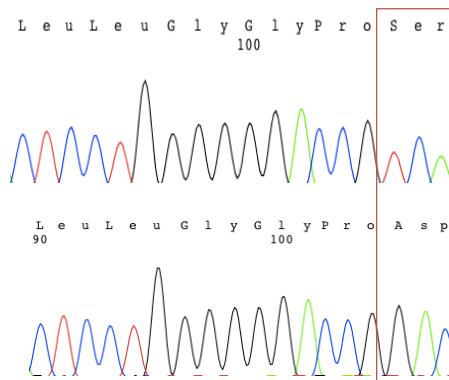


Figure 3-6: DNA sequencing result showing the second Fc variant with EF mutations.

(A) The S267E mutant, (B) the L328F mutant. The top sequence represents the wild type while the bottom sequence is mutant.

(A) S239D



(B) S267E

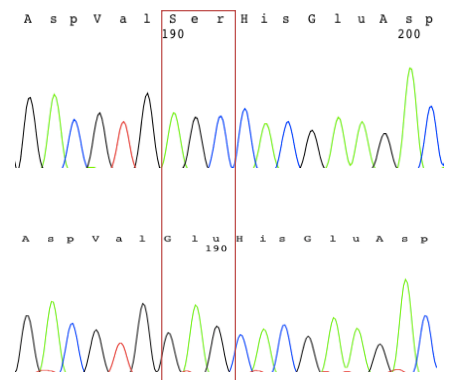


Figure 3-7: DNA sequencing result showing the third Fc variant with DE mutations.

(A) The S239D mutant, (B) the S267E mutant. The top sequence represents the wild type while the bottom sequence is mutant.

For protein purification and conjugation to micro particles, both tags (6xHis and AviTags) were added to the C-terminal of both IgG Fc variants and IgE Fc constructs using site directed mutagenesis. All IgG Fc variants and IgE Fc vectors were amplified using back-to-back synthetic primers (Figure 3-8). Amplified constructs were cleaned up, ligated and transformed into *E. coli*. DNA sequencing (data not shown) confirmed successful addition of tags to all vectors.

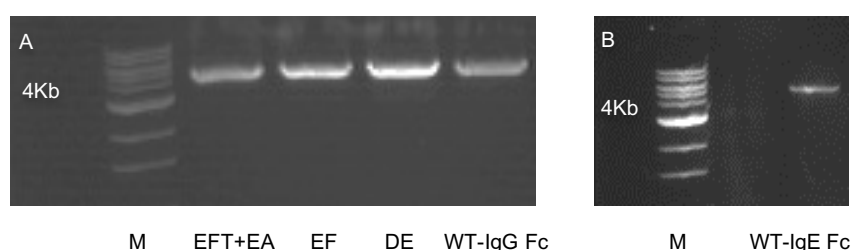


Figure 3-8: Gel electrophoresis separation after addition of an AviTag and a 6xHis tag to the C-terminus of Fc domains.

(A) Amplification of vector DNA for IgG Fc variants (EFT+E, EF, DE) and wildtype IgG Fc (pFUSE-hIgG1-Fc2, 4194bp), and (B) WT-IgE Fc (pFUSE-CHlg-hE, 4807 bp).

IgG Fc variants and IgE Fc gene fragments with a 6xHis and an AviTag were cloned successfully into *P. pastoris* expression vector (pPICZ alpha B) for expression in methylotrophic yeast. IgG and IgE Fc fragments (758 bp and 1297 bp, respectively) were amplified using primers with *Pst*I and *Not*I restriction sites. IgG and IgE Fc genes, along with the pPICZ alpha B vector were subjected to restriction digest, purified, and mixed, ligated and cloned into *E. coli* for plasmid propagation. After PCR screening of the colonies, the samples were sent for DNA sequencing to confirm insertion of the gene.

3.3.2 Recombinant expression in the yeast system

Expression was attempted using a yeast host to express the Fc recombinant proteins from pPICZ alpha B derivative constructs containing IgG Fc variants and IgE Fc genes generated as described above. These constructs contain alpha-factor signal peptides for secretion of expressed recombinant proteins (Figure 3-9).

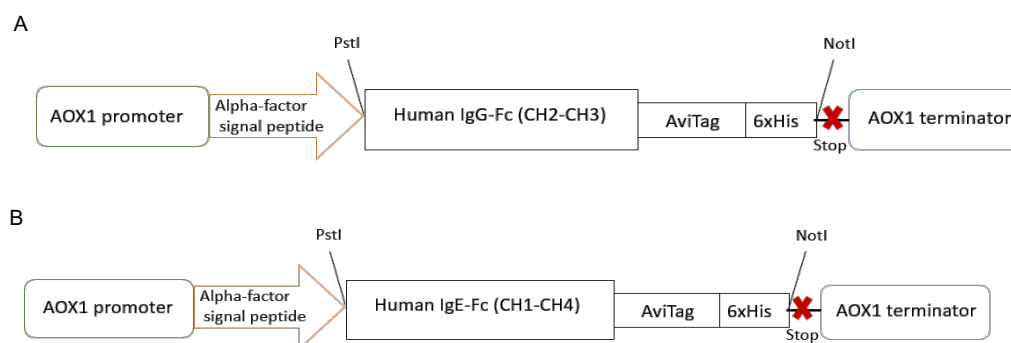


Figure 3-9: Schematic diagram of the pPICZ alpha B derivative expression cassette.

(A) IgG Fc variants, (B) IgE Fc genes. All Fc genes were cloned using *Pst*I and *Not*I restriction sites. The abbreviations represent the following: (Aox1) alcohol oxidase 1 gene; (IgG Fc CH2-CH3) IgG Fc gene fragment from domain 2 to 4; (IgE Fc CH1-CH4) IgE Fc gene fragment from domain 1 to 4; (6xHis) 6-histidine peptide tag; (AviTag) peptide tag to allow the biotinylation by BirA; (Stop/x) Stop codon.

The supernatants of eight transformed *P. pastoris* X33 cells of each IgG Fc variant were screened for secreted Fc recombinants protein by dot blot analysis, probed with antibodies against human IgG and IgE Fc portions as described in the experimental procedures; the blots show no protein expression for any Fc protein (Figure 3-10).

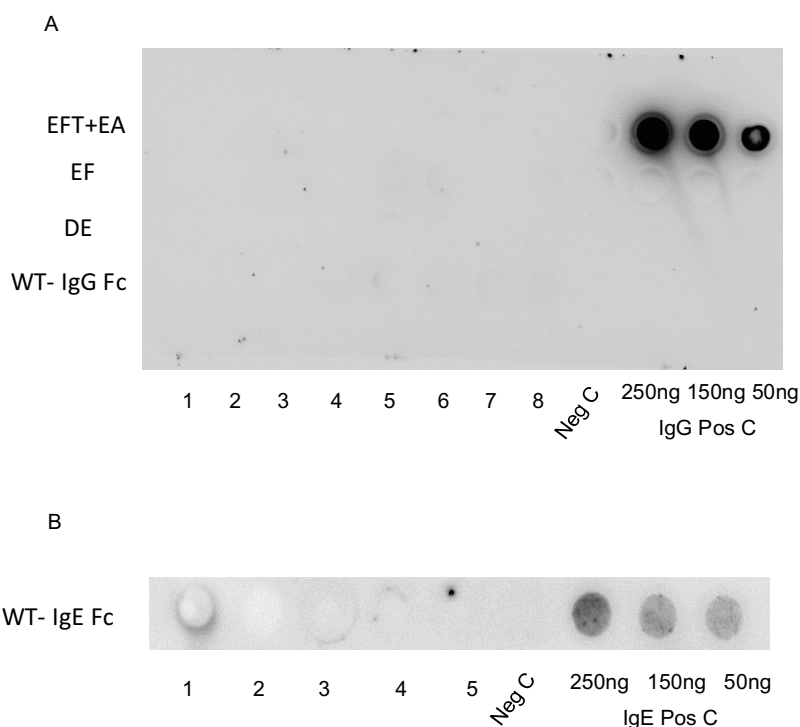


Figure 3-10: Dot blot results of recombinant IgG Fc variants and IgE Fc proteins.

(A) Dot blot screening of the supernatants of eight transformed *Pichia* expression cultures of each IgG Fc variant: EFT+EA, EF, DE and wild type (WT) IgG Fc. (B) Dot blot screening of the supernatants of 5 transformed expression cultures with wild type (WT) IgE Fc expression vector. The negative control was the supernatant of non-transformed *Pichia* cells; the positive control was human IgG and IgE proteins (A) and (B) respectively, at different concentrations (250, 150 and 50 ng). The primary antibodies used for detection were anti-human IgG, and IgE Fc region conjugated HRP antibodies.

After the dot blot screening, genomic DNA was extracted from grown yeast colonies to determine if the expression cassettes had integrated into the *Pichia* genome and colony PCR analysis conducted. Figure 3-11 shows that transformation of some colonies was successful, as bands are present of the expected sizes of the IgG and IgE Fc genes.

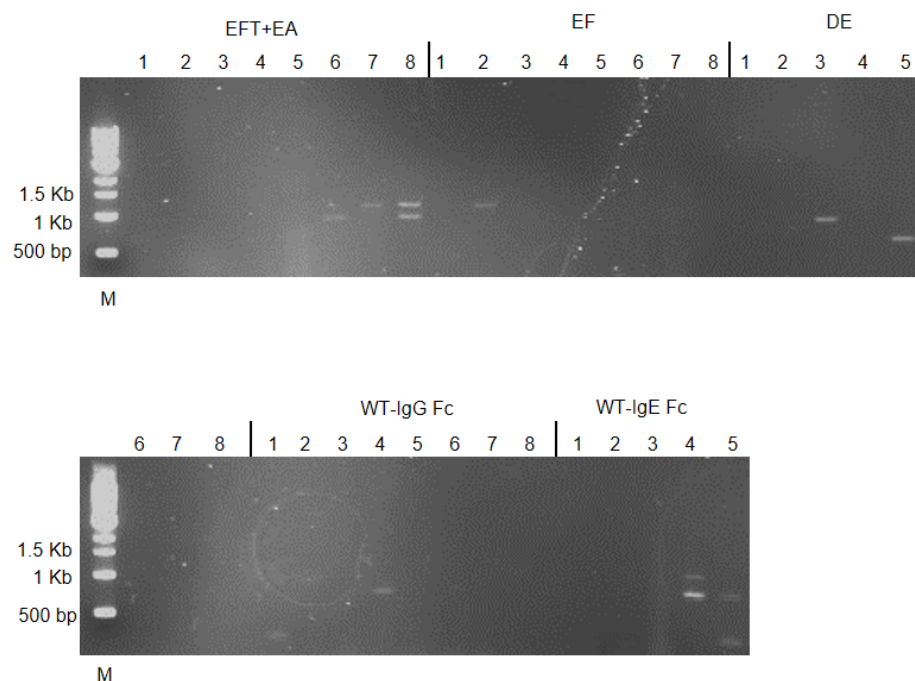


Figure 3-11: Gel electrophoresis results for PCR screening of transformed *Pichia* genomes.

Colony PCR analysis of extracted genomic DNA from transformed *Pichia* colonies (8 replicates of each IgG Fc variant genes and 5 IgE Fc gene replicates). M denotes the molecular weight ladder for fragment size estimation.

Pichia cell cultures from colonies which were shown by colony PCR to contain IgG or IgE Fc genes were screened for protein expression by Western blot. Antibodies against the hIgG Fc region and the 6xHis polypeptide tag (to detect the IgE Fc protein) were used to detect expression; revealing that, as seen in the dot blot analysis, expression of the recombinant proteins by yeast was unsuccessful (Figure 3-12).

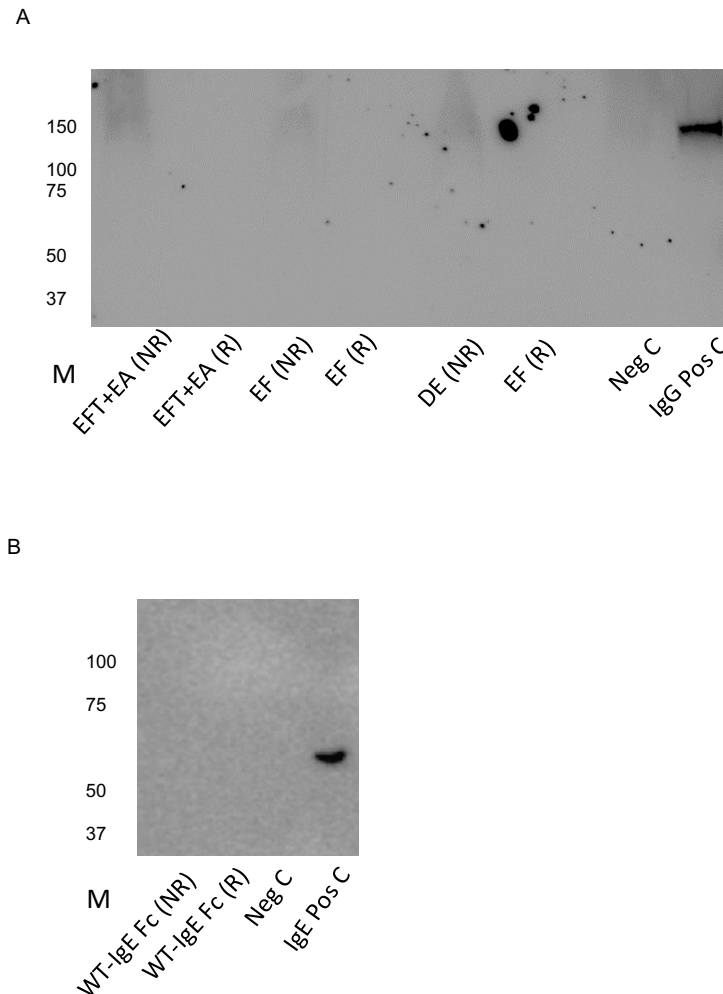


Figure 3-12: Western blot analysis results of recombinant IgG and IgE Fc proteins transformed in *Pichia* cells

(A) Western blot of IgG Fc variants protein under both non-reducing (NR) and reducing conditions (R). Lysate from non-transformed *Pichia* cells was used as a negative control and whole human IgG as a positive control (Molecular weight, 150 kDa). (B) Western blot analysis of wild type IgE Fc protein (WT-IgE Fc) under non-reducing and reducing conditions, non-transformed *Pichia* cells were used as a negative control and a 6xHis-tagged protein (Molecular weight 60 kDa) as positive control. Antibodies raised against the hIgG Fc region and the 6xHis polypeptide tag for detection of the IgE Fc protein were used.

3.3.3 Recombinant expression by adherent mammalian cells

The second expression strategy trialled utilised adherent HEK-293 cells. Expression constructs were formed of the pFUSE vector with inserted IgG and IgE Fc genes, and fused in frame with the nucleotides encoding the human interleukin 2 (IL-2) secretory signal peptide (Figure 3-13).

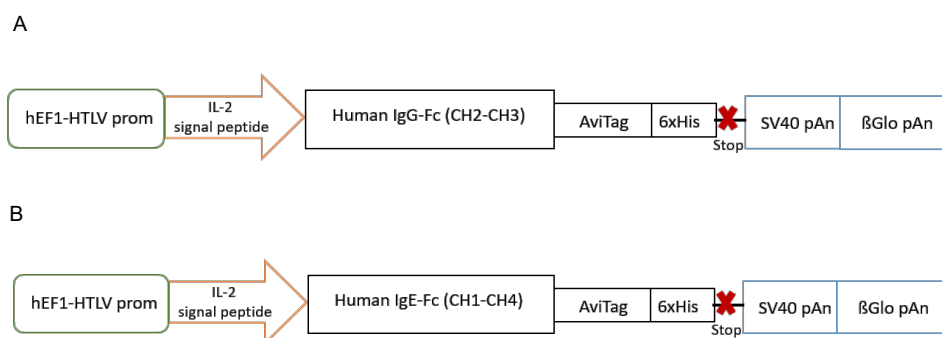


Figure 3-13: Schematic diagram of pFUSE derivatives expression cassette.

(A) General schematic of the IgG Fc variant constructs (B) Schematic of the IgE Fc construct. Abbreviations represent the following: (hEF1- HTLV prom) hybrid of elongation factor 1 α and human T-cell leukemia virus promoters; (IL-2) Interleukin 2; (IgG Fc CH2-CH3) IgG Fc gene fragment from domain 2 to 4; (IgE Fc CH1-CH4) IgE Fc gene fragment from domain 1 to 4; (SV40 pAn) Simian Virus 40 late polyadenylation signal for cleavage; (βGlo pAn) human beta-globin 3'UTR and polyadenylation sequence to arrest transgene transcription; (6xHis) 6-histidine peptide tag; (AviTag) peptide tag to allow biotinylation with BirA enzyme; (Stop/x) Stop codon.

3.3.3.1 Stable expression of IgG and IgE Fc recombinant proteins

HEK-293 cells were transfected with IgG and IgE Fc expression vectors using a commercial transfection reagent (X-tremeGENE 9 DNA) at a 6:1 ratio (v/w) of transfection reagent to DNA. To evaluate the transfection efficiency, pDsRed-Express-N1 vector, which encoded red fluorescent protein were used in parallel. After 24 hours, the cells were evaluated for transfection success using fluorescence microscopy (Figure 3-14).

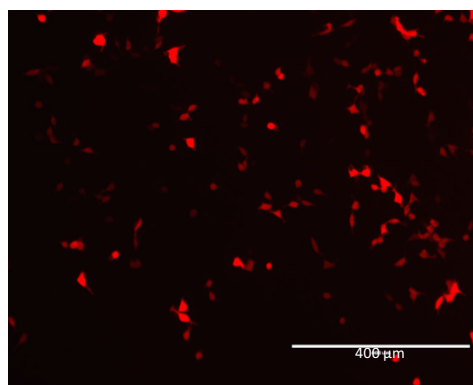
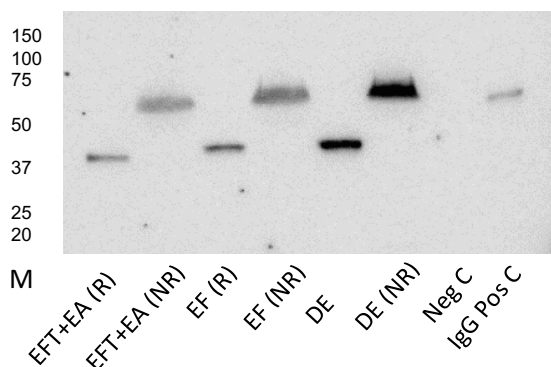


Figure 3-14: Fluorescence microscopy image of transfected HEK-293 cells with pDsRed-Express-N1 vector.

HEK-293 cells were transfected using X-tremeGENE 9 DNA transfection reagent at a ratio of 6:1 (v/w) (transfection reagent to pDsRed-Express-N1 vector).

Stably transfected HEK-293 cells capable of expressing IgG Fc variants and IgE Fc proteins were grown in DMEM media supplemented with 300 $\mu\text{g/mL}$ zeocin to establish the cell line. The culture supernatants (containing secreted proteins) were screened by Western blot using antibodies raised against the Fc regions of human IgG and IgE. All three Fc mutant variants of hIgG (EFT+EA, EF and DE) displayed bands of the expected size in both conditions (reducing and non-reducing), indicating successful expression (Figure 3-15A). The IgE Fc region (Ce1-Ce4) did not produce any bands with the expected size 100 kDa under either reducing or non-reducing conditions (Figure 3-15B), indicating expression was unsuccessful. The lack of expression results of the IgE Fc in both expression systems (yeast and mammalian) suggests that alternative strategies are required for expression, potentially based on modification of the sequence of the IgE Fc, rather than using a different expression system. This will be discussed later in this chapter.

A



B

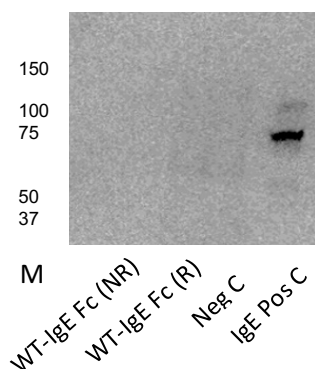


Figure 3-15: Western blot analysis results of the stable expression of IgG and IgE Fc regions from HEK-293 cells.

- (A) Expression of IgG Fc variants (EFT+EA, EF and DE) from stable transfection of HEK-293 cells, with an expected molecular weight of 60 kDa in non-reducing conditions (NR) and 30 kDa in reducing conditions (R). Detection was achieved with anti-human IgG Fc region antibodies. Non-transfected cells were used as a negative control and the positive control was whole IgG under reducing conditions.
- (B) Western blot for detection of expression of wild type (WT) IgE Fc under both non-reducing and reducing conditions, detection using an anti-human IgE Fc region antibody, non-transfected cells were used as the negative control and the positive control was whole IgE under non-reducing conditions.

After the successful expression of IgG Fc variant recombinant proteins was confirmed, the next step was the production of higher yields of purified IgG Fc proteins, sufficient for downstream experiment. To achieve this, when passaging, the media containing expressed protein was harvested from the cultures and fresh media added for the new passages. Collected media (400 ml) was applied to nickel columns for purification using the IMAC method described in the experimental procedures. Protein purity was evaluated by SDS-PAGE with Coomassie blue staining, and Western blot of IgG Fc variant (DE). These analyses revealed low yield of protein expression, and that some impurities remained after purification (Figure 3-16).

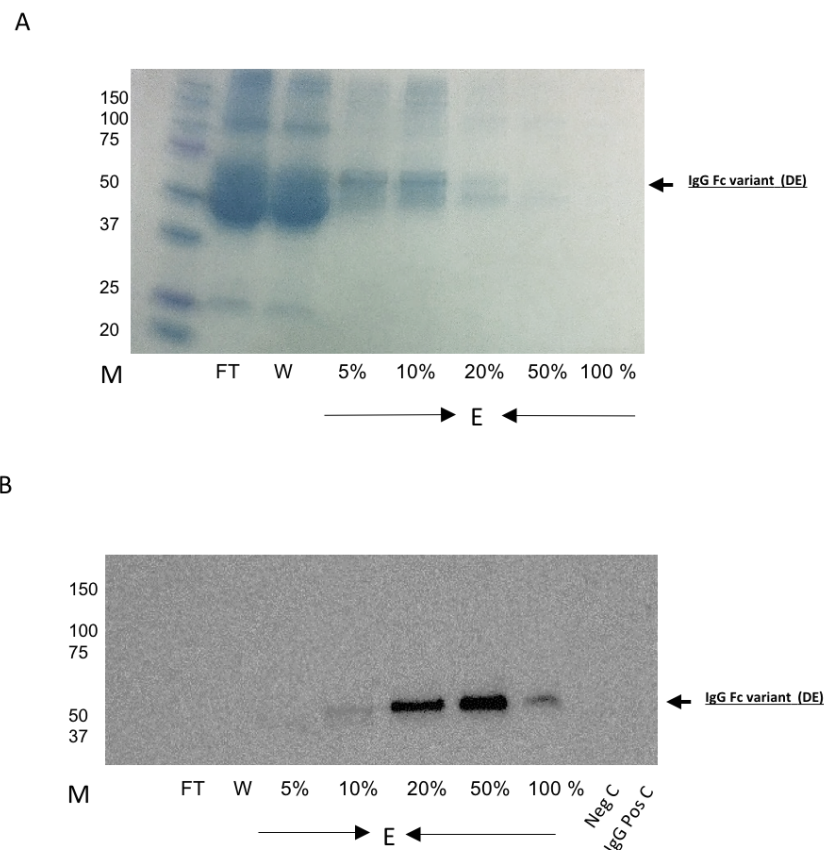


Figure 3-16: IMAC purification results of IgG Fc variant (DE) using nickel resin.

(A) Coomassie blue staining (B) and Western blot after IMAC purification with gradient elution method. Abbreviations are as follows: (FT) flow through fraction; (W) wash

fraction; (E) elution gradient fractions with 5, 10, 20, 50 and 100 % imidazole; M, molecular weight ladder. Western blot detection used an anti-human IgG Fc region antibody, whole IgG was used for the positive control, and non-transfected cells provided the negative control.

Using a similar IMAC purification concept, harvested supernatants of other IgG Fc variants (EFT+EA and EF) were concentrated using Vivaspin™ 20 Centrifugal Concentrators and applied to cobalt Dynabeads. SDS-PAGE stained by Coomassie blue clearly showed that the cobalt Dynabeads system produced purer protein than the nickel resin (Figure 3-17). However, there was still a low protein yield and the stable expression strategy was thus regarded as unsuitable.

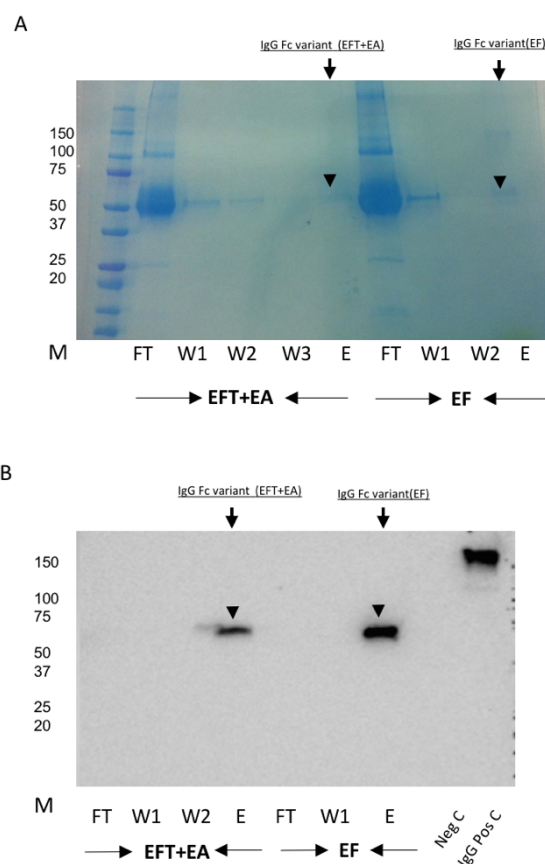


Figure 3-17: IMAC purification results of IgG Fc variants (EFT+EA and EF) using cobalt Dynabeads.

(A) Coomassie blue staining and (B) Western blot analysis of purification with cobalt Dynabeads. Abbreviations represent the following: (FT) flow through fraction; (W1) wash fraction 1; (W2) wash fraction 2; (W3) wash fraction 3; (E) elution fractions; M, molecular weight markers. The Western blot was probed with an anti-human IgG Fc region antibody, whole IgG was used as a positive control and non-transfected cells were used for the negative control.

3.3.3.2 Transient expression of IgG Fc recombinant proteins

Transient expression was used as an alternative expression strategy in an attempt to improve yield of IgG Fc variants. HEK-293 cells were transfected with pEGFP-Tub vector, encoding green fluorescent protein to assess the transfection efficiency at different ratios of Genejuice transfection reagent, in order to find the optimal ratio of transfection. Analysis using fluorescence microscopy revealed that no major difference could be detected between 3:1 and 6:1 ratios hence 3:1 was chosen as it requires less transfection reagent (Figure 3-18). 24 hours after transfection, 3.75 mM valproic acid was added to the medium and the supernatant harvested 6 days later to be concentrated and purified with cobalt Dynabeads. Coomassie staining and Western blot analysis of expression (Figure 3-19) did not suggest any major improvement in the yield of proteins.

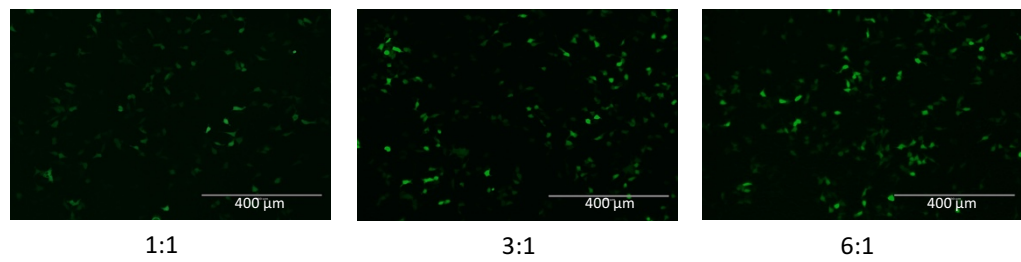


Figure 3-18: Fluorescence microscopy image of transfected with various Genejuice:DNA ratios.

Three different ratios of transfection reagent to DNA were used to transfect HEK-293 cells (1:1, 3:1, and 6:1) (v/w).

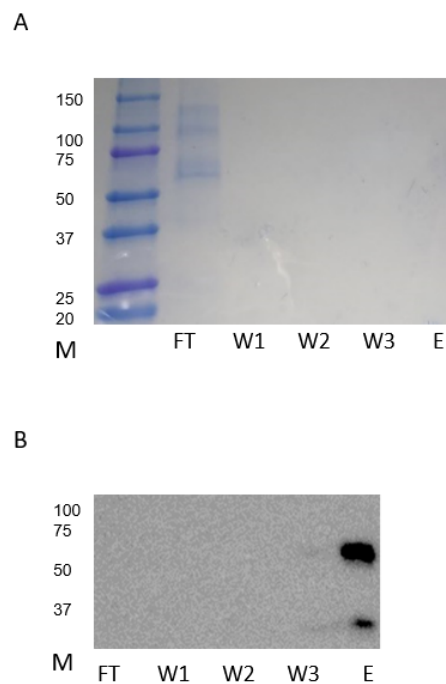


Figure 3-19: IMAC purification results of IgG Fc variant (EFT+EA) using Cobalt Dynabeads.

(A) SDS-PAGE stained with Coomassie blue and (B) Western blot after purification with cobalt Dynabeads. Abbreviations represent the following: (FT) flow through fraction; (W1) wash fraction 1; (W2) wash fraction 2; (W3) wash fraction 3; (E) elution fractions; M, molecular weight markers. Western blotting conducted with an anti-human IgG Fc region antibody.

3.3.3.3 Expression of IgE Fc (CH1-CH4) with Kappa light chain and without the variable region

Modifying the sequence of IgE Fc region to add the Kappa light chain constant region was another strategy attempted in order to improve expression. The rationale for this strategy will be described in detail in the discussion section. The expression construct (pVITRO1-dv-IgEK) has two expression promoters and two different secretory signal peptides for expression of the protein and extracellular secretion (Figure 3-20). An AviTag and a 6xHis-tag were added to the protein C-terminal using site directed mutagenesis, and confirmed by DNA sequencing. Two constructs were used to transfect HEK-293 cells, one with and one without the C-terminal tags. Six days post transfection the supernatant was harvested and expression evaluated by Western blotting.

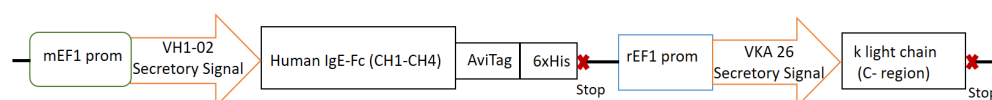


Figure 3-20: Schematic diagram of the pVITRO1-dv-IgEK-6xHis/Avi expression cassette.

Abbreviations represent the following: (mEF1 and rEF1 prom) elongation factor 1 α promoters from mouse and rat respectively; (VH1-02) immunoglobulin variable heavy chain secretory signal; (IgE Fc CH1-CH4) IgE Fc gene fragment from domain 1 to 4; (6xHis) 6-histidine peptide tag; (AviTag) peptide tag to allow biotinylation by BirA enzyme; (Stop/x) Stop codon; (VKA 26) immunoglobulin kappa chain variable region secretory signal; (k light chain C-region); constant region of kappa light chain.

Western blotting demonstrated no expression of proteins of the expected size (125 kDa) either with or without the added tags, indicating that this strategy is not suitable for IgE Fc region expression (Figure 3-21).

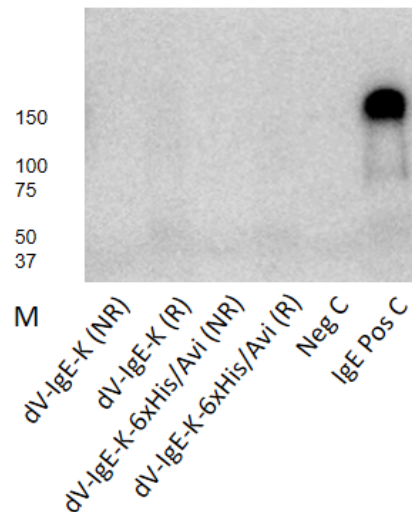


Figure 3-21: Western blot analysis results of the expression of IgE Fc with the Kappa light chain. constant region .

IgE Fc with kappa light chain without variable region (dv-IgE-K), either with or without added peptide tags (6XHis/Avi) is detected. The blot was probed with an anti-human IgE Fc region antibody. The negative control used was non-transfected HEK-293 cells and whole IgE in non- reducing condition is the positive control.

3.3.3.4 Expression of IgE Fc (CH2-CH4)

The IgE Fc final expression strategy was based on modification of the sequence of IgE Fc by deletion of the first domain (CH1). Site directed mutagenesis was used to produce this new construct, utilising two non-mutated primers that flank the area to be deleted. The newly created IgE Fc region gene started at the first amino acid of domain 2 (Valine, 224) (Figure 3-22). After confirming the new sequence of IgE Fc gene by DNA sequencing, the pFUSE-CHlg-hE(2-4)-6xHis/Avi construct was used to transfect HEK-293 cells and after expression supernatants were collected for analysis. Again no expression (expected size 80 kDa) was detected by Western blot (Figure 3-23).

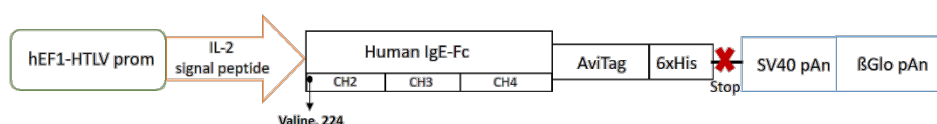


Figure 3-22: Schematic diagram of pFUSE expression cassette of IgE (CH2-CH4).

Abbreviations represent the following: (hEF1-HTLV prom) hybrid of elongation factor 1 α and human T-cell leukemia virus promoters; (IL-2) Interleukin 2; (IgE Fc CH2-CH4) IgE Fc gene fragment from domain 2 to 4; (SV40 pAn) Simian Virus 40 late polyadenylation signal for cleavage; (β Glo pAn) human beta-globin 3'UTR and polyadenylation sequence to arrest transgene transcription; (6xHis) 6-histidine peptide tag; (AviTag) peptide tag to allow the biotinylation by the enzyme BirA; (Stop/x) stop codon; (valine, 224) the first amino acid of the IgE Fc (CH2) gene at position of 224.

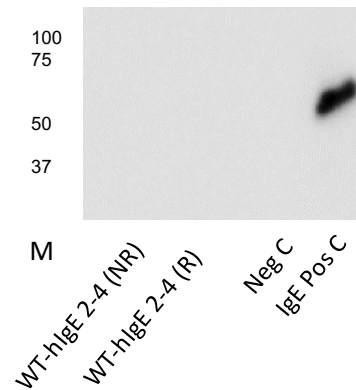


Figure 3-23: Western blot analysis results of the expression of IgE Fc domains CH2-CH4.

Western blot analysis of the expression of wild type (WT) IgE Fc (CH2-CH4) under both non-reducing and reducing conditions (NR and R). Detection performed with anti-6xHis antibody. Non-transfected HEK-293 cells were used for the negative control and the positive control was a His-tagged protein with a molecular weight of approximately 60 kDa.

3.4 Discussion

The aims of the work described in this chapter were twofold: firstly, the production of recombinant Fc variants of human IgG with enhanced binding affinity to the inhibitory Fc receptor (FcγRIIB), and secondly, to achieve high-yield expression and purity of IgE Fc proteins. To achieve these goals the process of expression and purification required careful optimisation. Three stages were involved in this optimisation process: engineering of expression vectors containing the Fc variants with the desired mutations and tags, production in a suitable expression host offering the correct post-translational modifications, and choice of an appropriate purification system to obtain highly pure protein yields.

Site-directed mutagenesis was used to genetically engineer Fc variants, this is a widely-used technique in generation of libraries of mutated proteins for studying structure-function relationships and site directed mutagenesis of whole plasmids is considered an efficient, simple and time saving approach compared to other mutagenesis methods (E.g. cassette and primer extension)^{190,191}. By applying this approach, IgG Fc variants with the desired mutations (EFT+EA, EF and DE) were created by amplifying a whole vector (pFUSE-hIgG1-Fc2) using tail-to-tail phosphorylated primers, one of which contained the desired mutations (Figure 3-4). The newly amplified, linear PCR products of Fc variant genes were ligated at the ends using DNA ligase and the mutation confirmed by DNA sequencing (Figures 3-5, 3-6, and 3-7).

A 6xHis tag and an AviTag were added to each Fc variant of IgG and IgE for protein purification and subsequent conjugation to micro particles respectively. These tags were added to the C-terminus of the protein to avoid any potential disruption of interaction between the IgG and IgE Fc regions and their receptors (Figure 3-8). The

6xHis-tag is commonly used for purification of proteins using IMAC as it is small, easily added and rarely has an effect on the protein activity. The AviTag is a peptide sequence of 15 amino acids (GLNDIFEAQ**K**IEWHE) with a lysine residue (highlighted in bold) that is covalently bound to biotin by a bacterial biotin ligase, BirA¹⁹². This tag is attached to allow the protein to be biotinylated and subsequently conjugated to streptavidin-coated micro particles. Enzymatic biotinylation via the AviTag has several advantages over a chemical biotinylation method: the enzymatic method results in site-specific conjugation between the biotinylated Fc variants and micro particles¹⁹³, and chemical biotinylation can result in random multiple biotinylation of any exposed lysine residue, which can potentially affect the binding properties of Fc regions with its receptors, or cause aggregation of the streptavidin coated micro particles due to multiple biotinylation. As Lysine is a very common amino acid, most proteins biotinylated chemically will carry multiple biotin groups.

The first expression attempted used the *P. pastoris* expression system. This is a eukaryotic system suitable for production of antibody fragments, and is recognised as an ideal host for expression of a wide variety of recombinant proteins due to the presence of the alcohol oxidase promoter (AOX1), which controls the expression of foreign proteins. AOX1 permits *P. pastoris* to utilise methanol in the medium as a source of carbon in the absence of glucose through a role in catalysis of the first step of the methanol metabolism. This promoter has been isolated, characterised and cloned into various *Pichia* expression constructs^{194–196}.

The *P. pastoris* expression system is becoming a good alternative platform to mammalian expression systems, especially in term of producing Fc fragments that require specific post-translational modifications. Moreover, it is considered faster,

easier to manipulate and less expensive than mammalian systems and is capable of producing a high yield of recombinant protein.

Several publications show successful *P. pastoris* expression of antibody fragments; either Fc region or fused with scFv^{197–200}. However, the dot blot results in this work show that IgG and IgE Fc variants were not expressed by the *P. pastoris* system (Figure 3-10). To confirm whether the IgG and IgE Fc genes were integrated into the *Pichia* genome, PCR was used to screen the colonies (Figure 3-11). Some, but not all colonies had the desired genes successfully integrated into their genomes. Colonies with successful integration were analysed for expression by Western blot, but still failed to produce protein (Figure 3-12). The lack of expression of Fc variants might be due to proteolysis of secreted proteins, the most common problem of expression proteins in a *Pichia* system²⁰¹. Inefficient secretion is another common cause and occurs when expressing some complex proteins such as GP 120, which is a glycoprotein exposed on the surface of the HIV envelope²⁰². Furthermore, yeast transcriptional terminators also have a role in preventing some proteins from being expressed to detectable levels due to truncated mRNA. The influence of transcriptional terminators has been previously observed in *Pichia* for two proteins, *B. sphaericus* Bsp2 insecticidal toxin [P1] and HIV-1 Env. However, this was solved by synthesis of a gene with high GC content^{202,203}.

Due to the lack of expression of Fc fragments in yeast, the expression system was switched to a mammalian system. HEK-293 cells were selected as the biochemical machinery present can express mature and functional proteins with the required post-translational modifications²⁰⁴. Several expression strategies were trialled in this cell line, the first being stable expression using Zeocin as selective marker. Although it is time-consuming to generate a stable cell line, stable expression is usually

preferable for large-scale expression as it allows better control over the quality and homogeneity of expressed proteins²⁰⁵. HEK293 cells produce a consistent yield of expressed protein and do not require large quantities of consumable reagents. However, when stably transfected the yield of recombinant IgG Fc variants from HEK293 cells was low (Figures 3-15A and 3-16). There are two main possibilities for this: the stable cell line was generated using a batch culture method without limiting dilution (a procedure used to generate clonal populations); therefore potentially resulting in a polyclonal cell population that can heterogeneously express proteins, or, the IgG Fc genes could have been integrated into the genome at a low copy number and/or unfavourable genetic loci.

In an effort to improve yield of IgG Fc variants, transient expression of IgG Fc regions was trialled in the HEK293 cell line. The aim was to improve the copy number by transfecting a large amount of IgG Fc construct DNA using a commercial transfection reagent (GeneJuice), widely used for transient transfection of HEK-293 cells^{206–208}. The optimal ratio of transfection reagent to the DNA was determined by investigating 3 different ratios (1:1, 3:1, and 1:6) of transfection reagent to GFP-expressing construct. The experiment was analysed by fluorescence microscopy (Figure 3-18). Roughly 40 to 50 % of cells were transfected showing GFP when transfected at two ratios (3:1 and 6:1). The ratio using the least reagents was chosen to maximise the number of possible transfections.

Transfected cells were supplemented with 3.75 mM valproic acid 24 hours after transfection. The concentration of valproic acid used was based on published studies^{209–211}. Valproic acid is a histone deacetylase inhibitor (iHDAC) used in transient transfections to increase the protein yield^{212,213}. The main impact of valproic acid is prevention of deacetylation of histones, which is important in regulation of gene

expression and can lead to more transcriptionally active chromatin²¹⁴. Moreover, valproic acid and other iHDACs can play a role in cell growth arrest and change in the distribution of the cell cycle^{215,216}. All these modifications add up to enhanced stability and increased production of mRNA to increase recombinant protein production²¹⁷. A minimum of a 4-fold increase in antibody expression level has been published in the literature when using iHDACs with transient expression²¹⁸. In this research, however, SDS-PAGE analysis of secreted proteins from transient transfection HEK293 cells did not show any improvement in the yield of IgG Fc proteins (Figure 3-19). There are a couple of possible explanations for this result. Recombinant protein yields could be limited by the available surface area of the adherent cell culture; in a suspension culture cell density is higher, therefore increasing the potential protein yield. A second explanation could be that although the reagent ratio was optimised, the transfection efficiency of GeneJuice could be limited as this was used at a low concentration.

Affinity chromatography with protein A, G, or L is regularly used to purify IgG fragments, however this is expensive and there is the potential for proteins to be degraded due to the harsh elution procedure or cleaning conditions, respectively. For those reasons, IMAC purification with two transition metal ions (Ni^{2+} and Cu^{2+}) was used to purify the IgG Fc variants. Supernatants containing the secreted IgG Fc variant (DE) were applied to the nickel resin while the other Fc variants (EFT+EA and EF) were applied to cobalt Dynabeads (Figures 3-16 and 3-17). Although the supernatants contain serum proteins which complicate the purification process, both metal ions show a specificity for 6xHis-tagged proteins. Based on SDS-PAGE analysis, cobalt Dynabeads showed better protein purification than nickel resin. This agrees with a previous study in which cobalt showed greater specificity than nickel when purifying a 6xHis-tagged protein from a mixture²¹⁹.

Three different strategies were used for expression of the IgE Fc fragment based on modification of the Fc gene sequence. Expression of the IgE epsilon chain (Ce1-Ce4) was the first strategy tested; Western blot analysis shows that this Fc fragment was not expressed by either yeast or mammalian cell lines (Figures 3-12B and 3-15B). However, this fragment has been successfully expressed in *E. coli*, by another research group and showing the same antigenicity of human IgE, and a molecular weight equal to that expected from the amino acid sequence²²⁰. The second expression trial was based around including the constant region kappa light chain in the Fc fragment. This addition was made as the heavy chain constant region 1 (CH1) is not stable in the absence of a constant region light chain (CL); the intermolecular disulphide bond between heavy and light chains provides stability²²¹. Although these fragments (CH1/CL) have been expressed in the periplasm of *E. coli*²²¹ indicating that this was a promising strategy, Western blot analysis detected no protein in the expression supernatant (Figure 3-21), suggesting no expression. The success in the literature of expressing IgE fragments (Ce2-Ce4) using a mammalian platform²²²⁻²²⁴, lead to the development of a third strategy. The first domain of the IgE Fc epsilon chain was deleted using site directed mutagenesis, beginning the chain at the first amino acid of the second domain (valine 224). Despite favourable outcomes in published work, no secreted IgE (Ce2-Ce4) was detected in this research (Figure 3-23). One explanation for the failure of the first two strategies is that the role of heavy chain binding protein (BiP) prevents CH1 from secretion until it assembles with the light chain²²⁵, and one potential reason why no expression was observed from the third strategy could be the presence of the thiol side-chain of cysteine 225²²⁶. Those reasons will be explained in detail in chapter 4.

3.5 Conclusion

In this chapter, expression constructs of IgG Fc variants (EFT+EA, EF and DE) with enhanced binding affinity to FcγRIIB were engineered using site-directed mutagenesis. To obtain a site-specific conjugation with the streptavidin coated microparticles, these fragments were designed with a 15 amino acid peptide sequence known as AviTag. Yeast expression of these fragments was unsuccessful, however Fc fragments were successfully expressed by HEK-293 cells and secreted into the supernatant. The yield of IgG Fc protein was low however, and varying the method of transfection (stable or transient) did not improve. Two IMAC purification methods were tested, of which cobalt Dynabeads were shown to remove more non-6xHis-tagged proteins than nickel, producing a purer protein yield. Finally, three different expression strategies were investigated for the expression of IgE Fc proteins, none of which were successful.

Chapter 4. Expression of recombinant IgG and IgE Fc domains in suspension growing mammalian cells

4.1 Introduction

The low yield of IgG Fc variants was the key problem that we faced in the optimisation experiments (see Chapter 3). Choosing the appropriate expression technique to produce sufficient amounts of the recombinant human antibody Fc proteins is crucial to the success of an expression experiment. The challenge in the expression of Fc protein is to establish a robust expression system that is able to produce highly purified recombinant proteins in yields suitable for the planned downstream experiments. During the past years, many developments in mammalian production procedures have emphasised four key elements to produce more recombinant proteins with proper folding and possessing the necessary post translational modifications:²²⁷ the genetic modifications of the host cells; the expression vector design; the transfection vehicle; and the culture conditions.

The Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) is one of the genetic modifications made on HEK 293 cells to increase the protein expression level²²⁸. EBNA1 is an EBV protein which is expressed in the proliferating infected cells and has a crucial role during the latent viral infection through the replication and the mitotic segregation of EBV genomic DNA²²⁹. Thus, the genetic variant of HEK293 cells (293-EBNA1) permits the episomal amplification of the vector comprising the viral EBV origin of replication (*oriP*). The expected role of EBNA1 as an enhancer in the expression of the recombinant protein is during the production phase by allowing more plasmid copies to be produced in transfected cells²³⁰. The second key of the development process is to design an expression vector with a highly active promoter in the mammalian cell line. The human cytomegalovirus promoter (pCMV) is a

powerful promoter, especially in the HEK293 cell line. It has been reported that consistently expressed adenovirus E1a can strongly activate the CMV promoter²³¹. The expression level of adenovirus recombinant proteins was reached up to 20 % of total cell protein when using different expression cassettes under the control of this promoter^{232,233}. The third aspect is associated with the efficacy of the transfection vehicle. Although many effective commercial transfection reagents are available, only the more cost-effective reagents are desirable in large-scale expression. There are many criteria regarding the transfection reagent which should be satisfied when operating a large-scale transient transfection. These are ease of use, minimal cytotoxicity, and effectiveness of use in suspension-grown cells. Polyethyleneimine (PEI) is one of the transfection reagents that meets most of these considerations as a cost-effective reagent, which is highly efficient in transferring the gene in many host cell lines with low cytotoxicity profile. The suspension HEK293-EBNA1 can be effectively transfected with this polymer^{234,235}. Two isoforms (linear and branched) are available for this polymer, with a broad range of polydispersity and molecular weights; these different physiochemical properties are important for the efficiency of the transfection activity²³⁶. The culture growth conditions are the last factor of the development procedure. The ability to adapt the cell to grow in suspension is preferable, as it allows easy scale-up and growth is not limited to surface area as in adherent cells. Higher cell density will result in much higher yields. The growth of the cells in serum-free medium is required for some transfection reagent, as it enhances the activity of transfection or is the only working option for other gene transfer reagents²³⁷. Development of culture medium suitable for both steps (transfection and growth of the cells) is also highly preferable as it removes the necessity to exchange

the medium before and after the transfection. Finally, the medium additive has a role in increasing the production level such as tryptone²³⁸.

In this chapter, we will demonstrate the successful expression of both recombinant IgG Fc variants and IgE Fc (CH2-CH4). The optimal expression and purification conditions were achieved by cloning in pTT vectors and transfection in suspension of HEK293-EBNA1 cells using Polyethyleneimine, and the purification of these Fc proteins by immobilised metal affinity chromatography method using the ÄKTA start system.

4.2 Experimental procedure

4.2.1 Generation of pTT and pYD constructs with IgG and IgE fragments

All pTT expression constructs are listed in Table 4-1

Table 4-1: Summary of IgG Fc and IgE Fc pTT constructs.

Construct Name	Mutations Positions	Vector Backbone	Fc variants ID
pTT5-EFT+EA-6xHis/Avi	S267E/ H268F/ S324T/ I332E/ G236A	pTT5	EFT+EA
pTT5-EF-6xHis/Avi	S267E/L328F	pTT5	EF
pTT5-DE-6xHis/Avi	S239D/ S267E	pTT5	DE
pTT5-hIgG1-Fc- 6xHis/Avi	-	pTT5	WT-IgG Fc
pTT5-IgE Fc (2-4) _{Phe229} - 6xHis/Avi	-	pTT5	WT-IgE Fc (2- 4) _{Phe229}
pYD5-IgG Fc-TEV-IgE Fc	-	pYD5	Fc γ - Fc ϵ

IgG and IgE Fc fragments were amplified using Q5® High-Fidelity DNA Polymerase (New England Biolabs, USA) with primers listed in Table 2-1. The cycling condition was set up with initial denaturation at 98°C for 30 seconds, followed by 25 cycles of denaturation at 95°C for 10 seconds, annealing at 72°C for 30 seconds and extension at 72°C for 30 seconds/kb with final extension at 72°C for 10 minutes. The amplified IgG Fc fragments with IL-2 signal peptide were cloned into pTT5 vectors (National Research Council, Canada). Both vector and insert were digested with *PmeI* and *NotI* restriction enzymes under the conditions stated by the manufacture. Both digested IgG Fc products and pTT5 vector were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, UK) and mixed in 3:1 molar ratio (insert to vector), then ligated with Quick ligation kit (New England Biolabs, UK) according to the manufacturer's protocol.

With the same protocol described for the cloning of IgG Fc, IgE Fc (CH2-CH4) fragment was first cloned into the pYD5 vector (containing the sequence of IgG Fc and a TEV protease cleavage site) with two different restriction enzymes – EcoRV and BamHI – to be expressed as IgG-IgE Fc fusion protein. The second cloning strategy involved using the same restriction enzymes (EcoRV and BamHI) to clone the amplified IgE Fc (CH2-CH4)_{Phe229} into the pTT5 vector. Following the ligation step, the ligated constructs were transformed using the heat shock method into DH5 alpha competent cells for plasmid propagation. The cells were then grown in LB medium agar supplemented with 100µg/ mL ampicillin (Sigma Aldrich, UK) for selection. To check the process of vectors cloning, separately grown colonies were analysed using colony PCR with JumpStart Taq DNA polymerase (Sigma Aldrich, UK) with the primers shown in Table 2-2 and an annealing temperature of 58°C. Agarose gel electrophoresis was the first step to confirm gene insertion, and then the positive colonies were grown in LB medium supplemented with 100µg/ mL ampicillin and the plasmid were purified. Finally, all samples were sent to Source Bioscience Lifesciences for DNA sequencing along with the sequencing primers (Table 2-2).

4.2.2 Expression procedure in suspension HEK 293-6E cells

4.2.2.1 Small-scale transfection of HEK 293-6E cells

At a cell density between $1.5\text{--}2.0 \times 10^6$ cells/ml of HEK293-6E, 1.8 ml of the cells was transferred to each well of a 6 well-plate. This plate was then transferred to an orbital shaker in a humidified incubator containing 5% CO₂ at 37°C. The transfection procedure was begun by preparing the complex of DNA-PEI using a ratio of (1:2) (w/w). For each well, the DNA solution was prepared by adding 100 µL transfection medium (F17 medium) to 6x1.5 mL Eppendorf tubes, followed by 2 µg DNA of each Fc construct to each tube. The PEI solution was prepared for the entire 6 well-plate by

adding 672 μ L of transfection medium to 1.5 mL tube followed by 28 μ L PEI with vortex. The following step was adding 100 μ L PEI solution to each DNA solution with immediate vortexing upon adding the PEI, and incubation for 3 minutes at room temperature. Once the complex of DNA-PEI was prepared, the whole complex mixture of each construct was adding dropwise to each well with swirling, and the plate returned to the orbital shaker in the incubator. For the secreted Fc construct, 20 % w/v of Tryptone N1 (Tekniscience Inc., Canada) in F17 medium without G418 was added to a final concentration of 0.5 % within 24 to 48 hours post- transfection. Finally, the supernatants of secreted Fc proteins were collected after 120 hours to be analysed by SDS-PAGE and Western blot (see Section 3.2.4).

4.2.2.2 Large-scale transfection of HEK 293-6E cells

Two days before the experiment, 125 mL of HEK293-6E with a density of 2×10^6 cells/mL were diluted to 0.5×10^6 cells/mL with F17 medium in a disposable 2 L Corning® Erlenmeyer flask (Sigma Aldrich, USA). On the day of transfection, the density of the cells was determined by counting in a haemocytometer and the density confirmed between $1.5\text{-}2.0 \times 10^6$ cells/mL. 1 μ g of each Fc construct was used to transfect 1 mL of cells at density of $1.5\text{-}2.0 \times 10^6$. PureYield™ Plasmid Midiprep System (Promega, UK) was used to purify the Fc constructs and obtain these vectors in high amount. For 0.5 L transfection volume, the DNA-PEI complex with a ratio of (1:2) (w/w) was prepared as follow; 0.5 mg of Fc construct DNA was added to 12.5 mL of F17 medium without G418 in Falcon 15 mL Conical Centrifuge Tube (Fisher scientific, USA) with vortexing. Then, in another 15 mL tube, the PEI solution was prepared by adding 1.5 mg to 12.5 mL of F17 medium with vortexing. To form the complex, PEI solution was added to the DNA solution with vortexing and incubation for 3 minutes followed by adding the complex mixture directly to the cells. Finally, the supernatants

of secreted Fc proteins were harvested and centrifuged at 2000 xg for 15 minutes at 4°C followed by filtration with 0.22 µm syringe filters and cOmplete®, Mini, EDTA-free protease inhibitor cocktail tablets (Roche, USA) were added to the supernatant, which was then stored at 4°C.

4.2.3 Immobilised Metal Affinity Chromatography (IMAC) method using ÄKTA system

Using the ÄKTA start system (GE Healthcare, USA), the harvested supernatants of polyhistidine-tagged Fc proteins from the previous step were mixed in 1:1 ratio with 2X binding buffer (100 mM sodium phosphate, 600 mM NaCl, pH 7.4) and loaded onto 1 ml HiTrap Columns prepacked with cobalt-based resin (GE Healthcare, USA). The flow rate of the loaded samples was 1ml/min and the elution method was a continuous gradient from 0-100 % of elution buffer with the same flow rate. The washing buffer consisted of 50 mM sodium phosphate, 300 mM NaCl and 5mM imidazole at pH 7.4, and the elution buffer consisted of 50mM sodium phosphate, 300 mM NaCl and 150 mM imidazole with pH 7.4. All elution fractions were analysed by SDS-PAGE gel electrophoresis and Coomassie staining.

4.2.4 Cleavage of IgG-IgE Fc fusion protein using Tobacco Etch Virus (TEV) protease

Pro TEV plus (Promega, UK) was used to cleave the purified IgG-IgE Fc fusion proteins. In a 100 µL reaction volume, the following components were assembled in 1.5 ml Eppendorf tubes: 5 µL of 20x ProTEV Buffer, 1 µL of the redox pair (3.0 mM reduced GSH/0.3 mM oxidized GSH), 20 µg of fusion protein, 2 µL of Pro TEV plus and filled up with water. The samples were incubated at 30°C and 20 µL aliquots were removed at 1, 2, 4 and 8 hours overnight at room temperature and at 4°C, before being analysed by SDS-PAGE gel. These aliquots were mixed with 1x SDS-PAGE sample buffer and stored at -20°C.

4.2.5 Protein quantification

Following the purification procedure, all eluted fractions containing Fc proteins were collected and loaded into Amicon® Ultra Centrifugal Filters (MWCO 10 kDa, Merck Millipore, UK) to remove the imidazole from the elution buffer and to concentrate the samples. Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, UK) was used to quantify the protein concentration by following the manufacturer's instructions.

4.3 Results

4.3.1 Cloning of IgG Fc and IgE Fc domains into expression vectors

To generate IgG Fc variants and IgE Fc expression constructs, PCR amplification was performed to amplify the IgG and IgE Fc fragments. In case of IgG Fc, four amplified gene products were obtained using primers with *PmeI* and *NotI* restriction in the expected size, and this corresponded to three IgG Fc variants with enhanced binding affinity to FcγRIIB receptor (EFT+EA, EF, DE) and wild type IgG Fc respectively (Figure 4-1A). For IgE Fc CH2-CH4 domain, two DNA products were amplified using primers with *EcoRV* and *BamHI* restriction; one encoded the whole CH2 domains while the other one was missing the first five amino acids of the same domain and was labelled as IgE Fc (2-4)_{Phe229} (Figure 4-2B and C). Following the amplification process, the IgG Fc variant and IgE Fc with a 6xHis and an AviTag along with their corresponding vectors (Table 4-1) were digested, purified, mixed, ligated according to the manufacture's instruction, and cloned into *E. coli* for plasmid propagation. After PCR screening of the colonies, DNA sequencing results showed the successful cloning of the inserted genes.

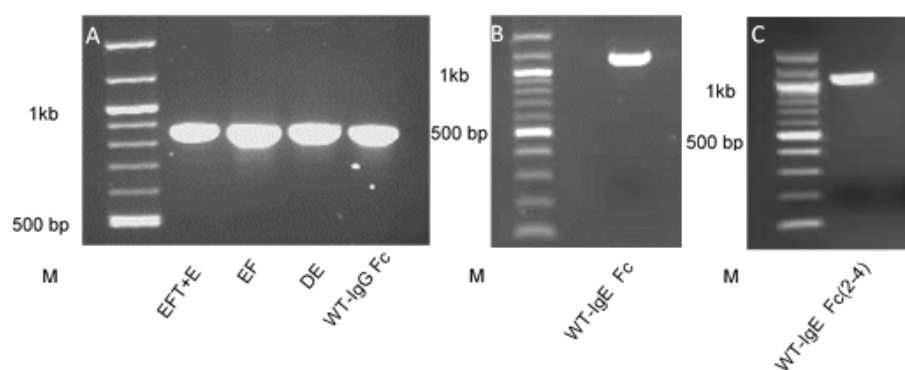


Figure 4-1: Gel electrophoresis separation of IgG and IgE Fc fragments.

EtBr-stained 1.2 % agarose gel with a 100 bp ladder showing the separation of following; (A) IgG Fc variants (EFT+E, EF, DE) and wildtype IgG Fc with the expected size (856 bp), (B) the wild type of IgE Fc fragment domain 2 to 4 and (C) the same IgE fragment but starting from Phenylalanine at position of Phe229 (WT-IgE Fc (2-4)_{Phe229}) with expected sizes 1120 bp and 1103bp, respectively.

4.3.2 Recombinant expression screening in Suspension growing HEK 293-6E cells

HEK 293-6E suspension cells were used as an expression platform to express the Fc recombinant proteins from expression constructs derivative containing IgG Fc variants and IgE Fc genes generated as described above. Initially, the cells were transfected with pTTo-GFPq vector (National Research Council, Canada) encoding a green fluorescent protein to evaluate the transfection efficiency of PEI transfection reagent at ratio of 1:2 (DNA to PEI). Using fluorescence microscopy, the cells were evaluated after 48 hours for transfection success. Roughly, 70 to 80 % of the cells were transfected with PEI transfection reagent, and this is considered a high transfection efficiency (Figure 4-2).

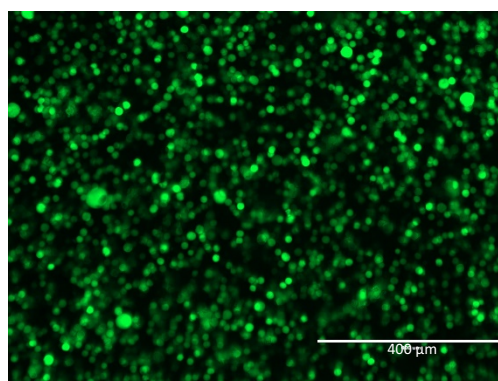


Figure 4-2: Fluorescence microscopy image of transfected HEK 293-6E cells with pTTo-GFPq vector.

HEK 293-6E cells were transfected using PEI transfection reagent at a ratio of 1:2 (pTTo-GFPq vector to PEI) (w/w). The picture was taken 48 hours post-transfection.

4.3.2.1 Expression screening of IgG Fc variants

IgG Fc variants expression constructs were transfected into HEK 293-6E cells to transiently express the Fc proteins. The expression cassette of pTT5 construct consisted of inserted IgG Fc variants genes, and a human interleukin 2 (IL-2) secretory signal peptide (Figure 4-3).

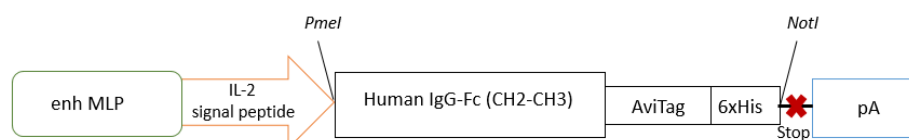


Figure 4-3: Schematic diagram of pTT5 derivatives expression cassette of IgG Fc.

General schematic of the IgG Fc variant constructs. Abbreviations represent the following: (enh MLP) adenovirus major late promoter enhancer; (IL-2) Interleukin 2; (IgG Fc CH2-CH3) IgG Fc gene fragment from domain 2 to 3; (pA) polyadenylation signal sequence; (6xHis) 6-histidine peptide tag; (AviTag) peptide tag to allow biotinylation with BirA enzyme; (Stop/x) Stop codon.

The supernatants of HEK 293-6E cells transfected with IgG Fc variants expression constructs were harvested five days post transfection and examined by Western blot using anti-IgG Fc region antibody. The data demonstrated successful expression of all IgG Fc variants (EFT+EA, EF, DE and wildtype IgG Fc) and bands were seen at the expected sizes (approximately 60 and 30 kDa in under non-reducing and reducing conditions, respectively) see Figure 4-4.

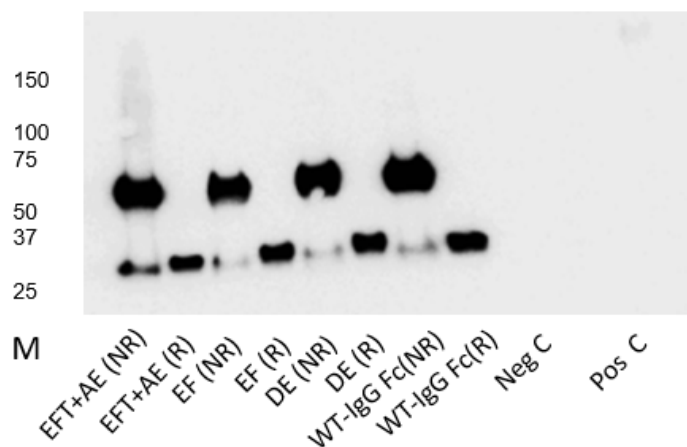


Figure 4-4: Western blot analysis results of the transient expression of IgG Fc variants from HEK 293-6E cells.

Expression of IgG Fc variants (EFT+EA, EF, DE and wild type IgG) from transient transfection of 293-6E cells, with an expected molecular weight of 60 kDa under non-reducing conditions (NR) and 30 kDa under reducing conditions (R). Detection was achieved with anti-human IgG Fc region antibodies. Non-transfected cells were used as a negative control and the positive control was whole IgG under non-reducing conditions.

An expression kinetics study was performed to establish the best time for harvesting the secreted Fc proteins. Supernatant samples of wild type IgG Fc were collected every 24 hours after the third day of transfection and screened by Western blot. The results in Figure 4-5 show the protein expression level is high at 144 hours post transfection. However, increasing putative protein degradation was observed at molecular weight of approximately 30 kDa. To avoid the problem of degradation, 120 hours post transfection was chosen as the best harvesting time.

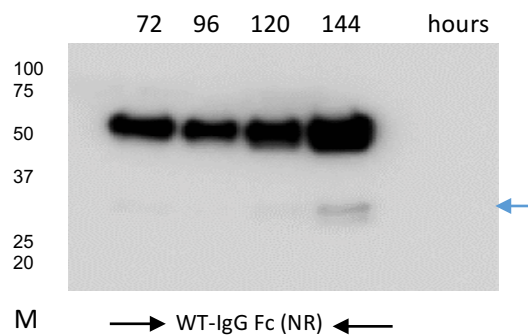


Figure 4-5: Time course analysis results of the expression of IgG Fc from HEK 293-6E cells.

Supernatant aliquots of secreted wild type IgG Fc protein were collected at 72, 96, 120 and 144 hours post transfection and examined by Western blot in non-reducing conditions (NR). Detection was achieved with anti-human IgG Fc region antibodies. (→) symbol indicates the site of protein degradation.

4.3.2.2 Expression screening of IgG-IgE Fc Fusion protein

One of IgE Fc expression strategies is based on the cloning of IgE (CH2-CH4) sequence to pYD5 vector (contain IgG Fc) to be expressed as a fusion protein, and then cleaved by using Tobacco Etch Virus (TEV) protease. The expression construct of this strategy was made of the signal secretory sequence of IgG kappa light chain, the sequence of IgG Fc then TEV protease sequence followed by IgE Fc with AviTag and poly histidine tags (Figure 4-6).

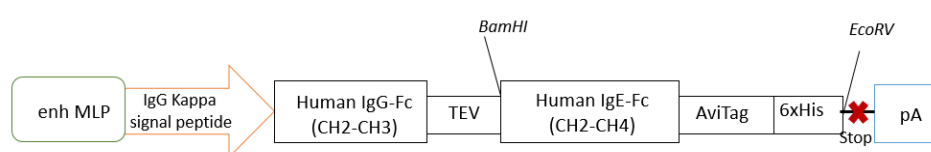


Figure 4-6: Schematic diagram of pYD5 derivatives expression cassette of IgE Fc.

Schematic of the IgE Fc construct. Abbreviations represent the following: (enh MLP) adenovirus major late promoter enhancer; (IgG Kappa) IgG Kappa light chain; (IgG Fc CH2-CH3) IgG Fc gene fragment from domain 2 to 3; (TEV) Tobacco Etch Virus protease sequence; (IgE Fc CH2-CH4) IgE Fc gene fragment from domain 2 to 4; (pA) polyadenylation signal sequence; (6xHis) 6-histidine peptide tag; (AviTag) peptide tag to allow biotinylation with BirA enzyme; (Stop/x) Stop codon.

Following the transfection with pYD5 derivate expression construct, the supernatant of transfected 293-6E were examined to test the expression of the fusion protein. The expression strategy of IgG-IgE Fc fusion protein was successful as demonstrated by the presence of bands of the expected sizes (146 and 73 KDa) under non-reducing and reducing conditions respectively. it was confirmed by using anti IgE Fc antibody as method of detection (Figure 4-7A). To increase the protein yield, supernatant aliquots were taken at different interval time and 120 hours post transfection was selected to be the best time of harvesting (Figure 4-7B).

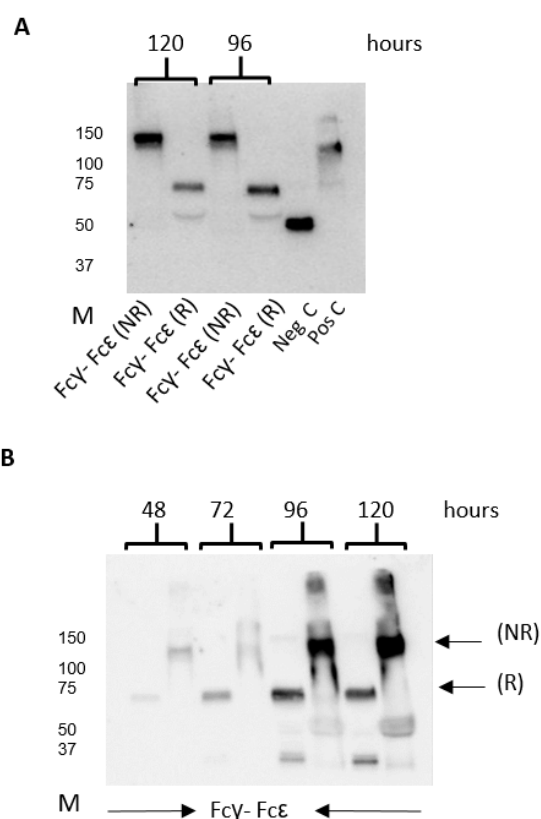


Figure 4-7: Western blot analysis results of the expression of IgG- IgE Fc fusion protein.

- A. Expression of IgG- IgE Fc fusion protein (Fcγ- Fcε) from transient expression of 293-6E cells with expected sizes (136 KDa and 68 kDa) under both non-reducing and reducing conditions (NR and R) respectively, at 120 and 96 hours post transfection. Non-transfected HEK-293 cells were used for the negative control and the positive control was whole IgE under non-reducing condition. Detection performed with anti-IgE Fc region antibody.
- B. Time course analysis of IgG- IgE Fc fusion protein (Fcγ- Fcε) expression under both non-reducing and reducing conditions (NR and R) at 120, 96, 72 and 48 hours post transfection and it was detected with anti-IgG Fc region antibody.

4.3.2.3 Expression screening of IgE Fc (CH2-CH4)_{Phe229} protein

In this expression strategy, we aimed to modify the sequence IgE Fc (CH2-CH4) coding DNA to start from Phenylalanine amino acid at position 229 of domain 2 (the rationale of this strategy will be found in the discussion section). The process of cloning was performed using *EcoRV* and *BamHI* restriction sites into the expression construct (pTT5 vector). By using this method, the first five amino acids of domain 2 including cysteine at position 225 were missed (Figure 4-8).

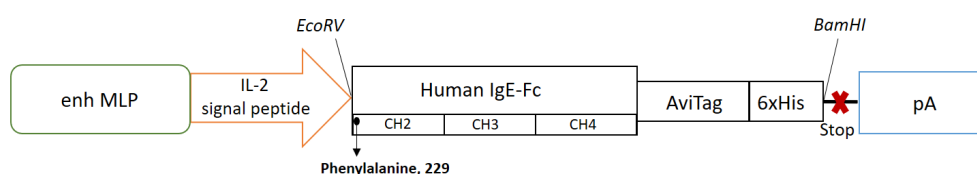


Figure 4-8: Schematic diagram of pTT5 derivatives expression cassette of IgE Fc (CH2-CH4)_{Phe229}.

Schematic of the IgE Fc (CH2-CH4)_{Phe229} construct. Abbreviations represent the following: (enh MLP) adenovirus major late promoter enhancer; (IL-2) Interleukin 2; (IgE Fc CH2-CH4) IgE Fc gene fragment from domain 2 to 4; (pA) polyadenylation signal sequence; (6xHis) 6-histidine peptide tag; (AviTag) peptide tag to allow biotinylation with BirA enzyme; (Stop/x) Stop codon; (Phenylalanine, 229) the first amino acid of the IgE Fc (CH2) gene at position of 229.

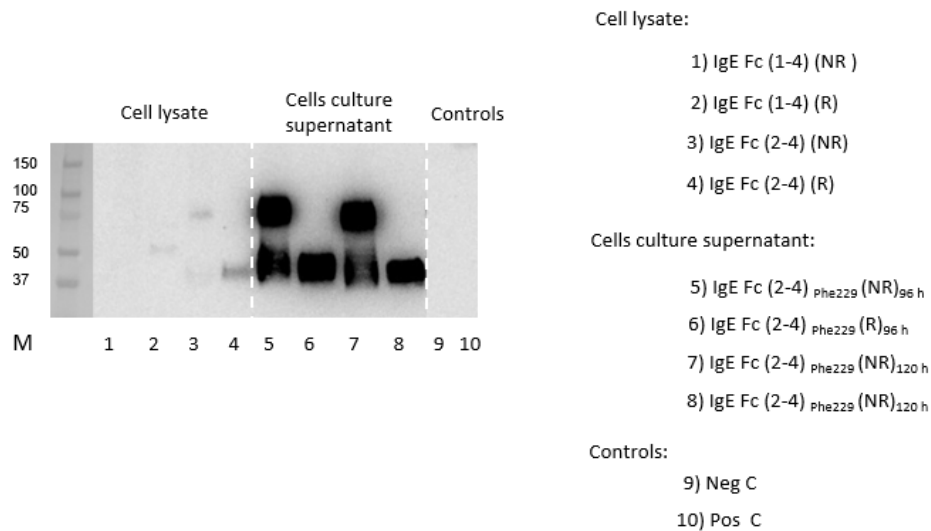


Figure 4-9: Western blot analysis results of the expression of IgE Fc (CH2-CH4)_{Phe229}.

Expression of IgE Fc (2-4)_{Phe229} from transient expression of HEK293-6E cells with expected sizes (80 and 40 kDa) under both non-reducing and reducing conditions (NR and R) respectively from supernatant and at 96 and 120 hours post transfection. Two other IgE Fc fragments (1-4) and (2-4) from the cell lysate show bands with their corresponding sizes (80 and 40 kDa) in non-reducing and reducing conditions of IgE Fc (2-4) and only one band in the reducing condition of IgE Fc (1-4) (50 kDa). Non-transfected HEK 293-6E cells were used for the negative control and the positive control was whole IgE in non-reducing condition. Detection performed with anti-IgE Fc region antibody.

Sample aliquots were taken from the cell supernatant of transfected 293-6E cells with IgE Fc (CH2-CH4)_{Phe229} expression construct at 96 and 120 hours post transfection. The result of Western blot clearly shows bands of the expected size of Fc (CH2-CH4)_{Phe229} under both non-reducing and reducing conditions (80 and 40 kDa, respectively). The cell lysate of transfected cells with two different fragments of IgE Fc (1-4) and (2-4) were also tested. As described previously in Sections 3.3.3.3 and 3.3.3.4, the expression constructs containing these IgE fragments gene did not give positive results when the cells' supernatants were examined. However, bands were observed

in the expected sizes in cell lysates samples of IgE Fc (2-4) in the non-reducing and reducing conditions, while one band of IgE Fc (1-4) was found in the reducing condition. This data reflects that the previous IgE Fc regions were expressed but failed to be secreted in the cells' supernatants (Figure 4-9).

4.3.3 Large-scale purification of recombinant IgG Fc variant and IgE Fc proteins

After the expression screening step, the next was to perform large scale transfection and purification of all recombinant Fc proteins. To achieve this, Fc expression constructs were prepared and purified using Midiprep purification scale to be used in 0.5L transfection volume of HEK 293-6E cells (see Section 4.2.2.4). The harvested supernatant containing Fc proteins was performed through their polyhistidine tags by using IMAC purification method.

Although the data obtained from the column purification of IgG Fc protein shows that some target Fc protein was lost in the flow through-fraction, which was confirmed by Western blot (Figures 4-10A and B), the eluted Fc protein was purified and the yield was better compared to the previous method of expression and purification (see Sections 3.3.3.1 and 3.3.3.2). following the purification procedure, the eluted fractions of purified IgG Fc protein were collected and quantified using BCA assay kit (Table 4-2).

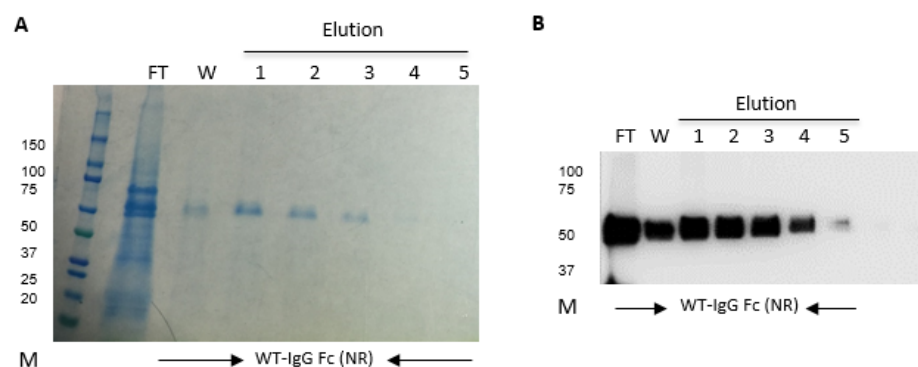


Figure 4-10: Purification results of wild type IgG Fc using IMAC method.

(A) SDS-PAGE stained with Coomassie blue and (B) Western blot after purification with Cobalt TALON superflow (cobalt resin). Abbreviations represent the following: (FT) flow through fraction; (W) wash fraction; (E) elution fractions; M, molecular weight markers.

(B) Western blotting conducted with an anti-human IgG Fc region antibody.

Regarding the purification of IgG-IgE Fc fusion protein, the SDS-PAGE showed that elution fractions contained a purified fusion protein with no loss of protein in the flow-through compared with the purification of IgG Fc protein (Figure 4-11A). Following the purification and quantification process, TEV protease was used to cleave the IgG-IgE Fc fusion protein by applying the enzyme to the protein and incubating at 30°C for 0.5 - 8 hours and overnight at room temperature and at 4°C. The results demonstrated that fusion protein was efficiently cleaved with TEV protease by observing the bands of IgE Fc protein after the overnight incubation at room temperature and at 4°C (Figure 4-11B).

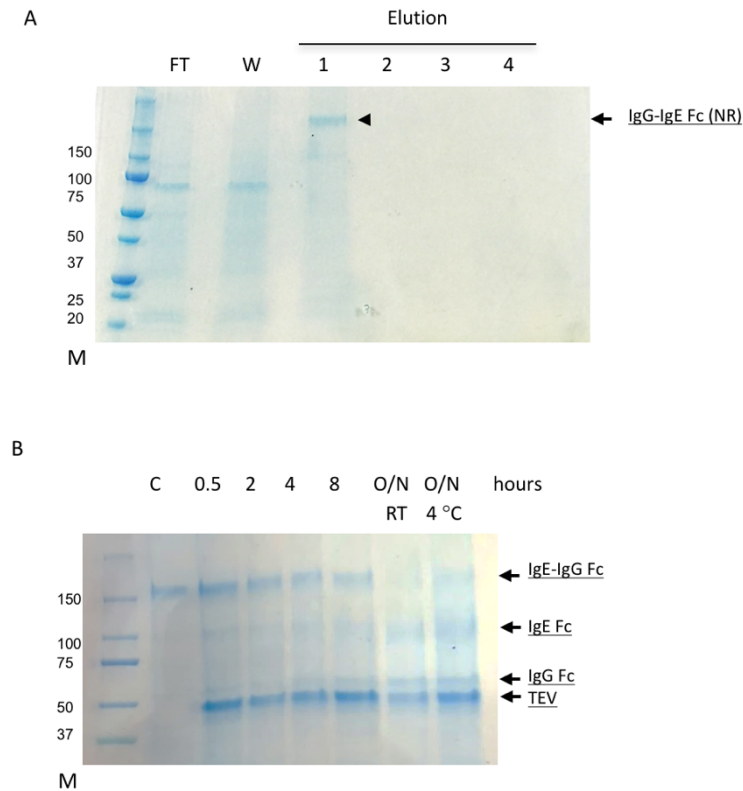


Figure 4-11: Purification and cleavage results of IgG-IgE Fc protein.

- (A) SDS-PAGE stained with Coomassie blue showing the purification of IgG-IgE Fc fusion protein with Cobalt TALON superflow (cobalt resin). Abbreviations represent the following: (FT) flow through fraction; (W) wash fraction; (E) elution fractions; M, molecular weight markers.
- (B) SDS-PAGE stained with Coomassie blue demonstrates the cleavage process of IgG-IgE Fc fusion protein using Tobacco Etch Virus (TEV) protease enzyme at different interval time 0.5, 2, 4, 8 hours, overnight (O/N) at room temperature (RT) and at 4°C. (→) symbol represents the position of the proteins, (c) symbol is the control.

Similar to the previous the recombinant Fc proteins, IgE Fc (CH2-CH4)_{Phe229} protein was purified from the supernatant of 293-6E by IMAC using the ÄKTA start system. The data shows that IgE Fc protein was successfully purified with no visible contaminants in the elution fraction of IMAC (Figure 4.12). Following the purification

all the eluted fractions containing the IgE Fc protein were collected and quantified (Table 4-2).

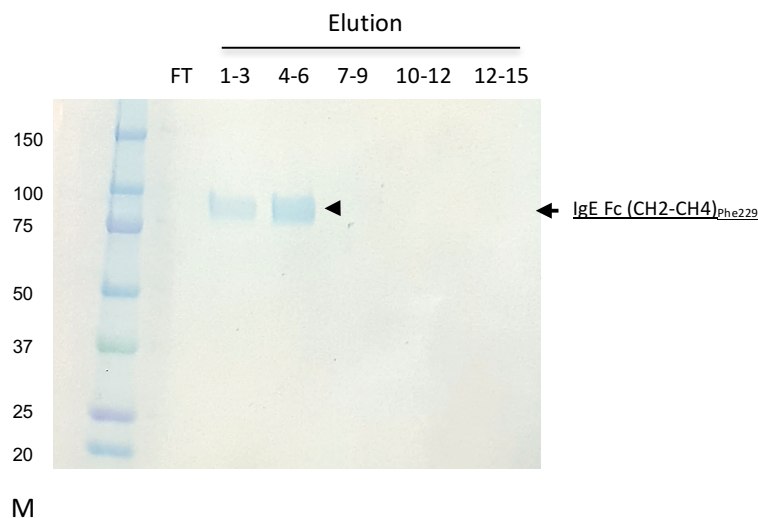


Figure 4-12: Purification results of IgE Fc (CH2-CH4)_{Phe229} protein.

SDS-PAGE analysis showing the purification of IgE Fc (CH2-CH4)_{Phe229} protein from AKTA start system with 1ml HiTrap Columns prepacked with cobalt-based resin. Abbreviations represent the following: (FT) flow through fraction; (E) elution fractions; M, molecular weight markers.

Table 4-2: Summary of recombinant Fc protein expression yields.

Protein ID	Molecular weight (kDa)	Volumetric yield (mg/L)
WT-IgG Fc	60	8
DE	60	6.22
Fcγ- Fcε	146	0.270
WT-IgE Fc (2-4) _{Phe229}	80	2

- Protein molecular weight was calculated theoretically based on the sequence of their amino acids. This does not include any posttranscriptional modifications known to occur, such as glycosylation.

4.4 Discussion

The aim of this chapter was to choose a robust, versatile and simple expression platform with optimal conditions to overcome the expression challenges of IgG and IgE Fc. To achieve this, a new expression strategy was applied by using HEK 293-6E cells as an expression host with pTT5 and pYD5 vectors and Polyethyleneimine (transfection vehicle). The experimental procedures of this expression strategy were carried out in three steps: vector cloning, expression screening and large-scale purification.

Two vectors pTT5 (4.4 kb) and pYD5 (5.1 kb) were cloned with recombinant Fc genes using digestion and ligation cloning. They are a family of expression constructs derived from the pTT vector licenced from National Research Council, Canada (NRC). These plasmids encompass viral EBV origin of replication (*oriP*), and an expression cassette of enhanced cytomegalovirus (CMV) promoter which can lead to a high expression level in 293-6E cells²²⁸. The suggested mechanism for increasing the protein expression is the role of the EBV replicon-based constructs in maintaining the episomal replication²³⁰. Another likely mechanism explains the high expression of *oriP*-mediated vectors is arising from the dependent enhancer activity of EBNA1- *oriP*^{239–241}. As EBV *oriP* has 24 binding sites for EBNA1²⁴², constructs containing an *oriP* can be coated at specific sites by EBNA1 nuclear localisation signal to form protein-plasmid complex which could lead to increase the nuclear retention of these vectors^{243,244}.

Following the cloning of recombinant Fc genes with their corresponding vectors (confirmed by DNA sequencing), pTT5 and pYD5 derivative constructs were transfected into 293-6E using Polyethyleneimine (PEI) as transfection reagent. Two isoforms (linear and branched) of PEI are available to be used as a gene carrier²⁴⁵.

Some published studies demonstrated the differences between the two forms of PEI in terms of transfection efficiency and cytotoxicity profiles. In comparison to the branched PEI, Linear PEI has an improved transfection efficiency and preserves higher cell viability. Based on these findings, the 25 kDa linear PEI was chosen as transfection reagent^{246–249}.

Generally, the transfection complex's formulation is equal to 10% of the cell culture volume and it has important effect on the transfection efficiency. For HEK 293 cells growing in suspension culture, the transient transfection with a ratio of 1:2 (DNA:PEI) is regarded as an optimal ratio in different published reports^{228,250,251}, so we kept the same ratio in the small and large expression scale. This transfection vehicle shows higher gene transfer efficacy (Figure 4-2) compared to the other commercial transfection reagent (X-tremeGENE 9 and GeneJuice) which were used in Chapter 3. It is a highly positively-charged cationic polymer and is considered as one of the most efficient polymers used in gene delivery²³⁴. It works by the condensation of anionic DNA into polyplexes (nano-size complexes with positive charge), and enter the cells by endocytosis²⁵². The buffering capacity property of PEI over a wide range of pH is linked to its many amino groups²⁵³. At lower pH, PEI mediates the chloride influx due to high level of protonation which in turn lead to rupture the endosomal membrane by the effect of osmotic pressure. This process known as a sponge proton effect of PEI. As a result, the endosomal vesicles' acidification is prevented, and also polyplexes will be escaped from the endosome²⁵⁴. At the end, this polyplexes will help to avoid the enzymatic degradation of DNA in the lysosome²⁵⁵.

Before the production of the recombinant IgG Fc variants protein from large-scale expression, two steps should be completed; small-scale screening and selecting the best time for protein harvesting. The positive results from the expression screening

of transfected cells (Figure 4-4) confirm the process of IgG Fc gene cloning as well as the successful cleavage of Interleukin-2 (IL-2) signal peptide, which in turn directs the proteins to be secreted into the supernatant. This signal peptide is composed of 20 amino acids and it is cleaved after serine (Ser) by proteases (signal peptidases) in the ER lumen before secretion. It also shares the common features of other secretory signal peptides in having an abundant hydrophobic core between its polar N and C-terminal sites²⁵⁶. To improve the protein yield level as well as to limit the protein degradation at the time of harvest, the secreted IgG Fc proteins were monitored by the time course expression analysis using Western blotting (Figure 4-5). Although protein expression levels increased continuously over time, we found a monomeric degradation product from the dimer IgG Fc protein at 140 hours post-transfection, which might be due to disulphide bridge reduction that holds the Fc region²⁵⁷. After optimising the expression screening condition, the IgG Fc protein was purified from the large-scale transfection using the integrated polyhistidine tag on the C-terminal. The results obtained from SDS-PAGE gel showed that some proteins were lost in the flow-through, and that suggests that the target protein could not bind efficiently with the cobalt resin (Figure 4-10). This might be due to suboptimal binding conditions such as pH or salt concentration between the His-tagged protein and the cobalt resin or the binding capacity of the resin was exceeded. To optimise these conditions, the protein sample were diluted 1:1 (v/v) in 2 x binding buffer for the better interaction and good protein separation and this gave better results with the purification of other IgE Fc fragments (Figures 4-11 A and 4-12).

In an attempt to express the IgE Fc (CH2-CH4) protein, an IgG-IgE Fc fusion protein expression strategy was employed. The idea behind this approach is to make IgE Fc fuse with IgG Fc protein which exhibited good gene expression and secretion from the

HEK 293-6E cells. This plan was used by other research groups as they demonstrated the gene construction design of this fusion protein which consists of IgG hinge region-CH2-CH3-linker-IgE CH2-CH3-CH4, and is expressed and secreted from the transfected host cells^{132,140,141}. In our design (Figure 4-6), the TEV protease recognition sequence was used as a cleavable link. It is composed of seven amino acids EXXYXQ(G/S) and cleavage occurs between glutamine and glycine or serine when it is recognised by TEV protease enzyme²⁵⁸. Other protease enzymes (factor Xa, enteropeptidase, and thrombin) display the same proteolytic action when they were applied to remove the affinity tags. However, TEV protease offers highly specific site cleavage activity over a wide range of pH with no frequent cleavage at noncanonical sites^{259–261}.

The expression screening shows that the fusion protein was expressed with the predicted molecular weight (Figure 4-7 A), and the best time of harvesting fusion protein was also determined (Figure 4-7 B). The promising data achieved from the screening led us to express the fusion in a large-scale, to be purified through a polyhistidine tag (Figure 4-11 A). Following the purification, the fusion IgG-IgE Fc protein was prepared to be cleaved using TEV protease. As this enzyme is active under reducing conditions (DTT), the disulphide bond of the Fc dimer would be more susceptible to being cleaved, which in turn would lead to protein inactivation. The challenge of this experiment was to maintain the activity of TEV protease without affecting the structure stability of the fusion protein. To overcome this challenge, a redox pair of reduced and oxidized glutathione (GSH/GSSG) were used as an alternative to DTT in the reaction mix with concentration of 3.0 mM of GSH and 0.3 mM GSSG²⁶². This redox buffer condition should preserve the cleavage efficiency with minimising shuffling in the disulphide bond in the fusion protein²⁶³. By applying this special condition, we found that efficiency of TEV in the cleavage were observed when

the fusion protein was incubated overnight at room temperature or at 4 ° C (Figure 4-11 B).

The successful expression of recombinant IgE Fc (CH2-CH4) as a fusion protein drew attention to the sequence of IgE heavy chain, particularly domains 1 and 2, and to investigating the reason behind the failure of all previous expression trials. By performing a thorough literature search, some potential explanations were found to the issue of not obtaining the IgE Fc protein, described below.

Heavy chain-binding protein (Bip) is a glucose-regulated protein with molecular weight of 78 KDa (GRP 78) which is expressed in the endoplasmic reticulum (ER) and has a regulatory role in protein folding and assembly, newly synthesized protein translocation, and protein degradation^{264,265}. It has been found that Bip associates with immunoglobulin (Ig) heavy chain to make a complex which remains in the ER until these heavy chains assemble with their light chains. Without this, the Bip would stay with the heavy chain in the ER, or lead to intracellular degradation of the heavy chain. The ultimate effect of Bip will be to block the transport of unassembled antibody from ER to the Golgi body, and secretion of the protein will be prevented^{225,266,267}. To determine which site on the heavy chain is binding with Bip, researchers have screened different Fc fragments synthesised from 30 mouse myelomas and hybridomas. They found that mutant Ig Fc without the first domain (CH1) could not associate with Bip, while other mutant Ig Fc lacking the second and the third domains (CH2 and CH3) were still able to bind²²⁵. Those findings explained the expression failure of IgG (CH1-CH4).

Regarding IgE Fc (CH2-CH4) expression, the first attempt was to express the entire domains (2-4) starting from the first amino acid of the second domain (Valine 224)

(see Section 3.3.3.4). The reason for not detecting this fragment in the supernatant could be related to the cysteine at position 225 (Cys225) which forms a disulphide bridge with another cysteine in the first domain of the heavy chain (CH1). Thus, In the absence of CH1, Cys225 might causes aggregates from unwanted disulphide interaction²²⁶. Another reason was the free thiol group of Cys225 which might block the secretion of IgE Fc from mammalian cells²⁶⁸. Suggested solutions to avoid Fc protein aggregation are e.g. to delete or substitute Cys225 with alanine. According to Young RJ *et al*, the predicted framework of IgE Fc (CH2) starts from Phe229, as no protein is secreted in the supernatant if that fragment begins from isoleucine at position 235 (Ile235)^{223,226}.

Based on all previous outcomes, the IgE Fc (CH2-CH4)_{Phe229} fragment was designed to be cloned into pTT5 vector (Figure 4-8) and transfect to the 293-6E cells. To test whether the IgE Fc fragments (IgE Fc (CH1-CH4) and IgE Fc (CH2-CH4)_{Val224}) were expressed but not secreted in the supernatant, the corresponding vectors were transfected and the cell lysate analysed. The data achieved from the Western blot showed that both previous IgE Fc fragments were expressed but not secreted while IgE Fc (CH2-CH4)_{Phe229} was expressed and successfully secreted from the cells (Figure 4-9). This data confirmed the suggested explanations for the lack of recombinant protein in the cell supernatant described above. The purification of IgE Fc (CH2-CH4)_{Phe229} from the large-scale transfection showed the benefit of using FreeStyle™ F17 Expression Medium which does not contain a serum, and allows easier purification of Fc (CH2-CH4)_{Phe229} (Figure 4.12) using only a single chromatographic step (IMAC). Overall, the protein yield from all expressed recombinant Fc proteins was sufficient to run the planned subsequent experiments.

4.5 Conclusion

One of the objectives of this study was to produce recombinant IgG and IgE Fc proteins in high yield and purity. This objective was achieved by choosing 293-6E cells (mammalian cells grown in suspension) as the appropriate expression platform to produce the recombinant Fc proteins. All Fc genes were cloned into pTT-derivative expression constructs and transfected with a highly efficient gene transfer vehicle (polyethyleneimine, PEI). For the expression of IgE Fc protein, two successful strategies were developed, one of which was IgG-IgE Fc fusion protein with the TEV recognition site as a cleavable linker. Two methods of IMAC purification (manual and AKTA start system) with cobalt resin were applied to purify the FC protein. Finally, the yield of the purified recombinant Fc proteins was high enough to be used for the next application.

Chapter 5. Assessing Fc variants and particle conjugates on rat basophil leukaemia cells and human peripheral blood basophils

5.1 Introduction

Having successfully achieved the recombinant expression of IgG and IgE Fc regions, the final objective of this study focused on evaluating the Fc-particle conjugates and their delivery to the inflammatory cells. Different approaches can be applied to achieve targeted drug delivery, from local administration to advanced drug vehicles with specific ligands. Generally, targeted drug delivery can be categorised as passive or active targeting. For active targeting purposes, antibody fragments, particularly (Fc regions) is widely used for directing ligands towards target cells which selectively express Fc receptors^{269–271}. These Fc-directed particle conjugates can be used for many *in vitro* and *in vivo* purposes, such as imaging and therapeutic applications^{272–274}.

The problem with allergic reactions is that there are many cells underlying the disease symptoms, which makes the ability to target several sites difficult as this would potentially cause more adverse effects. Despite this, basophils and mast cells are considered to be the main effector cells in the disease^{275,276}. There are many potential sites that can be targeted in those important cells by the therapeutic agents, including mediators, receptors and signalling proteins. One of these potential targets is the inhibitory IgG receptor (FcγRIIB)²⁷⁷. Our Fc-particle conjugates (Figure 5.1A) are designed to have a high target specificity and affinity for basophils through crosslinking of the activating and inhibitory Fc receptor (FcεRI and FcγRIIB) on the surface of the cells. This selectivity feature due to the high selective expression of FcεRI receptor on basophilic cells²⁷⁸, while FcγRIIB provides the inhibitory function

resulting from the phosphorylation of the ITIM motif on FcγRIIB. The net result of this co-crosslinking is suppression of the activation signals of FcεRI which results in down-regulation of the activation of basophils and decreased release of inflammatory mediators (Figure 5.1B)¹²⁷.

Drug vehicles such as microspheres offer a promising method in terms of drug targeting and reducing drug side effects²⁷⁹. Normally, the modified microspheres offer a convenient means of attachment for ligands or molecules to their surface. There are many ways for antibody fragments to be attached to the microsphere's solid surface, including adsorption, covalent coupling and non-covalent avidin-biotin binding²⁸⁰.

In this study, we used microspheres modified with streptavidin, which allow the attachment of ligand via the streptavidin biotin non-covalent bond method. In case of IgG and IgE Fc proteins, the microspheres help to control the conjugation of biotinylated Fc proteins of different binding affinities to the surface of streptavidin-coated microspheres in a desired, rather than random, stoichiometric ratio. The functionality of IgE Fc variants expressed recombinantly was evaluated before and after the biotinylation step, followed by the assessment of the Fc-particle conjugates on reporter rat basophilic leukaemia (RBL) cell lines (RS-ATL8 and NFAT-DsRed) and human peripheral basophils.

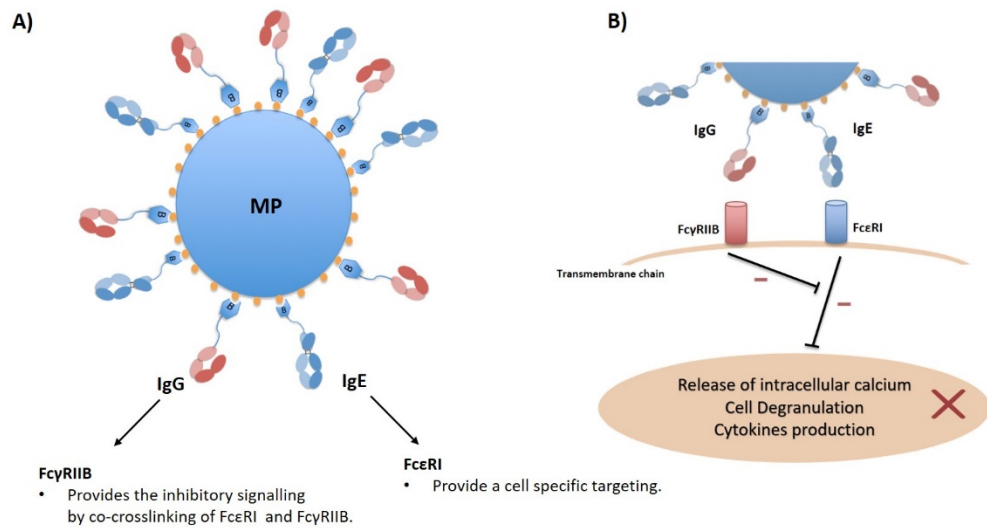


Figure 5-1: The proposed drug delivery system with the role of its ligands.

The design of Fc-particle conjugates, which consists of engineered IgG1 and IgE Fc chains conjugated to microparticle using Avi tag technology to facilitate the conjugation of antibodies to the microparticle. (B) representing the engagement of Fc ligands with their corresponding receptors on the surface of the allergic cells.

5.2 Experimental procedures

5.2.1 Assessment of Fc variants and particles conjugates

5.2.1.1 *In vitro* biotinylation of Fc variants

BirA biotin ligase kits (Avidity, USA) were used to biotinylate the Fc variant proteins. The purified Fc fragments were first suspended in 10 mM Tris-HCl, pH 8, and concentrated to 40 μ M. Following the manufacturer's instructions, 2.5 μ g of BirA enzyme were used for every 10 nmol of Fc protein at a concentration of 40 μ M. Final concentrations of 1X Biomix A and 1X Biomix B solutions were added to the reaction. The reaction mix was then incubated for one hour at room temperature. Following the biotinylation step, excess biotin was removed by spinning in 0.5 mL Amicon centrifugal filter with a molecular weight cut-off 10 kDa.

5.2.1.2 Testing the extent of Fc proteins bitoinylation by streptavidin gel-shift

In a 600 μ l Eppendorf tube, 5 μ L of 10 μ M biotinylated Fc proteins were mixed with 2X SDS-PAGE non-reducing sample buffer (NRSB) and incubated for 5 minutes at 95°C followed by cooling the sample to room temperature and briefly centrifuging it. Next, 5 μ L of excess streptavidin was added to the protein sample with incubation at room temperature for 5 minutes. Other samples were used as controls; one of them contained only streptavidin without biotinylated protein, and the other only biotinylated protein. All samples were loaded onto SDS-PAGE gel and stained with Coomassie stain for visualisation. For Western blotting, the membrane was probed with streptavidin, HRP conjugate at dilution of 1:5000 (Sigma Aldrich, UK).

5.2.1.3 Conjugation of Fc variants to microparticles

Streptavidin conjugated microparticles, 0.5, 1,2 and 6 μ m with stock particle concentration of 1.25 % w/v, were used to be coated with biotinylated Fc proteins. Different amounts of the microparticle suspension were dispensed into 1 ml

Eppendorf tube and centrifuged at 10,000 xg for 5 minutes at 4°C. Next, the supernatant was removed and discarded and the particles were re-suspended in 900 µL Dulbecco's phosphate buffered saline (DPBS) with 1 % bovine serum albumin (BSA) and washed using the same buffer three times. After the last wash, the particles were re-suspended in 1 mL DPBS/BSA binding buffer and different amounts of biotinylated IgE Fc at a concentration of 1mg/mL were added to be incubated at 4°C for 30 minutes. Finally, the mixture was centrifuged at 10,000 xg for 5 minutes, and the particle pellet was washed twice more with PBS/BSA binding buffer, re-suspended in the same buffer, and stored on ice until use.

5.2.2 Creation of an RBL cell line expressing human FcγRIIB

5.2.2.1 Optimise the antibiotic concentration for stable transfection

To transfect RS-ATL8 luciferase reporter cells and NFATp-DsRed-Express2 fluorescence reporter cells with the human inhibitory Fc receptor (FcγRIIB), a kill curve experiment with puromycin was performed to determine the concentration for selecting the stable cell colonies. In a 12 well plate, 1.5×10^5 cells/mL were plated and incubated overnight before addition of the antibiotic. The next day, an increasing amount of puromycin was added – 0.5, 1, 2, 4, 6, 8 and 10 µg/ mL – to each well plate and untreated cells used as a control. The culture medium was changed every 2-3 days for 7 days with daily observation to determine the optimal antibiotic concentration, which is defined as the lowest dose at which all cells are dead a week after the puromycin addition.

5.2.2.2 Cloning of (FcγRIIB) gene into mammalian construct

The gene sequence of the human FcγRIIB receptor was obtained from National Centre for Biotechnology Information (NCBI) under accession number (NG_023318) and sent to be synthesised (Sigma Aldrich, USA). The synthesised gene was cloned into the pcDNA4-TO-Puromycin-mVenus-MAP vector (Addgene plasmid # 44118) using EcoRV and XbaI restriction sites and successful cloning was verified by DNA sequencing.

5.2.2.3 Transfection procedure

Two methods of transfection were used to transfect the RBL-2H3 derivative reporter cell lines, one with commercial transfection reagents and the other with electroporation using Nucleofector™ Technology. For the commercial transfection reagents, 1.5×10^5 cells/mL were plated in each well of a 6 well-plate and incubated overnight at 37°C. On the day of the transfection, *TransIT*-2020 Transfection Reagent (Mirus Bio LLC, USA) and X-tremeGENE 9 DNA transfection reagent (Roche, USA) were used in parallel and mixed with DNA according to the manufacturer's instructions. 2 µg of the transfected cDNA (FcγRIIB receptor) was diluted in Opti-MEM I Reduced-Serum Medium at ratios of 1:1, 1:2 and 1:3 (DNA: *TransIT*-2020, w/v) and 1:1,1:3 and 1:6 (DNA: X-tremeGENE 9, w/v). After an incubation for 15 minutes at room temperature, the complexes were added drop-wise to the well. Then the plates were placed in the incubator for 48 hr before addition of the puromycin antibiotic. While in the nucleofection experiment, pellets of 2×10^6 cells were re-suspended in SF cell line nucleofector solution (Lonza, Switzerland) followed by addition of 2 µg of the transfected cDNA (human FcγRIIB receptor in plasmid vector) to the solution, and then transferred to the single 100 µL nucleocuvette vessel (Lonza, Switzerland). Next, the nucleofection was performed by placing the vessels into 4D- nucleofector Y unit (Lonza, Switzerland) which was set up with a pulse code (DS-150). After the run, the

vessels were removed from the retainer and incubated for 10 minutes at room temperature. Finally, the cells were re-suspended in 500 μ L of pre-warmed medium before being added to each well of the 6 well-plate which contains 1.5 mL of the culture medium.

5.2.3 Evaluating the IgE Fc-particle conjugates on reporter cell lines

5.2.3.1 RS-ATL8 luciferase assay

RS-ATL8 luciferase reporter cells is an RBL-SX-38 cell line expressing the human Fc ϵ RI $\alpha\beta\gamma$ subunits and transfected with NFAT-regulated luciferase reporter gene plasmid. The cells were seeded at 1×10^5 cells per well in a Nunc™ F96 MicroWell™ White Polystyrene Plate (Thermo Fisher scientific, UK) with or without sensitisation using Human IgE isotype control (IgE k, Clone HE1, Invitrogen, UK) at a final concentration of $1 \mu\text{g/mL}$ in culture medium and incubated for 16 hours. Next, the cells were stimulated with selected concentrations of IgE Fc microparticle conjugates resuspended in the cell culture medium, and the plate placed in the incubator. For positive control, cells were sensitised with IgE and stimulated with goat anti-human IgE (ϵ chain) antibody (Sigma Aldrich, UK). 50 μ L of Dual-Glo luciferase assay reagent (Promega, USA) were added to each well and left for 3 minutes to allow complete cell lysis. Luminescence was measured using Spark 10M multimode microplate reader (Tecan, Switzerland).

5.2.3.2 NFATp-DsRed-Express2 fluorescence reporter

A NFATp-DsRed-Express2 fluorescence reporter is an RBL-703/21 cell line expressing human Fc ϵ RI α subunit and stably transfected with NFAT enhancer-DsRed-Express2 reporter plasmid. The cells was seeded at 5×10^4 cells per well into a corning 96 clear well, flat-bottomed tissue-culture (Sigma Aldrich, USA) treated plate with or without sensitisation using human IgE (Invitrogen, UK) at final concentration of $1 \mu\text{g/mL}$ in

culture medium with overnight incubation at 37°C in a humidified 5 % CO₂ incubator. The following day, the stimulation step was performed with selected concentrations of IgE Fc microparticle conjugates diluted in the cell culture medium and the plate was incubated overnight. Next, the cells were inspected visually using fluorescence microscopy (EVOS fl, Advanced Imaging Group, ThermoScientific Fisher).

5.2.4 Evaluating the IgE Fc -particle conjugates on purified human basophils cells

5.2.4.1 Human basophils purification

Peripheral blood was harvested from healthy volunteer by venepuncture in several BD Vacutainer Lithium Heparin containing tubes. In a sterilised 50 mL tube, the blood sample were pooled and one part of HetaSep (STEMCELL Technologies, Canada) was added to five parts blood with careful inversion and without shaking. Next, the mixture of HetaSep-blood was split into sterilised 15 mL tubes and centrifuged for 6 minutes at 110 xg with the break off. After the centrifugation step, the tubes were allowed to stand for 10 minutes at room temperature and then the upper phase containing the white blood cells was collected, pooled into new 50 ml tubes and topped up with chilled Robosep isolation buffer (DPBS without Ca²⁺ or Mg²⁺ with 2% FBS (heat inactivated; v/v) and 1 mM EDTA pH 7.3). Two washing steps were performed with the same buffer, one with 120 xg for 10 minutes and the second with 450 xg for 7 minutes. The supernatant was removed and the cell pellets reconstituted in 0.5 mL Robosep isolation buffer. For cell counting using a haemocytometer, 20 µL of the cell suspension was mixed 1:1 with a prefiltered 0.4% Trypan blue solution (Sigma Aldrich, UK). The whole volume of 0.5 ml Robosep buffer was transferred to a 5 mL FACS tube, and 25 µL of Human Basophil Enrichment Cocktail (STEMCELL Technologies, Canada) were added and mixed with incubation at room temperature for 10 minutes. Then, 50 µL of EasySep nanoparticles (STEMCELL Technologies,

Canada) were added after resuspension by pipetting more than five times and incubated at room temperature for another 10 minutes. Following the incubation with EasySep nanoparticles, the suspension was topped up to 2.5 mL with the isolation buffer and transferred to an Easy-Sep purple magnet (STEMCELL Technologies, Canada) and incubated at room temperature for 5 minutes. The unbound cells were decanted into a fresh FACS tube and incubated again in Easy-Sep purple magnet for 5 minutes. This incubation was repeated 3 times in total. Finally, the FACS tube was removed from the magnet, the cells centrifuged and washed with isolation buffer or the assay medium. Purity of the obtained basophils was checked on Cytospins (Cytospin 3 cytocentrifuge, Shandon, UK) stained with modified May-Gruenwald solution (Sigma Aldrich, UK) for 3 minutes using 100x magnification and immersion oil.

5.2.4.2 Cell stimulation by Fc-particle conjugates and histamine release assay

In DPBS buffer with Ca^{2+} and Mg^{2+} , 1×10^4 purified peripheral blood basophils were stimulated with WT-IgG-IgE Fc MP conjugates, IgG_{DE}-IgE Fc MP conjugates, IgE Fc MP conjugates, PBS Fc conjugate, anti-human IgE and cells with buffer only at 37 °C for 15 minutes, followed by cooling on ice for another 10 minutes to stop the reaction. Next, all samples were centrifuged and the supernatants transferred to new Eppendorf tubes. The pellets of the cells were lysed at the same time with 4% perchloric acid. Histamine contents of both supernatant and cell pellets were determined by Dr Bernhard F Gibbs at the Medway School of Pharmacy, using a histamine autoanalyser machine. Finally, the percentage of histamine release was calculated from the amount of histamine in both supernatant and cell pellet.

5.3 Results

5.3.1 Biotinylation of IgG and IgE Fc proteins

Following the in vitro biotinylation using BirA enzyme, the biotinylation efficiency of IgG and IgE Fc proteins was evaluated using two methods, a streptavidin gel-shift assay (SDS-PAGE) and Western blotting. The streptavidin gel-shift verifies that the target Fc protein is biotinylated and determines whether the reaction has gone to completion. For IgE Fc protein, complete biotinylation was achieved as no band was observed in the expected size of IgE Fc (≈ 80 kDa) after addition of excess streptavidin to the biotinylated protein (Figure 5-2A). This step was further confirmed by Western blot using streptavidin HRP conjugate to detect the biotinylated IgE Fc fragments, with or without the addition of Streptavidin (Figure 5-2B).

In case of IgG Fc protein, the same process was carried out using in vitro enzymatic biotinylation method. The data show that a successful biotinylation was achieved and no band was detected after incubation of an excess of streptavidin with IgG Fc protein (≈ 60 kDa) in lane 3 of both SDS-PAGE gel (Figure 5-3A). The biotinylation was confirmed further by Western blot (Figure 5-3B).

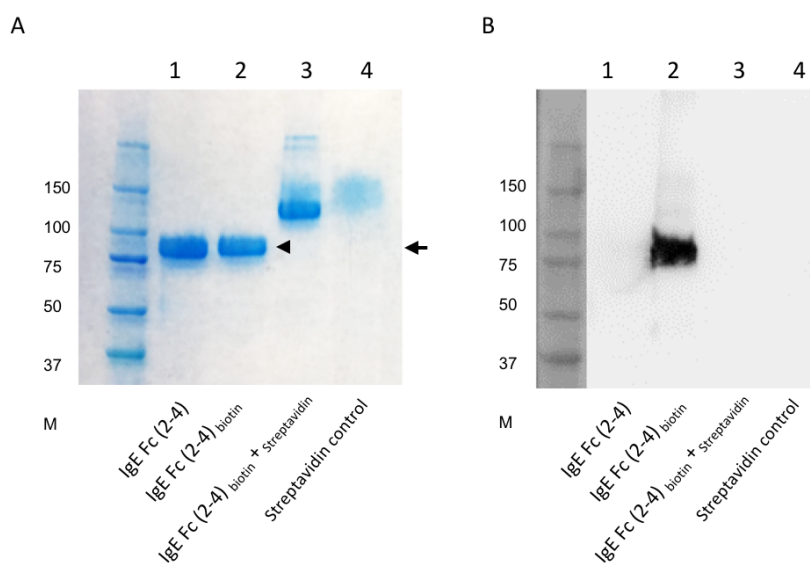


Figure 5-2: Biotinylation analysis of IgE Fc protein.

(A) SDS-PAGE gel stained with Coomassie blue and (B) Western blot after the biotinylation of IgE Fc fragment using BirA enzyme. Lanes (1 and 2) represent the IgE Fc protein with or without the biotinylation respectively. Lanes (3) represent the biotinylated IgE Fc with streptavidin and lane (4) represent the streptavidin only. M, indicates molecular weight markers. Western blotting conducted with streptavidin, HRP conjugate. (→) symbol indicates that no band was observed after the binding with streptavidin.

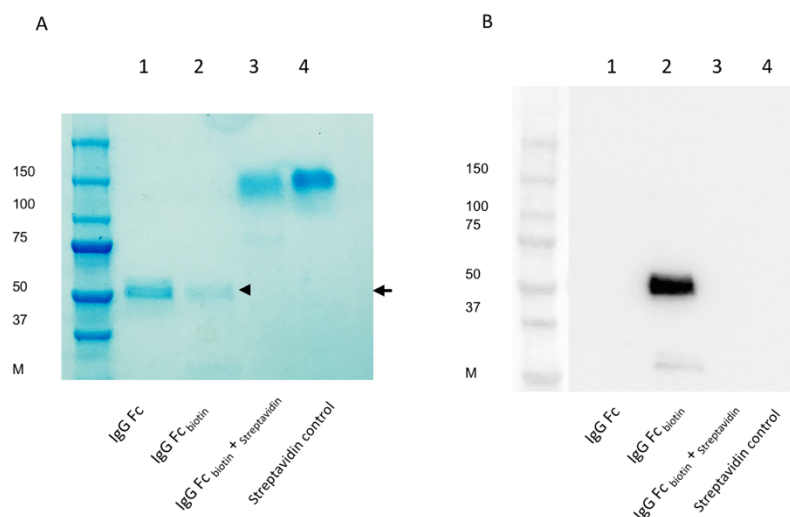


Figure 5-3: Biotinylation analysis of IgG Fc protein.

(A) SDS-PAGE gel stained with Coomassie blue and (B) Western blot after the biotinylation of IgG Fc fragment using BirA enzyme. Lanes (1 and 2) represent the IgG Fc protein before and after the biotinylation respectively. Lanes (3) represent the

biotinylated IgG Fc with streptavidin and lane (4) represent the streptavidin only. M, indicates molecular weight markers. Western blotting conducted with streptavidin, HRP conjugate. (→) symbol indicates no band was observed after the binding with streptavidin.

5.3.2 Functionality of IgE Fc protein on the reporter RBL cell line

Before the conjugation to the particles, it is important to test the functionality of IgE Fc protein on the cells to see if it engage its receptor and cause activation. To perform this step, a rat basophil leukaemia cell line (RS-ATL8) with stable surface expression of the human high affinity IgE receptor (FcεRI) was used. The cells were sensitised with 0.0125 µM non-biotinylated IgE Fc (2-4) and a commercial human IgE, full length (Bio-Rad, USA) as a gold standard, followed by stimulation using goat anti-human IgE (ε chain) antibody. The data in Figure 5-4 demonstrate the ability of IgE Fc (2-4) to activate the cells close the commercial full length human myeloma IgE as indicated by luciferase production.

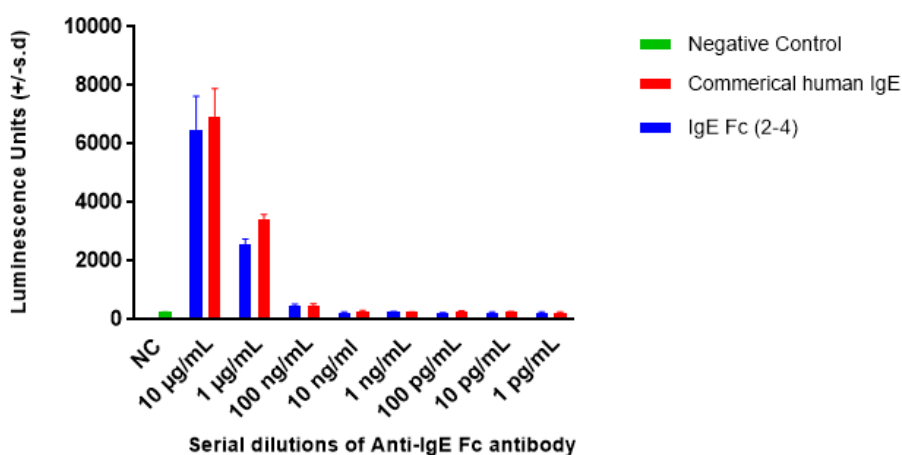


Figure 5-4: Luminescence response of the comparison between IgE Fc (2-4) protein and commercial human full length IgE.

This data was determined by sensitising the RS-ATL8 cells overnight with both IgE Fc (2-4) and a commercial human full length IgE, then challenged using serial dilutions of goat anti-human IgE (ε chain) antibody. Data represent the mean value with ± SD from readings of three different wells.

The next step was to assess whether the biotinylation of IgE Fc (2-4) could influence the activation of the cells. The same luminescence experiment was applied to IgE Fc (2-4) before and after biotinylation. Again, the data shows no difference between the two forms of IgE Fc (2-4), which suggests that biotin does not affect the binding to receptor (Figure 5-5).

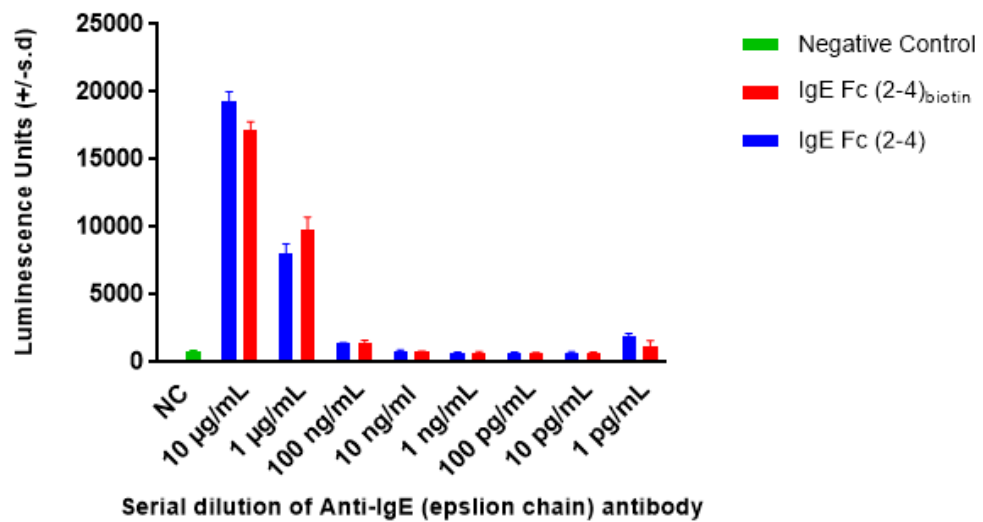


Figure 5-5: Luminescence response of IgE Fc (2-4) protein with or without biotinylation.

RS-ATL8 cells were sensitised overnight with both biotinylated and non-biotinylated IgE Fc (2-4) then challenged using serial dilutions of goat anti-human IgE (ε chain) antibody. Data represent the mean value with $\pm$ SD from readings of three different wells. N=2.

5.3.3 Conjugation of biotinylated Fc proteins to Streptavidin-microparticles

The binding of biotinylated Fc proteins with streptavidin-coated particles was assessed in order to determine what amount of protein is needed to saturate the particles' surface. Various amounts of biotinylated IgE Fc (2-4) protein were used for capture by particles of two different diameters (0.5 μ m and 1 μ m), and analysed by SDS-PAGE gel before and after the capture process. The obtained data showed that attachment of IgE Fc protein to the particles can be detected. 2.5 μ g and 1 μ g of protein was needed to saturate 50 μ g of 0.5 μ m and 1 μ m particles, respectively (Figure 5-6 A and B).

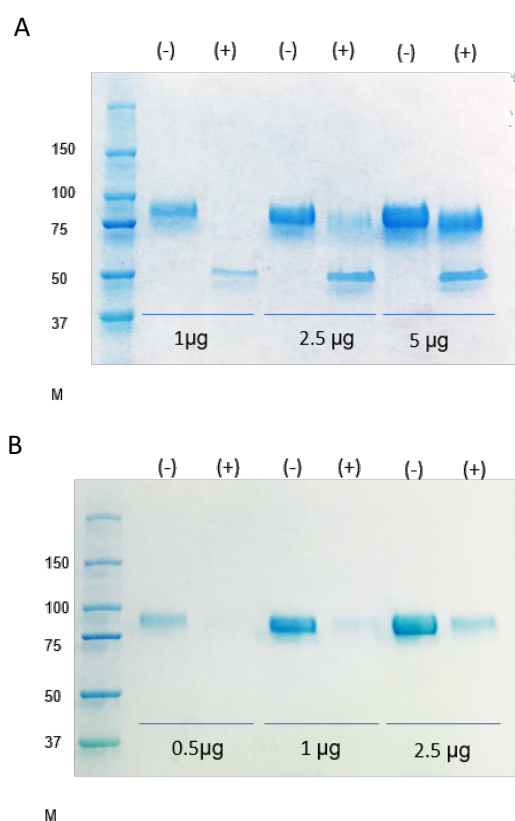


Figure 5-6: Optimisation of IgE Fc protein conjugation with streptavidin-microparticles.

SDS-PAGE analysis of loading of different amounts of IgE Fc protein to 0.5 μ m particles (A) and 1 μ m particles (B). (-) represents 100% of IgE Fc protein before the binding with streptavidin-particles. (+) represents the unbound protein after the same amount of IgE Fc protein was captured with 50 μ g of streptavidin –microparticles.

5.3.4 Functionality of IgE Fc -particle conjugates on the reporter RBL cell lines

Various parameters need to be optimised before applying the designed delivery system (IgG/IgE Fc microparticles conjugate) on the basophils. To achieve this, modified particles with IgE Fc (2-4) only were tested on RBL reporter cells (RS-ATL8) to see whether they could activate the cells and to evaluate the effects of the following conditions; particle size, particle concentration and the density of Fc ligands on the surface of the particles.

5.3.4.1 Testing different concentrations of modified 0.5µm particles

Firstly, different concentrations of modified 0.5µm particles were exposed to sensitised and non-sensitised cells with human IgE and luciferase production was measured after 4 hours. The results show the ability of modified particles to engage with IgE receptors and mediate cell activation. The data displays a concentration-dependent activation in both cell conditions. In addition, unsensitised cells consistently showed a higher activation value than the sensitised cells (Figure 5-7). Based on these results, 250, 100 and 50 µg/mL were chosen for all subsequent experiments.

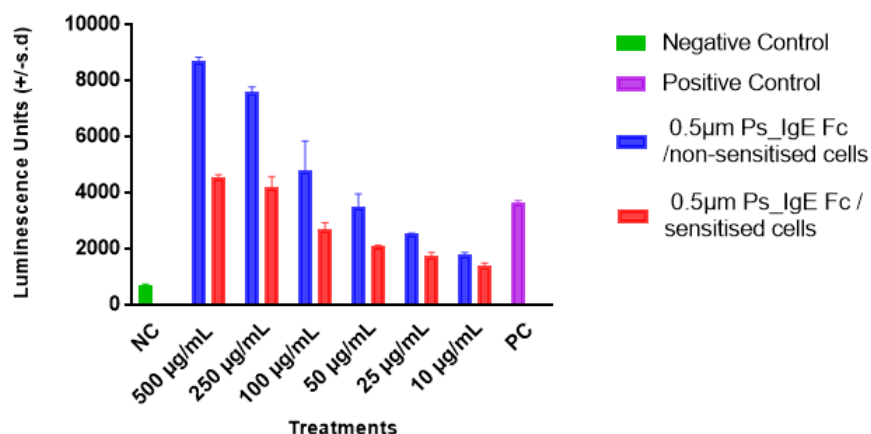


Figure 5-7: Luminescence response of different concentration of modified 0.5 μm particles.

RS-ATL8 cells in two conditions were used in this experiment, cells sensitised overnight with 1 $\mu\text{g/mL}$ IgE and non-sensitised cells. modified particles were added to sensitised (red bar) and non-sensitised cells (blue bar). Data represent the mean value with $\pm$ SD from readings of three different wells. the negative control was unsensitised cells stimulated with anti Ig. The positive control was cells sensitised with human IgE and stimulated with anti-human IgE (epsilon chain) antibody.

To confirm the luciferase data from the RS-ATL8 cell line, the same modified particles were applied on another RBL reporter cell line (NFAT-DsRed). In this experiment, Fc ϵ RI receptors were crosslinked after stimulation and the cells produced red fluorescent protein (NFAT-DsRed-Express2) which acts as a reporter for cell activation. This can be visualised using fluorescence microscopy 24 hours post-stimulation. Similar to luciferase assay data, the images show more red fluorescent cells with Fc-modified particles in non-sensitised cells compared to sensitised cells (Figure 5-8).

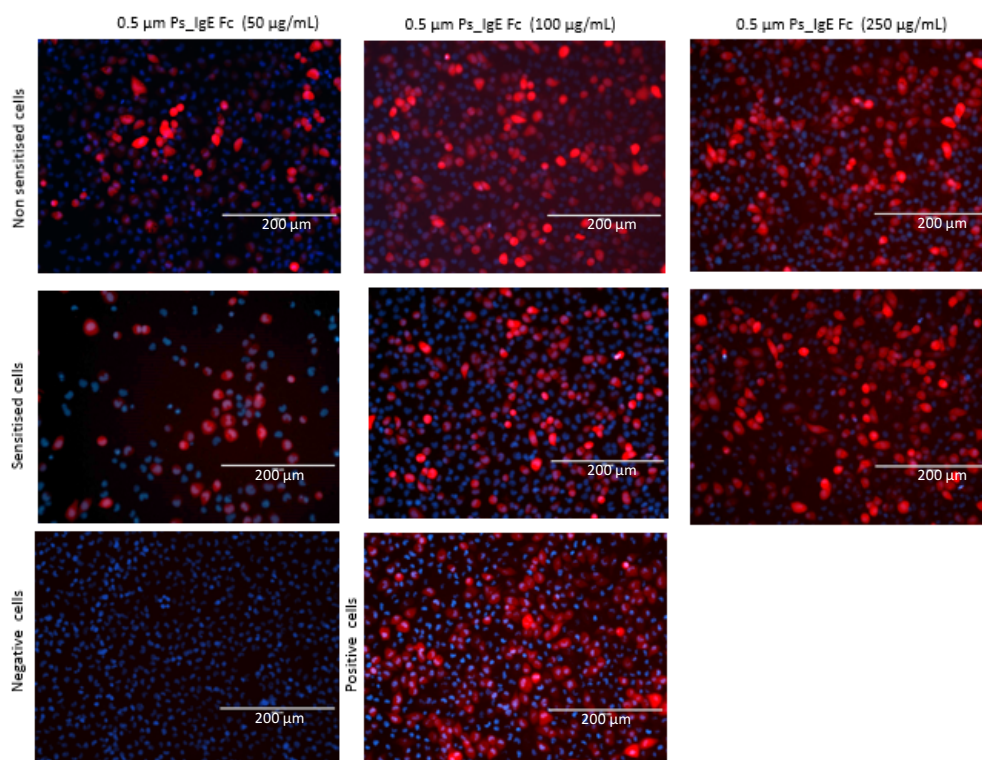


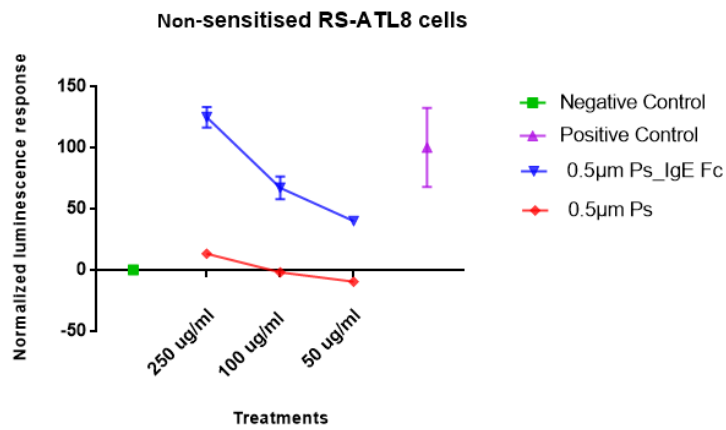
Figure 5-8: Images from fluorescence microscopy for 0.5 μm Ps_IgE Fc conjugates in both cells conditions.

Cells were exposed to different concentrations (50,100 and 250 $\mu\text{g/mL}$). Negative control was non-sensitised cells that stimulated with anti-IgE, while positive control was sensitised cells stimulated with anti-IgE. The cell's nucleus was stained with Hoechst dye (blue staining).

5.3.4.2 Comparing modified and non-modified 0.5 μm particles

The next step was comparing the modified to un-modified particles to confirm that the effect of cell activation is seen only for the particles coated with IgE Fc. The previously determined concentrations (50, 100 and 250 $\mu\text{g/mL}$) were used, and the same design of experiment was applied. The results of normalised luminescence response showed that un-modified 0.5 μm particles could not activate the cells compared to modified particles in non-sensitised and sensitised conditions as demonstrated in Figure 5-9 A and B.

A



B

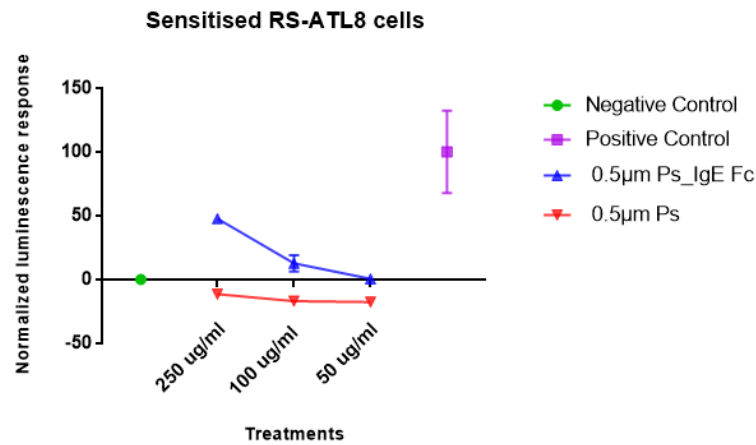


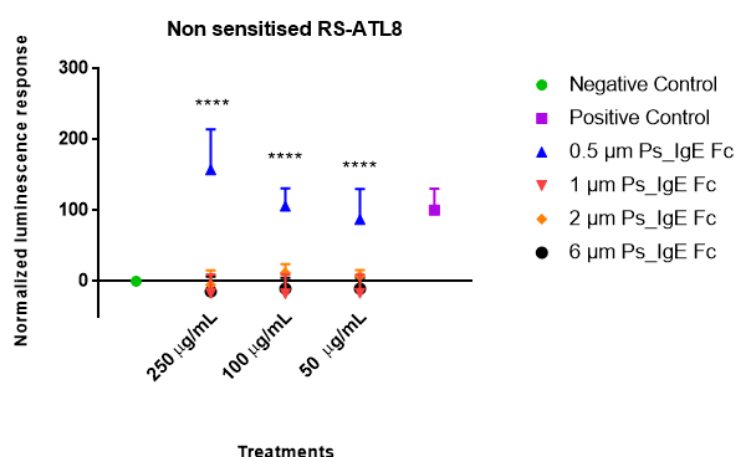
Figure 5-9: luminescence response of modified and un-modified 0.5µm particles.

In this experiment, modified and un-modified 0.5µm particles were added to RS-ATL-8 cells. (A) cells were sensitised overnight with 1 µg/mL IgE (A) and (B) non-sensitised cells. Data represent the mean value $\pm$ SD from readings of three different wells. the negative control was unsensitised cells stimulated with anti-IgE. the positive control was cells sensitised with human IgE and stimulated with anti-human IgE (epsilon chain) antibody. N=2.

5.3.4.3 Effect of particle sizes on the activation of the cells

The effect of particle size on cell activation has been emphasised in this study. To know if the size of the particles could play a role in cell activation, four different sizes of particles (0.5, 1, 2 and 6 μm) coated with IgE Fc protein at a concentration of 250 $\mu\text{g}/\text{mL}$ were exposed to the cells. It can be clearly observed from the data in Figure 5-10 that particle size has a major effect on cell activation. Particles of 0.5 μm diameter were the only size that could activate non-sensitised and sensitised cells.

A



B

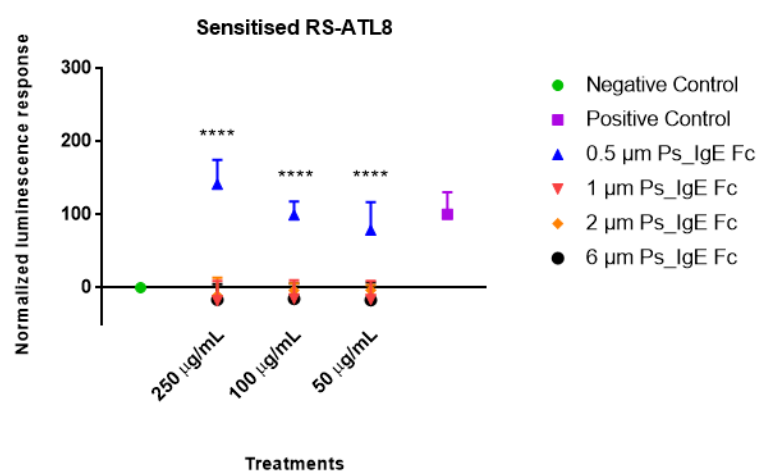


Figure 5-10: Effect of the particle sizes on the cell activation.

Comparison of four different particle sizes in non-sensitised (A) and sensitised RS-ATL8 cells (B). negative and positive control was non-sensitised and sensitised cells stimulated with Anti-IgE respectively. Data are presented as mean with $\pm$ SD (N=3). ****p < 0.0001 (Dunnett's test).

5.3.4.4 Effect of IgE Fc density on cell activation

Following the comparison of particle size, the effect of 1 μ m particles coated with different IgE Fc densities was also explored. The purpose of this experiment was to evaluate the role of Fc density on cell activation. At a concentration of 250 μ g/ml of 1 μ m particles, several ratios of IgE Fc protein were prepared starting from 1:1 which represented the maximum coverage of the particles' Fc protein. Overall, the results show that the highest activation value was achieved with a lower Fc density ratio (1:50) compared to high Fc density (1:1) and this means that Fc density plays a role in the activation of the cells (Figure 5-11).

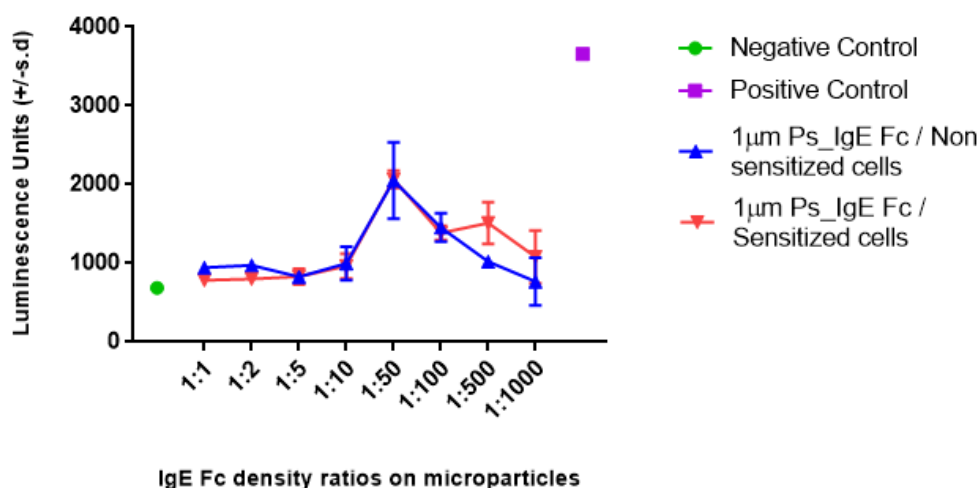


Figure 5-11: The effect of the IgE Fc density on the cell activation.

Different Fc density ratios of IgE Fc ligand on the surface of 1 μ m particle were tested on RS-ATL-8 cells in non-sensitised and sensitised conditions.

5.3.5 Development of RBL cell lines expressing human FcγRIIB

Establishing a reporter cell line that contains both human IgE receptors (FcεRI) and the inhibitory IgG receptor (FcγRIIB) is required to test the functionality of the designed delivery system, and three steps are essential to develop such stably transfected cell line: cloning of the FcγRIIB gene into a mammalian expression vector; optimising the selective antibiotic concentration to generate stably transfected cells; and transfection the RBL cells with cloned FcγRIIB vector.

Firstly, the synthesised human FcγRIIB coding DNA was cloned successfully into pcDNA4-TO-Puromycin-mVenus-MAP using EcoRV and XbaI restriction sites and DNA sequencing was used to confirm successful cloning. Following the cloning procedure, the RS-ATL8 cells were grown in medium containing different concentrations of puromycin to determine the lowest concentration that can kill all non-transfected cells within a week. After a week of adding the treatment, 1 µg/mL was the minimal concentration observed to kill all cells, and this concentration was chosen for the selection after the transfection (Figure 5-12).

Next, two ways of transfection were used to transfect RS-ATL8 cells; using commercial transfection reagents (X-tremeGENE 9 and TransIT-2020) and an electroporation method (nucleofection). Initially, the transfection efficiency obtained from both transfection reagents was low compared to and all cells were dead after the selection with puromycin (Figure 5-13 A and B). Later, nucleofection method was applied to obtain a higher transfection efficiency. The data of transfection efficiency was promising as it was much higher compared to the transfection reagent (Figure 5-13 C). Unfortunately, after the selection with puromycin, all cells were dead.

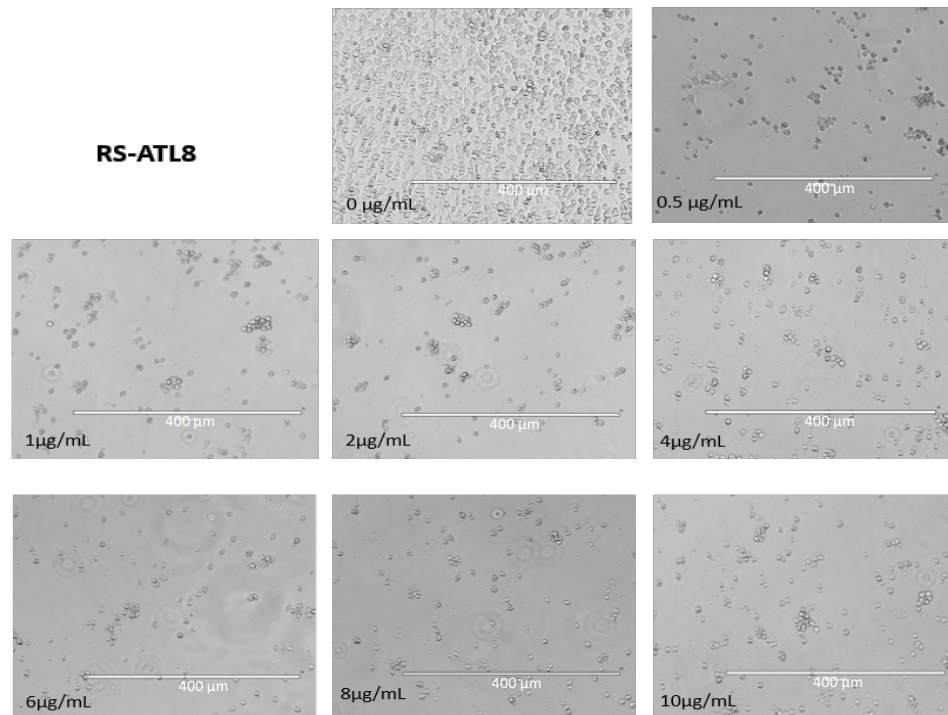


Figure 5-12: Microscopy image RS-ATL8 cells treated with different concentrations of puromycin.

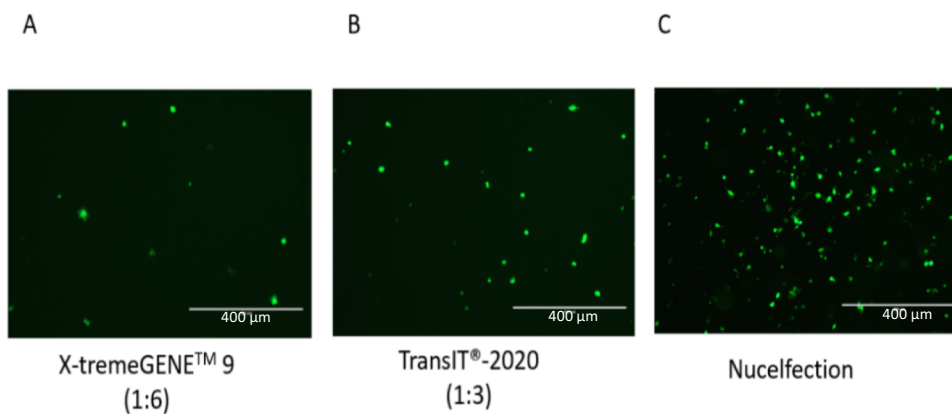


Figure 5-13: Fluorescence microscopy image of transfected RS-ATL8 cells using commercial transfection reagents and nucleofection.

Two different ways to transfect RS-ATL8 were used, (A) and (B) represent the transfection of green fluorescent protein with X-tremeGENE 9 and TransIT®-2020 with 1:6 and 1:3 ratios (w/w) respectively. while (C) show the transfection using nucleofection method.

Due to unavailability of dual humanised Fc ϵ RI/Fc γ RIIB RBL cells to test the delivery system, Fc-particle conjugates were applied directly to purified human peripheral blood basophils. The basophils were purified and stimulated with concentration of 250 μ g/mL of different coated 0.5 μ m particles as follows: IgG_{WT}-IgE Fc conjugates, IgG_{DE}-IgE Fc conjugates, IgE Fc conjugates, BSA conjugates, anti-human IgE and PBS buffer only. The applied microparticles had different stoichiometric ratios of attached IgE Fc to IgG Fc regions; $\approx$ 1:1000 with wild type IgG and $\approx$ 1:7 with the mutant IgG with improved binding affinity (IgG_{DE}) see section (3.3.1). No differences in histamine release were observed between all Fc-particle conjugates. This indicates that all types of the designed delivery systems were unable either to activate the basophils (IgE Fc conjugate) or inhibit the activation (IgG_{WT}-IgE Fc and IgG_{DE}-IgE Fc conjugates) before and after cell stripping (see Figure 5-14 A and B).

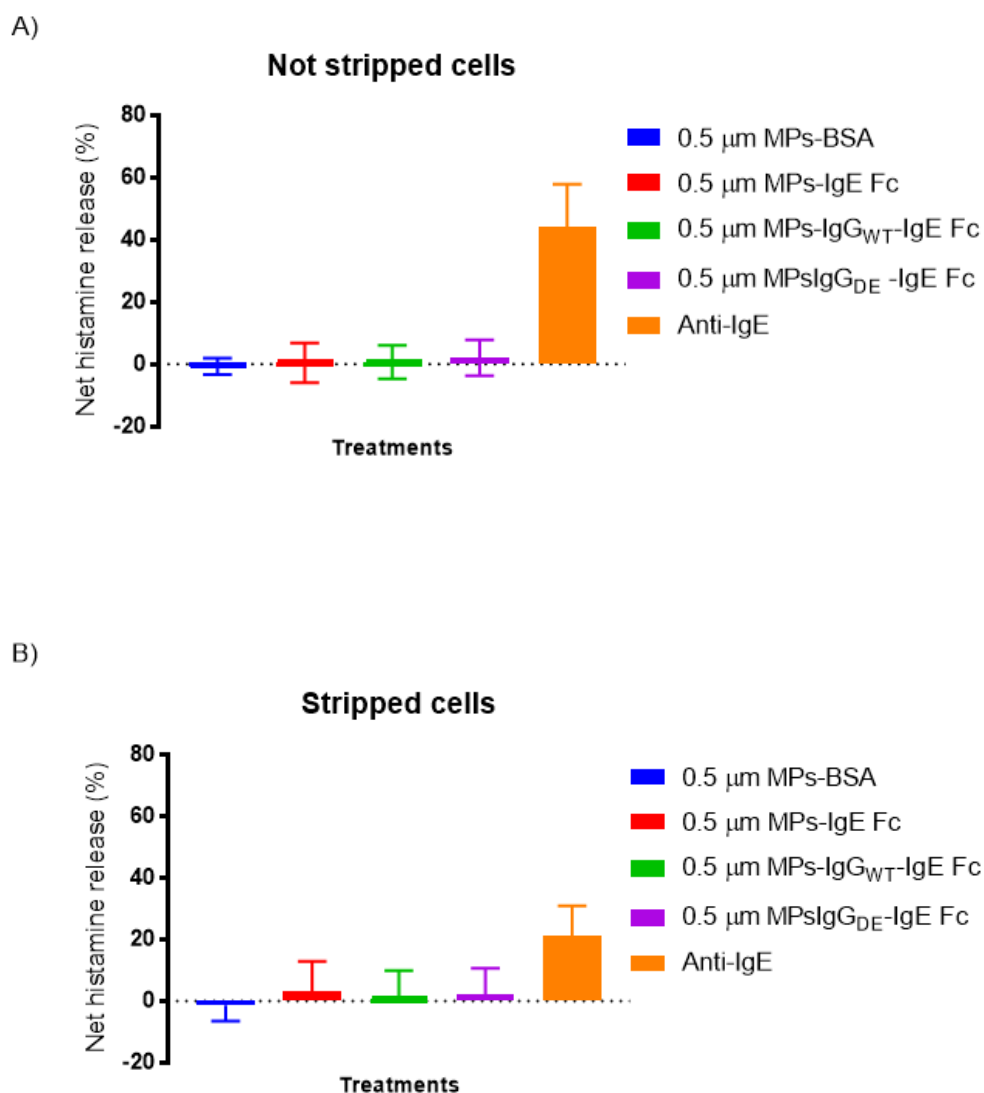


Figure 5-14: Histamine release from basophils by using different Fc-particle conjugates.

Differential histamine release from basophils was assessed were IgE not-stripped in (A) and stripped in (B). Stripping refers to a short incubation in lactic acid buffer, followed by a fast centrifugation, which removes a large proportion of receptor-bound IgE, without damaging the cells. 0.5 μ m particles coated with BSA, IgE, IgG_{WT}-IgE, IgG_{DE}-IgE were added in both conditions while Positive control was cells stimulated with anti-IgE. Data are corrected for spontaneous histamine release. N=1, this experiment was performed in collaboration with Dr Bernhard F Gibbs (Medway School of Pharmacy, University of Kent).

5.4 Discussion

After establishing an effective strategy to express Fc recombinant proteins in a good yield and high purity, the ultimate goal of this chapter was to evaluate the ability of Fc-particle conjugates to control allergic cell activation, and to know whether this therapeutic system could be a good option to treat or prevent allergies. To perform these experiment, two essential steps were conducted; *in vitro* biotinylation of Fc protein and conjugation to streptavidin microparticles. Some characterisation procedures were also used to identify the optimal condition before applying the designed system to the basophils.

Protein bitoinylation was the first stage. To generate a biotinylated protein using BirA enzyme, *in vivo* and *in vitro* methods can be used; The *in vitro* method takes longer, but also gives a better efficiency compared to *in vivo*. Due to several cellular factors, it is difficult to control the degree of *in vivo* biotinylation as it is different from batch to batch^{281–283}. Both IgE and IgG Fc were completely biotinylated after incubation with BirA enzyme at room temperature. These results support the advantage of using *in vitro* biotinylation as the reaction can be controlled by concentrating the protein in a lower volume with less incubation time (Figures 5-2 & 5-3). Another aspect in this experiment was using SDS-PAGE gel as a method to assess the extent of biotinylation. The current methods such as HABA and FluorReporter assays have many drawbacks in terms of requiring more time, consuming a large amount of protein, the high cost of the reagents, and the need for a biotin calibration curve for the analysis. A streptavidin gel-shift assay can overcome these limitations and is a good alternative method of biotinylation detection.

Next, it was important to study the functionality of Fc protein in inducing the signalling pathway after the insertion of Avi Tag in the immunoglobulin construct and the

biotinylation with BirA enzyme at room temperature. As the binding profile before and after the biotinylation was the same, this finding allows us to go to the next step (Figures 5-4 and 5-5).

The luminescence data observed after applying the IgE Fc-particle conjugate on the reporter cell line reveals aspects that should be taken into consideration. Firstly, the ability of the IgE Fc protein to bind to its FcεRI receptor reflects the stability of Fc-particles' conjugate and the proper presentation of these proteins on the surface of microparticles (figure 5-7). This supports the benefit of using biotin-streptavidin conjugation via BirA enzyme as it allows the biotinylated Fc proteins to bind to one microparticle in a precise position rather than the random attachments to many particles which caused by the chemical method^{284,285}. Our data also represents the ability of the designed system to be directly involved in the crosslinking of FcεRI receptors which in turn initiate the downstream signalling pathway detected by the release of the luciferase, which is an indicator of the cell activation. These findings were further validated by the positive outcomes that were obtained when applying the same designed system to another RBL reporter cell (NFAT-DsRed) (Figure 5-8).

It is believed that there is a direct relationship between the quantity of IgE molecules and the increased expression levels of FcεRI receptor on the surface of the basophils and mast cells⁵¹. This has been explained by a stabilising effect of IgE on occupied receptors. In the absence of bound IgE, the FcεRI complex is rapidly internalised and degraded²⁸⁶. Thus, overnight incubation with IgE results in increased surface expression of FcεRI, as shown also by our group²⁸⁷. However, a stronger activation response was consistently detected when the non-sensitised cells were exposed to IgE Fc-particle conjugates compared to sensitised RBL cells. This perhaps unexpected result could be explained by the idea of presence a competition between the IgE

molecule which bound to basophils from the sensitisation step and IgE Fc-particle conjugates on the receptors site. The binding affinity between IgE and its receptor is high (giving the receptor its name: High affinity IgE receptor); IgE molecules show a remarkably slow dissociation rate which makes it difficult for other competing ligands to occupy the receptor⁴⁴. However, this effect might have been counterbalanced by the high valency of multiple IgE ligands immobilised on the MP surface, resulting in cooperative binding of the particle and displacement of monomeric IgE. Our results indicate that IgE Fc-particle conjugates were unable to displace the receptor bound IgE molecules from the receptor binding site. Our results also show that there is no significant cellular activation with non-modified 0.5 μm particles when compared to the modified particle with IgE. This result is expected as the nanoparticle itself could not cause any activation without any attached IgE and at the same time proves the necessity of IgE on the surface of the particle in promoting the receptor clustering and inducing downstream signalling events (Figure 5-9).

The influence of particle size on regulating the immune response has also been explored in this part of the work. Although all particle sizes (0.5, 1, 2 and 6 μm) were loaded with the same maximum IgE Fc density, those with a diameter of 0.5 μm were considered the ideal size to mediate the cells' activation (Figure 5-10). This finding shows the importance of particle size as a key factor in the stimulation activity. The reason why other particle sizes could not produce any activation effect might be due to the difference in the distance between the attached ligands on particles and the average spacing between the two receptor binding sites on the cell surface not allowing for a proper binding²⁸⁸.

A study by Pierini and co-authors examined the role of particle size on the activation of mast cells or basophils, particularly at the micron scale. The results described support our findings, as they show that when the 6µm beads coated with biotinylated IgE were added to RBL-2H3 cells, no stimulation effect (as measured by mediator release) could be detected²⁸⁹. The authors of this study offer a very different explanation for the lack of activation by 6µm beads. They suggest that clearance of some plasma membrane proteins such as GD_{1B} gangliosides, Thy-1 and p53/56^{lyn} from the interaction site between particles and cells might cause the limiting of cells response. They also suggest that the loss of these membrane components is related to the process of beads phagocytosis that produces changes in the cells response.

Another study by Pacheco and co-authors focused on testing the level of complement activation. They used different particle sizes (0.5, 1, 2 and 4 µm) functionalised with IgG Fc ligands. Their results showed that although the 4 µm particle was saturated with IgG Fc ligand, it gave a lower complement activation level compared to other sizes²⁹⁰. In contrast with our results, a high activation response was noticed by the aforementioned Pierini when using beads with a diameter size $\approx 100\mu\text{m}$. Surprisingly, this degranulation effect was with a very low particle ratio to the cells (1 particle per 19 cells)²⁸⁹.

A less sophisticated, but related approach was taken by several other groups^{291–294}. This approach does not use humanised RBL reporter cell lines or engineered Fc regions of IgE. It mainly takes advantage of the existence of a murine myeloma IgE obtained in 1980 by Eshhar²⁹⁵. This widely used clone SPE-7 myeloma secretes IgE which is specific for the Dinitrophenyl hapten. Dinitrophenyl in itself is too small to act as an allergen, but becomes an allergenic epitope when conjugated e.g. to BSA or

nanoparticles. RBL-2H3 cells can be used, as the rat IgE receptor is able to bind murine IgE (but not human, hence our use of humanised cell lines). Activation of RBL-2H3 via the IgE receptor can be measured by measuring release of beta-hexosaminidase, which is stored in granules.

Using this system, Huang and co-authors could show that DNP-conjugated gold nanoparticles with a diameter of 25.8 nm could induce approximately 25% degranulation, which was more than the activation levels obtained with soluble DNP-BSA. The amount of degranulation also depended on DNP-ligand densities²⁹¹. However, larger Au nanoparticles are not available, and this type of particles is too small for conjugation with very large molecules such as IgE, thus a direct comparison with our results is not possible. Similarly, Joralemon and colleagues used DNP-decorated amphiphilic diblock copolymers obtained from different acrylates. The authors obtained high (up to 50%) levels of degranulation from RBL-2H3 cells with particle diameters ranging from 13 to 24 nm²⁹². Again, such sizes are not amenable to conjugation with large molecules such as IgE or the IgE Fc region. Interestingly another group found a direct activation effect of undecorated, 20 nm diameter silver nanoparticles on RBL-2H3, resulting in high (35-50%) levels of degranulation with or without IgE sensitization, suggesting potential issues of toxicity. The authors identified a role for scavenger receptor B1 (SR-B1) in Ag-dependent mast cell activation²⁹³. In contrast, Ortega and co-authors used polyacrylic acid (PAA) functionalized metal-oxide NP (50 -140 nm), found an inhibition of RBL-2H3 degranulation induced by DNP conjugated with human serum albumin in the presence of the metal-oxide NP²⁹⁴. However, another group described induction of histamine secretion by TiO₂ nanoparticles and suggest that TiO₂ NP exposure and allergen-independent histamine release could exacerbate allergic responses²⁹⁶.

Overall, due to the partially conflicting and relative scarcity of data in the literature, it is difficult to draw a definitive or generalised conclusion regarding the optimal particle size and other properties for the activation of the allergic cells; this clearly needs further investigations. However, not only their small size, but also the direct degranulation-inducing effects of metals (e.g. Ag) or metal oxides (e.g. TiO₂) nanoparticles may preclude these nanomaterials from use in anti-allergic therapies

The size of particle was not the only factor that gained attention in this study. The ratio of Fc density on the surface of the particles can also determine the level of cells activation. The role of IgE Fc density when presented on a microparticle carrier has not been investigated in the literature. Based on our experimental data with 1 µm particle size, IgE Fc density (1:50) gives more activation response than the higher density ratios. This finding is not similar to other types of immunoglobulin Fc regions. In the case of IgG Fc ligand, many groups of researchers have examined the effect of the Fc density on the immune response. a higher IgG Fc density leads to higher internalisation efficiency (phagocytosis) and higher activation of complement system^{290,297}. It was clear from the previous information that many variable factors contribute to the process of cell activation and need to be adjusted. We only examine two factors in this study, but there are other elements that need to be examined such as the ligand orientation, distance between Fc ligands, and the shape of the particle.

The technique of human basophils purification has been developed by many researchers over the last decade, including our own laboratory²⁹⁸, but there remain some limitations as a tool for studying degranulation, mainly the limited yield of purified basophils and the high cost of the purification kit^{299,300}. For that reason, the need to develop a RBL cell line with reporter genes to evaluate the IgG/IgE Fc-particles conjugates was one of the objective in this work. This would enable us to generate a

stable cell line, enabling a viable screening tool with the following advantages; easy to perform, cheap and less time consuming.

The current available luciferase RBL reporter system was developed by Ryosuke Nakamura³⁰¹. These cell lines were humanised with the FcεRI receptor only. To test the IgG/IgE Fc-particle conjugates, the reporter system needs to be transfected with the human FcγRIIB receptor. After choosing the minimum concentration of the selective antibiotic (puromycin), two transfection methods were used to transfect human FcγRIIB construct; cationic-lipid transfection and electroporation. For the first method, the transfection efficiency was too low and the cells were killed by adding the selective antibiotic. Although a higher transfection efficiency was achieved with electroporation, surprisingly, the cells did not survive after incubation with Puromycin. RBL cells are considered relatively difficult to transfect, and so perhaps as the cells were previously transfected with two genes under the selection of two different antibiotics, they become more sensitive to a third selective antibiotic. As the transfected plasmid was not linearised, this might have reduced the chances of the vector being integrated into the genome, a prerequisite for stable transfectants³⁰².

The result from the activation of the RBL cell line by 0.5µm particle was promising to continue for the next step. The functionality of IgG-IgE Fc-particle conjugates as a novel therapeutic option was evaluated with purified human basophils cells. In contrast to what we found *in vitro*, the histamine release data obtained from the purified human basophils did not show any inhibition caused by IgG-IgE Fc-particle conjugates, nor did it show any activation with IgE Fc-particle conjugates. There is no obvious reason why the Fc-particle conjugate does not work with human basophils

sample. However, with IgE Fc-particle conjugates, since FcεRI receptors are occupied with IgE in the purified human basophils, it will be difficult for IgE to be displaced (similar observations were made with the sensitised RBL cell line). Additionally, for IgG-gE Fc-particle conjugates, it is not clear whether the negative result is related to improper engagements of both Fc ligands with their receptors or the inability to initiate the inhibitory signalling even after the ligands-receptors interaction. In any case, the experiment has only been performed once with a single donor (albeit, based on the good activation with anti-IgE, not a non-responder), so more experiments will be needed before drawing a firm conclusion, according to the proverb “once is nothing at all”.

Finally, although the optimisation steps were performed for obtaining a better response in RBL cell line, this reporter system does not completely represent the situation with human basophils³⁰³.

5.5 Conclusion

In this chapter, purified IgE and IgG Fc recombinant proteins were biotinylated using BirA enzyme recognising an Avi-Tag on the C-terminal of the immunoglobulin construct. In terms of the biological activity of protein, no decline was detected in the activity of IgE Fc after biotinylation. Following this step, biotinylated Fc proteins were conjugated to streptavidin-coated particles to be evaluated using luciferase reporter RS-ATL8 and fluorescence NFAT-DsRed cell lines. Our result show that the cells' activation is dependent on the size of the particles and the density of IgE Fc density. The ideal particle size and the IgE density ratio that gives the highest activation was 0.5 μm and 1:50. Although different transfection methods were used in an attempt to establish a reporter system as screening tool to evaluate the functionality of the IgG-IgE Fc-particle conjugates, none of the methods was successful. Finally, to evaluate the activity of the proposed delivery system, different formulations were applied to purified human basophils and the level of the histamine release was measured. These preliminary data indicated that none of the drug delivery formulations was working in both stripped and non-stripped basophil cells, but more experiments are needed to confirm and validate these findings.

Chapter 6. General discussion and conclusion

6.1 Research significance

This research was inspired by the idea of developing a novel immunotherapy to overcome the limitations of current allergy treatments. Although there is an increasing understanding of the molecular and cellular basis that contributes to allergic disorders, this has not helped in the development of new and effective treatments with acceptable side effects. This may be due to the difficulty in understanding the chronicity of allergic inflammation, allergen exposure, and the influence of the environmental determinants on the development of allergies¹⁴⁶. Management that is based on avoiding the allergen is not a good alternative as in many cases, it is difficult to apply, insufficient, and most patients prefer to use the medication³⁰⁴. Currently, an allergy is not a curable disease, and patients are advised to avoid the triggers and to follow the medical advice before starting pharmacological therapy. Initially more or less benign allergic manifestations can progressively develop into severe asthma, which is a life-threatening condition. Similarly, food allergy can have a strong negative impact on the quality of life of patients and, in the case of children, their carers.

It is important in the allergic reaction to understand the activating or the inhibitory signalling pathways to create new therapeutic strategies. Some signalling molecules are considered as upstream master regulators, and this is an interesting site to target. Intervention with or control of these molecules could lead to the regulation of many inflammatory processes. In this study, the designed system (IgG/IgE Fc-particle conjugates) aimed to inhibit multiple signalling events and this is based on inhibition of the initial signalling pathways of the activated mast cells and basophils, using the

principle of co-aggregation of the inhibitory IgG receptor with the activating IgE Fc receptor.

6.2 Observations of the production of recombinant IgE and IgG Fc fragments

We have demonstrated a successful strategy for producing recombinant IgE and mutant IgG Fc regions (see Chapter 4). These Fc proteins were an important part in the design of the Fc-particle conjugate system. There are some aspects which need to be emphasized. The first is the difficulty faced in achieving these Fc recombinant proteins, such as low protein yield, and choosing the best purification technique, and optimising the sequence of IgE construct to be expressed (see Chapter 3). These led us to further consider the importance of choosing the ideal expression vector and host system in determining the quantity and purity of the desired protein.

The second aspect is the complexity of Fc protein structure compared to any other antibody fragments (ScFv, Fab and VHH). The changes in the structure of Fc region in terms of protein glycosylation could significantly impair the activity and affinity of the antibody for the receptor. Fc-glycosylation is essential in stabilising the antibody structure through CH2 domains to be less susceptible to unfolding and aggregation³⁰⁵. The conformational structure of CH2 is mainly controlled by the size of N-glycan which results in the development of two structures, open and closed. These formations will have a huge effect on the effector function following the binding of Fc protein with its receptor^{306,307}. For that reason, choosing the ideal expression system for producing functional Fc recombinant protein is crucial to the success of the Fc expression. Although it is costly, time consuming, and the risk of bacterial or fungal contamination is high, we chose to use the mammalian expression platform in this study as it offers post-translational modifications (e.g. glycosylation, disulphide bond formation, chaperones) that conserve the recombinant Fc protein activity.

Finally, there is the issue of expressing a customised Fc fragment instead of full sized antibody. Depending on the aim of the study, the expression of an Fc fragment is preferable for several reasons. First, both effector functions and the pharmacokinetic profile such as half-life of the IgE and IgG molecule will be retained, avoiding any undesirable immunogenicity effect caused by the presence of the Fab region. From the biopharmaceutical application perspective, the lower molecular weight of Fc protein is a good antibody candidate as it is more resistant to the changes in glycan patterns (proportion of mannose, galactose and sialic acid) or to the removal of N-glycosylation compared to the whole molecule³⁰⁸. In term of particle conjugation, the low molecular weight of the Fc fragment allows for a more convenient and precise orientation of Fc region to interact with the corresponding receptors. Finally, unlike the full-length immunoglobulins, the lower molecular weight hence smaller special requirements of Fc will allow the particle to be attached to a higher density on microparticles.

6.3 Observations of the effect of the delivery vector size

The influence of microparticle size on the immunological response is one of the main findings of this study. Many reports have investigated the role of particle size in targeted drug delivery, and small particle size (100 nm or less) is regarded as a more efficient carrier due to the ability to pass through capillaries after administration, and greater efficiency in crossing biological barriers^{309–311}. However, since there are few studies exploring the effect of particles size with the IgE Fc region, and depending on the type and the level of the immune response, it is difficult to predict the range of particle size that gives the optimal response.

It is probable that 0.5 μm is the ideal particle size when it is coated completely with IgE. However, it is not certain whether the lack of or low activation of other modified

particles is due to the particle size. It may be that they reach the supra-optimal level of IgE-crosslinking, as the degranulation effect in mast cells and basophils is a bell-shaped response and at supra-optimal IgE or antigen level, the degranulation response will be decreased^{312–314}. This assumption was supported by the data of Fc density ratios which demonstrated that at a lower ratio of IgE to particles (1:50), the activation level was higher, while both lower and higher ratios than (1:50) resulted in lower levels of activation. This study gives a first insight into the role of particle size and density of IgE Fc on the surface of particles in the engagement with basophil receptors (FcεRI).

6.4 Fc-particles conjugates as a therapeutic strategy of allergy

In Chapter 5, we describe an immunotherapeutic system composed of monobiotinylated IgG and IgE Fc-attached to streptavidin particles. The mechanism of this system as a therapeutic strategy is based mainly on the known inhibitory effect of the ITIM-bearing receptor (FcγRIIB) on the surface of allergic cells as a way to control the reaction of mast cells and basophils. Unlike the current approaches of allergy treatment, the special orientation of the Fc-particle conjugate system is optimised for binding to the Fc receptor and thus work on the signalling pathway of the allergic cells, which allow us to treat the allergic diseases from the initial steps in signal transduction. As it is discussed in Section 1.9, a number of current treatments are available to treat allergies, including corticosteroids, bronchodilators, mediator antagonists (anti-histamine and anti-leukotriene), cytokine modulators and mast cells stabilisers (Sodium cromoglycate). However, apart from the unpleasant side effects that accompany the current therapies due to lack of selectivity, there are other important limitations. Some of the conventional approaches are symptomatic treatments which do not treat the underlying cause (bronchodilators and

corticosteroid), while others are directed to target only one specific inflammatory mediator (anti-histamine, anti-leukotriene and cytokine modulators) or are not effective in acute anaphylaxis (Sodium cromoglycate). Patient compliance is another of the problems with allergen immunotherapy, as the treatment course is long (over years) and there is a small, but tangible potential that patients might suffer an anaphylactic attack.

The effect delivering the inhibitory receptor (FcγRIIB) to the allergic cells is mainly on regulating tyrosine phosphorylation of the syk protein, which represents upstream regulation of the signalling cascades and is a key factor in activating the ITAM signalling pathway. The recruitment of SHIP and SHIP1 proteins driven by the phosphorylation of FcγRIIB controls the early signalling events by inhibiting phosphorylation of syk-kinase enzyme^{315,316}. The net result of this intervention is a shutdown of all pro-inflammatory events, such as the release of histamine during degranulation, cytokine production, or arachidonic acid metabolite synthesis (leukotrienes and prostaglandins). Unlike the current allergy treatments, the ability to control all the parameters of cell activation with Fc-particles conjugate would create a better system as an immunotherapy.

Different groups of researchers have exploited the idea of FcεRI-FcγIIB co-crosslinking to develop an immune-based therapy either by creating fusion proteins consisting of Fc regions of IgG and IgE with a linker (GE2) or IgG Fc region fused allergen such as Fcγ-Fel d I and Fcγ-Der f2 (see Section 1.8). We have introduced two vital modifications to maximise the usefulness of the suggested therapy: incorporating the microparticles as the drug delivery platform, and insertion of specific mutations in the sequence of wild-type IgG Fc. The benefits of these modifications are: 1) specific

mutations in the IgG Fc fragment will minimise the otherwise severely biased stoichiometric ratios between IgE and IgG Fc fragments by improving the binding affinity of IgG Fc to FcγRIIB receptor, bringing the affinities for their respective receptors closer to each other ; 2) the microparticle vehicle will help in controlling the attachment of recombinant Fc proteins in a defined stoichiometric ratio and special orientation; and 3) the effect of Fc-particles conjugates will be broad, and target all basophils or mast cells and not be limited to a single allergen-specific reaction.

6.5 Conclusion

In this work, different IgG Fc variants were developed with improved binding affinity to FcγRIIB using site directed mutagenesis method. For the protein purification and particles conjugation purposes, two tags (Avi and His) were incorporated successfully to the C-terminal of IgG and IgE Fc variants constructs . All Fc recombinant proteins were expressed using a mammalian expression platform (293-6E cell line) and purified with cobalt resin through IMAC purification procedure. The purified protein yield was good enough for the next application. Regarding the particles conjugation step, purified IgG and IgE Fc domains were mono-biotinylated via in vitro enzymatic approach (BirA enzyme). To optimize the concentration, size and ligand density of the suggested immunotherapy, particles conjugated with IgE Fc region were evaluated on two reporter RBL cell lines (RS-ATL8 and NFAT-DsRed).

In term of the efficacy of Fc particle conjugates on peripheral blood basophils purified from human donor, the preliminary data demonstrated that the suggested immunotherapy could not either activate or inhibit the release of histamine from the basophils. However, more experiments are required to confirm the validity of these data.

In the future work, there is a need to investigate other parameters (CD63, CD203c SHIP protein) which represent the early stages of cell activation or inhibition to have a complete picture of the functionally of the Fc particle conjugates.

References

1. Urbanek, R. Allergy and asthma. *Lung* **168 Suppl**, 263–7 (1990).
2. Sohi, D. K. & Warner, J. O. Understanding allergy. *Paediatr. Child Health (Oxford)*. **18**, 301–308 (2008).
3. Martinez, F. D. Complexities of the genetics of asthma. *Am. J. Respir. Crit. Care Med.* **156**, S117-22 (1997).
4. Martinez, F. D. Gene by environment interactions in the development of asthma. *Clin. Exp. Allergy* **28 Suppl 5**, 21-5-8 (1998).
5. Abbey, D. E. Environmental determinants of asthma. *Lancet* **346**, 129–30 (1995).
6. Flokstra-de Blok, B. M. J. *et al.* Development and validation of the self-administered Food Allergy Quality of Life Questionnaire for adolescents. *J. Allergy Clin. Immunol.* **122**, 139–44, 144–2 (2008).
7. Resnick, E. S. *et al.* Development of a questionnaire to measure quality of life in adolescents with food allergy: the FAQL-teen. *Ann. Allergy. Asthma Immunol.* **105**, 364–8 (2010).
8. Van der Velde, J. L. *et al.* Food allergy-related quality of life after double-blind, placebo-controlled food challenges in adults, adolescents, and children. *J. Allergy Clin. Immunol.* **130**, 1136–1143.e2 (2012).
9. Sicherer, S. H., Noone, S. a & Muñoz-Furlong, a. The impact of childhood food allergy on quality of life. *Ann. Allergy. Asthma Immunol.* **87**, 461–4 (2001).
10. Pearce, N. *et al.* Worldwide trends in the prevalence of asthma symptoms: phase III of the International Study of Asthma and Allergies in Childhood (ISAAC). *Thorax* **62**, 758–66 (2007).
11. Weiland, S. K. *et al.* Phase II of the International Study of Asthma and Allergies in Childhood (ISAAC II): rationale and methods. *Eur. Respir. J.* **24**, 406–12 (2004).

12. Burney, P. *et al.* The distribution of total and specific serum IgE in the European Community Respiratory Health Survey. *J. Allergy Clin. Immunol.* **99**, 314–22 (1997).
13. Kimber, I. Allergy, asthma and the environment: an introduction. *Toxicol. Lett.* **102–103**, 301–6 (1998).
14. Sicherer, S. H., Muñoz-Furlong, A., Godbold, J. H. & Sampson, H. a. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J. Allergy Clin. Immunol.* **125**, 1322–6 (2010).
15. Gupta, R., Sheikh, A., Strachan, D. & Anderson, H. R. Increasing hospital admissions for systemic allergic disorders in England: analysis of national admissions data. *BMJ* **327**, 1142–3 (2003).
16. Gupta, R., Sheikh, a, Strachan, D. P. & Anderson, H. R. Time trends in allergic disorders in the UK. *Thorax* **62**, 91–6 (2007).
17. Johansson, S. G. O. *et al.* Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J. Allergy Clin. Immunol.* **113**, 832–6 (2004).
18. National Institutes of health. *Guidelines for the diagnosis and management of asthma.* (1997).
19. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. a & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. 1986. *J. Immunol.* **175**, 5–14 (2005).
20. Robinson, D. & Hamid, Q. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* (1992).
21. N. Franklin Adkinson Jr. *Middleton’S Allergy Principles and Practice. Middleton’s Allergy 2-Volume Set* (2014).
22. Spector, S. L. Overview of comorbid associations of allergic rhinitis. *J. Allergy Clin. Immunol.* **99**, S773-80 (1997).

23. Brozek, J. L. *et al.* Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines: 2010 revision. *J. Allergy Clin. Immunol.* **126**, 466–76 (2010).
24. Werfel, T. The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis. *J. Invest. Dermatol.* **129**, 1878–91 (2009).
25. Younker, J. & Soar, J. Recognition and treatment of anaphylaxis. *Nurs. Crit. Care* **15**, 94–8 (2010).
26. Simons, F. E. R. *et al.* Risk assessment in anaphylaxis: current and future approaches. *J. Allergy Clin. Immunol.* **120**, S2–24 (2007).
27. Kozel, M. M. a & Sabroe, R. a. Chronic urticaria: aetiology, management and current and future treatment options. *Drugs* **64**, 2515–36 (2004).
28. Zuberbier, T. *et al.* EAACI/GA(2)LEN/EDF/WAO guideline: management of urticaria. *Allergy* **64**, 1427–43 (2009).
29. Tharp, M. D. Chronic urticaria: pathophysiology and treatment approaches. *J. Allergy Clin. Immunol.* **98**, S325–30 (1996).
30. Rajan, T. V. The Gell–Coombs classification of hypersensitivity reactions: a re-interpretation. *Trends Immunol.* **24**, 376–379 (2003).
31. Abbas, A. K. & Lichtman, A. H. Basic immunology : functions and disorders of the immune system / Abul K. Abbas, Andrew H. Lichtman. (2010).
32. Kornbrust, D., Eydeloth, R. & Garratty, G. Investigations of the potential for five beta-lactam antibiotics to elicit type II hypersensitivity reactions in rats and monkeys. *Fundam. Appl. Toxicol.* **12**, 558–66 (1989).
33. Ellsworth, J. L. *et al.* Targeting immune complex-mediated hypersensitivity with recombinant soluble human Fcγ_{1A} (CD64A). *J. Immunol.* **180**, 580–9 (2008).
34. Coico, R. & Sunshine, G. *Immunology : a short course* / Richard Coico, Geoffrey Sunshine. (Wiley-Blackwell, 2009).
35. Rajan, T. V. The Gell–Coombs classification of hypersensitivity reactions: a re-

interpretation. *Trends Immunol.* **24**, 376–379 (2003).

36. Nosbaum, A., Vocanson, M., Rozieres, A., Hennino, A. & Nicolas, J.-F. Allergic and irritant contact dermatitis. *Eur. J. Dermatol.* **19**, 325–32 (2009).
37. Spies, J. R. Allergens. *J. Agric. Food Chem.* **22**, 30–36 (1974).
38. Mackay, I. R., Rosen, F. S. & Kay, A. B. Allergy and Allergic Diseases. *N. Engl. J. Med.* **344**, 30–37 (2001).
39. Miescher, S. M. & Vogel, M. Molecular aspects of allergy. *Molecular Aspects of Medicine* **23**, 413–462 (2002).
40. Bønnelykke, K., Pipper, C. B. & Bisgaard, H. Transfer of maternal IgE can be a common cause of increased IgE levels in cord blood. *J. Allergy Clin. Immunol.* **126**, 657–663 (2010).
41. Dullaers, M. *et al.* The who, where, and when of IgE in allergic airway disease. *J. Allergy Clin. Immunol.* **129**, 635–645 (2012).
42. Gould, H. J. *et al.* The biology of IgE and the basis of allergic disease. *Annu. Rev. Immunol.* **21**, 579–628 (2003).
43. Kenneth Murphy, Paul Travers, Mark Walport, Michael Ehrenstein, Claudia Mauri, Allan Mowat, A. S., Hsu, D. C., Murphy, K. M., Travers, P. & Walport, M. *Janeway's Immunobiology. Garland Science* **7**, (2008).
44. Kinet, J. P. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annu. Rev. Immunol.* **17**, 931–72 (1999).
45. Novak, N., Kraft, S. & Bieber, T. Unraveling the mission of FcεRI on antigen-presenting cells. *J. Allergy Clin. Immunol.* **111**, 38–44 (2003).
46. Bieber, T. *et al.* Human epidermal langerhans cells express the high affinity receptor for immunoglobulin E (FcεRI). *J. Exp. Med.* **175**, 0–5 (1992).
47. Daëron, M. Fc receptor biology. *Annu. Rev. Immunol.* (1997).
48. Wurzburg, B. a, Garman, S. C. & Jardetzky, T. S. Structure of the human IgE-Fc

- C epsilon 3-C epsilon 4 reveals conformational flexibility in the antibody effector domains. *Immunity* **13**, 375–85 (2000).
49. Garman, S. C., Wurzburg, B. a, Tarchevskaya, S. S., Kinet, J. P. & Jardetzky, T. S. Structure of the Fc fragment of human IgE bound to its high-affinity receptor Fc epsilonRI alpha. *Nature* **406**, 259–66 (2000).
 50. Garman, S. C., Kinet, J. P. & Jardetzky, T. S. Crystal structure of the human high-affinity IgE receptor. *Cell* **95**, 951–61 (1998).
 51. Kraft, S. & Kinet, J.-P. New developments in FcεRI regulation, function and inhibition. *Nat. Rev. Immunol.* **7**, 365–378 (2007).
 52. Malveaux, F. J., Conroy, M. C., Adkinson, N. F. & Lichtenstein, L. M. IgE receptors on human basophils. Relationship to serum IgE concentration. *J. Clin. Invest.* **62**, 176–81 (1978).
 53. Quarto, R., Kinet, J.-P. & Metzger, H. Coordinate synthesis and degradation of the α-, β- and γ-subunits of the receptor for immunoglobulin E. *Mol. Immunol.* **22**, 1045–1051 (1985).
 54. Furuichi, K., Rivera, J. & Isersky, C. The receptor for immunoglobulin E on rat basophilic leukemia cells: effect of ligand binding on receptor expression. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1522–5 (1985).
 55. Yamaguchi, M. *et al.* IgE Enhances Mouse Mast Cell FcεRI Expression In Vitro and In Vivo: Evidence for a Novel Amplification Mechanism in IgE-dependent Reactions. *J. Exp. Med.* **185**, 663–672 (1997).
 56. MacGlashan, D. W. *et al.* Down-regulation of Fc(epsilon)RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J. Immunol.* **158**, 1438 LP-1445 (1997).
 57. Lantz, C. S. *et al.* IgE regulates mouse basophil Fc epsilon RI expression in vivo. *J. Immunol.* **158**, 2517–2521 (1997).
 58. Yano, K. *et al.* Production of macrophage inflammatory protein-1alpha by human mast cells: increased anti-IgE-dependent secretion after IgE-dependent

- enhancement of mast cell IgE-binding ability. *Lab. Invest.* **77**, 185–93 (1997).
59. Novak, N. *et al.* Evidence for a differential expression of the Fc ϵ RI γ chain in dendritic cells of atopic and nonatopic donors. *J. Clin. Invest.* **111**, 1047–1056 (2003).
 60. Riley, J. & West, G. The presence of histamine in tissue mast cells. *J. Physiol.* **120**, 528–537 (1953).
 61. Wedemeyer, J., Tsai, M. & Galli, S. J. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* **12**, 624–31 (2000).
 62. Williams, C. M. & Galli, S. J. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. *J. Allergy Clin. Immunol.* **105**, 847–59 (2000).
 63. White, M. Mediators of inflammation and the inflammatory process. *J. Allergy Clin. Immunol.* **103**, 378–381 (1999).
 64. Anderson, D. M. *et al.* Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* **63**, 235–43 (1990).
 65. Stone, K. D., Prussin, C. & Metcalfe, D. D. IgE, mast cells, basophils, and eosinophils. *J. Allergy Clin. Immunol.* **125**, S73-80 (2010).
 66. Metcalfe, D., Baram, D. & Mekori, Y. Mast cells. *Physiol. Rev.* (1997).
 67. Brightling, C. E. *et al.* Mast-cell infiltration of airway smooth muscle in asthma. *N. Engl. J. Med.* **346**, 1699–1705 (2002).
 68. Bingham, C. O. & Austen, K. F. Mast-cell responses in the development of asthma. *J. Allergy Clin. Immunol.* **105**, S527–S534 (2000).
 69. Kepley, C. & Pfeiffer, J. The identification and characterization of umbilical cord blood-derived human basophils. *J. Leukoc. Biol.* **64**, 474–483 (1998).
 70. Denburg, J., Telizyn, S. & Messner, H. Heterogeneity of human peripheral blood eosinophil-type colonies: evidence for a common basophil-eosinophil

progenitor. *Blood* **66**, 312–318 (1985).

71. Eglite, S., Plüss, K. & Dahinden, C. a. Requirements for C5a receptor-mediated IL-4 and IL-13 production and leukotriene C4 generation in human basophils. *J. Immunol.* **165**, 2183–9 (2000).
72. Zwirner, J. *et al.* Evaluation of C3a receptor expression on human leucocytes by the use of novel monoclonal antibodies. *Immunology* **97**, 166–72 (1999).
73. Gibbs, B. F. *et al.* Purified human peripheral blood basophils release interleukin-13 and preformed interleukin-4 following immunological activation. *Eur. J. Immunol.* **26**, 2493–8 (1996).
74. Arock, M. & Merle-Beral, H. IL-4 Release by Human Leukemic and Activated Normal Basophils. *J. Immunol.* **151**, 1441–1447 (1993).
75. Ochensberger, B., Daepf, G. C., Rihs, S. & Dahinden, C. a. Human blood basophils produce interleukin-13 in response to IgE-receptor-dependent and -independent activation. *Blood* **88**, 3028–37 (1996).
76. Liu, M. C. *et al.* Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral airways in allergic asthmatics. Cellular, mediator, and permeability changes. *Am. Rev. Respir. Dis.* **144**, 51–8 (1991).
77. Charlesworth, E. N. *et al.* Cutaneous late-phase response to allergen. Mediator release and inflammatory cell infiltration. *J. Clin. Invest.* **83**, 1519–26 (1989).
78. Bascom, R. *et al.* Basophil influx occurs after nasal antigen challenge: effects of topical corticosteroid pretreatment. *J. Allergy Clin. Immunol.* **81**, 580–9 (1988).
79. Thomas, L. L. Basophil and eosinophil interactions in health and disease. *Chem. Immunol.* **61**, 186–207 (1995).
80. Knol, E. F., Mul, F. P., Lie, W. J., Verhoeven, A. J. & Roos, D. The role of basophils in allergic disease. *Eur. Respir. J. Suppl.* **22**, 126s–131s (1996).
81. Naclerio, R. M. *et al.* Inflammatory mediators in late antigen-induced rhinitis. *N. Engl. J. Med.* **313**, 65–70 (1985).

82. Jouvin, M. H., Numerof, R. P. & Kinet, J. P. Signal transduction through the conserved motifs of the high affinity IgE receptor Fc epsilon RI. *Semin. Immunol.* **7**, 29–35 (1995).
83. Torigoe, C., Goldstein, B., Wofsy, C. & Metzger, H. Shuttling of initiating kinase between discrete aggregates of the high affinity receptor for IgE regulates the cellular response. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 1372–7 (1997).
84. Siraganian, R. Mast cell signal transduction from the high-affinity IgE receptor. *Curr. Opin. Immunol.* **15**, 639–646 (2003).
85. Parravicini, V. *et al.* Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* **3**, 741–8 (2002).
86. Turner, H. & Kinet, J. P. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* **402**, B24-30 (1999).
87. Jabril-Cuenod, B. & Zhang, C. Syk-dependent phosphorylation of Shc. A potential link between FcepsilonRI and the Ras/mitogen-activated protein kinase signaling pathway through SOS and Grb2. *J. Biol. Chem.* **271**, 16268–72 (1996).
88. Song, J. S., Gomez, J., Stancato, L. F. & Rivera, J. Association of a p95 Vav-containing Signaling Complex with the FcεRI γ Chain in the RBL-2H3 Mast Cell Line. *J. Biol. Chem.* **271**, 26962–26970 (1996).
89. Gordon, J. & Galli, S. Mast cells as a source of both preformed and immunologically inducible TNF-α/cachectin. *Nature* **346**, (1990).
90. Gordon, J. & Galli, S. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. *A. J. Exp. Med.* **174**, 1–5 (1991).
91. Gri, G. *et al.* Mast cell: An emerging partner in immune interaction. *Front. Immunol.* **3**, 1–11 (2012).
92. Ohtsu, H. *et al.* Mice lacking histidine decarboxylase exhibit abnormal mast cells. *FEBS Lett.* **502**, 53–6 (2001).

93. MacGlashan, D. W. & Lichtenstein, L. M. The purification of human basophils. *J. Immunol.* **124**, 2519–21 (1980).
94. Schulman, E. S. *et al.* Human lung mast cells: purification and characterization. *J. Immunol.* **129**, 2662–7 (1982).
95. Abe, Y. *et al.* Histamine content, synthesis and degradation in human nasal mucosa. *Clin. Exp. Allergy* **23**, 132–136 (1993).
96. Yamauchi, K. & Sekizawa, K. Structure and function of human histamine N-methyltransferase: critical enzyme in histamine metabolism in airway. *Am. J. Physiol. Gastrointest. Liver Physiol.* **267**, L342–349 (1994).
97. Atsushi, Y., Kazutaka, M. & Takehiko, W. Tissue distribution of histamine in a mutant mouse deficient in mast cells: Clear evidence for the presence of non-mast-cell histamine. *Biochem. Pharmacol.* **31**, 305–309 (1982).
98. Schwartz, L. B., Bradford, T. R., Littman, B. H. & Wintroub, B. U. The fibrinogenolytic activity of purified tryptase from human lung mast cells. *J. Immunol.* **135**, 2762–2767 (1985).
99. Cairns, J. A. & Walls, A. F. Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. *J. Immunol.* **156**, 275–283 (1996).
100. Lewis, R. A. *et al.* Prostaglandin D₂ generation after activation of rat and human mast cells with anti-IgE. *J Immunol* **129**, 1627–1631 (1982).
101. Warner, J. A. *et al.* Differential release of mediators from human basophils: differences in arachidonic acid metabolism following activation by unrelated stimuli. *J. Leukoc. Biol.* **45**, 558–571 (1989).
102. Salmon, J. a & Higgs, G. a. Prostaglandins and leukotrienes as inflammatory mediators. *Br. Med. Bull.* **43**, 285–96 (1987).
103. Samuelsson, B. Arachidonic acid metabolism: role in inflammation. *Z Rheumatol* **50 Suppl 1**, 3–6 (1991).

104. Sullivan, S. O. & Dahlen, B. increased urinary excretion of the prostaglandin D2 metabolite 9 α ,11 β -prostaglandin F2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. *J. Allergy Clin. Immunol.* **98**, 421–432 (1996).
105. Benyon, R. C., Robinson, C. & Church, M. K. Differential release of histamine and eicosanoids from human skin mast cells activated by IgE-dependent and non-immunological stimuli. *Br. J. Pharmacol.* **97**, 898–904 (1989).
106. Triggiani, M., Schleimer, R. P., Warner, J. a & Chilton, F. H. Differential synthesis of 1-acyl-2-acetyl-sn-glycero-3-phosphocholine and platelet-activating factor by human inflammatory cells. *J. Immunol.* **147**, 660–6 (1991).
107. Braquet, P., Touqui, L., Shen, T. & Vargaftig, B. perspectives in platelet activating factor research. *Pharmacol. Rev.* (1987).
108. Stafforini, D. M. *et al.* Platelet-activating factor acetylhydrolase deficiency. A missense mutation near the active site of an anti-inflammatory phospholipase. *J. Clin. Invest.* **97**, 2784–91 (1996).
109. Gordon, J. R., Burd, P. R. & Galli, S. J. Mast cells as a source of multifunctional cytokines. *Immunol. Today* **11**, 458–464 (1990).
110. Echtenacher, B., Männel, D. & Hültner, L. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* **381**, 75–7 (1996).
111. Malaviya, R., Ikeda, T., Ross, E. & Abraham, S. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF- α . *Nature* **381**, 77–80 (1996).
112. Bradding, P., Feather, I. H., Wilson, S., Holgate, S. T. & Howarth, P. H. Cytokine immunoreactivity in seasonal rhinitis: regulation by a topical corticosteroid. *Am. J. Respir. Crit. Care Med.* **151**, 1900–6 (1995).
113. Benson, M., Strannegård, I. L., Wennergren, G. & Strannegård, O. Cytokines in nasal fluids from school children with seasonal allergic rhinitis. *Pediatr. Allergy Immunol.* **8**, 143–9 (1997).

114. Luster, A. D. Chemokines--chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* **338**, 436–445 (1998).
115. Alam, R. *et al.* Monocyte chemotactic and activating factor is a potent histamine-releasing factor for basophils. *J. Clin. Invest.* **89**, 723–8 (1992).
116. Dahinden, C. A. *et al.* Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine. *J. Exp. Med.* **179**, 751–6 (1994).
117. Schur, P. H. IgG subclasses. A historical perspective. *Monogr. Allergy* **23**, 1–11 (1988).
118. Jefferis, R. & Kumararatne, D. S. Selective IgG subclass deficiency: quantification and clinical relevance. *Clin. Exp. Immunol.* **81**, 357–67 (1990).
119. Potter, M. Structural correlates of immunoglobulin diversity. *Surv. Immunol. Res.* **2**, 27–42 (1983).
120. Wu, T. Te, Johnson, G. & Kabat, E. A. Length distribution of CDRH3 in antibodies. *Proteins: Structure, Function, and Bioinformatics* **16**, 1–7 (1993).
121. Kabat, E. A., Wu, T. T. & Bilofsky, H. Variable region genes for the immunoglobulin framework are assembled from small segments of DNA--a hypothesis. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2429–33 (1978).
122. Vidarsson, G., Dekkers, G. & Rispen, T. IgG subclasses and allotypes: From structure to effector functions. *Front. Immunol.* **5**, (2014).
123. Roux, K. H., Strelets, L. & Michaelsen, T. E. Flexibility of Human IgG Subclasses. *J. Immunol.* **159**, 3372–3382 (1997).
124. Bindon, C. I. Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. *J. Exp. Med.* **168**, 127–142 (1988).
125. Hamilton, R. G. The Human IgG Subclasses. *Calbiochem Handb.* 1–64 (2001).
126. Ravetch, J. & Bolland, S. IGG FC RECEPTORS. *Annu. Rev. Immunol.* (2001).
127. Nimmerjahn, F. & Ravetch, J. V. Fcγ receptors as regulators of immune

- responses. *Nat. Rev. Immunol.* **8**, 34–47 (2008).
128. Raghavan, M. & Bjorkman, P. J. Fc receptors and their interactions with immunoglobulins. *Annu. Rev. Cell Dev. Biol.* **12**, 181–220 (1996).
 129. Hulett, M. Chimeric Fc receptors identify immunoglobulin-binding regions in human FcγRII and FcεRI. *Eur. J. Immunol.* **23**, 640–645 (1993).
 130. Hulett, M. Multiple regions of human Fc gamma RII (CD32) contribute to the binding of IgG. *J Biol. Chem* **270**, 21188–94 (1995).
 131. Moore, G. L., Chen, H., Karki, S. & Lazar, G. a. Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. *MAbs* **2**, 181–9 (2010).
 132. Cemerski, S. *et al.* Suppression of mast cell degranulation through a dual-targeting tandem IgE-IgG Fc domain biologic engineered to bind with high affinity to FcγRIIb. *Immunol. Lett.* **143**, 34–43 (2012).
 133. Chu, S. Y. *et al.* Inhibition of B cell receptor-mediated activation of primary human B cells by coengagement of CD19 and FcγRIIb with Fc-engineered antibodies. *Mol. Immunol.* **45**, 3926–33 (2008).
 134. Malbec, O. *et al.* Fc epsilon receptor I-associated lyn-dependent phosphorylation of Fc gamma receptor IIB during negative regulation of mast cell activation. *J. Immunol.* **160**, 1647–58 (1998).
 135. Ono, M., Bolland, S., Tempst, P. & Ravetch, J. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(γ)RIIB. *Nature* **383**, 263–6 (1996).
 136. Scharenberg, A. & El-Hillal, O. Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *EMBO J.* **17**, 1961–1972 (1998).
 137. Rohrschneider, L., Fuller, J. & Wolf, I. Structure , function , and biology of SHIP proteins. *Genes Dev.* **14**, 505–520 (2000).

138. Daeron, M., Malbec, O., Latour, S., Arock, M. & Fridman, W. H. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *J. Clin. Invest.* **95**, 577–585 (1995).
139. Kepley, C. L. *et al.* Negative regulation of FcεRI signaling by FcγRII costimulation in human blood basophils. *J. Allergy Clin. Immunol.* **106**, 337–348 (2000).
140. Zhu, D., Kepley, C. L., Zhang, M., Zhang, K. & Saxon, A. A novel human immunoglobulin Fcγ1–Fcε1 bifunctional fusion protein inhibits Fcε1-mediated degranulation. *Nat. Med.* **8**, 518–521 (2002).
141. Zhang, K. *et al.* Inhibition of allergen-specific IgE reactivity by a human Ig Fcγ1–Fcε1 bifunctional fusion protein. *J. Allergy Clin. Immunol.* **114**, 321–7 (2004).
142. Zhu, D. *et al.* A chimeric human-cat fusion protein blocks cat-induced allergy. *Nat. Med.* **11**, 446–449 (2005).
143. Cemurski, S. *et al.* Suppression of mast cell degranulation through a dual-targeting tandem IgE-IgG Fc domain biologic engineered to bind with high affinity to FcγRIIb. *Immunol. Lett.* **143**, 34–43 (2012).
144. Lin, L. H. *et al.* Prevention and treatment of allergic inflammation by an Fcγ2b–Fcε1 fusion protein in a murine model of dust mite-induced asthma. *Immunol. Res.* **52**, 276–283 (2012).
145. Wigginton, S. J. *et al.* An immunoglobulin E-reactive chimeric human immunoglobulin G1 anti-idiotypic inhibits basophil degranulation through cross-linking of FcεRI with FcγRIIb. *Clin. Exp. Allergy* **38**, 313–319 (2008).
146. Holgate, S. T. & Polosa, R. Treatment strategies for allergy and asthma. *Nat. Rev. Immunol.* **8**, 218–30 (2008).
147. Maggi, E. T-cell responses induced by allergen-specific immunotherapy. *Clin. Exp. Immunol.* **161**, 10–8 (2010).
148. Cady, C. T. *et al.* IgG antibodies produced during subcutaneous allergen immunotherapy mediate inhibition of basophil activation via a mechanism

- involving both FcγRIIA and FcγRIIB. *Immunol. Lett.* **130**, 57–65 (2010).
149. Schulman, E. S. Development of a monoclonal anti-immunoglobulin E antibody (omalizumab) for the treatment of allergic respiratory disorders. *Am. J. Respir. Crit. Care Med.* **164**, S6-11 (2001).
 150. Ishizaka, T., Sterk, A. R., Daeron, M., Becker, E. L. & Ishizaka, K. Biochemical analysis of desensitization of mouse mast cells. *J. Immunol.* **135**, 492–501 (1985).
 151. Meng, Y. G., Singh, N. & Wong, W. L. Binding of cynomolgus monkey IgE to a humanized anti-human IgE antibody and human high affinity IgE recepto. *Mol. Immunol.* **33**, 635–642 (1996).
 152. Meltzer, E. O., Berkowitz, R. B. & Grossbard, E. B. An intranasal Syk-kinase inhibitor (R112) improves the symptoms of seasonal allergic rhinitis in a park environment. *J. Allergy Clin. Immunol.* **115**, 791–6 (2005).
 153. Reber, L., Da Silva, C. a & Frossard, N. Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. *Eur. J. Pharmacol.* **533**, 327–40 (2006).
 154. Wilson, D. R., Lima, M. T. & Durham, S. R. Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. *Allergy* **60**, 4–12 (2005).
 155. Lewis, D. B. Allergy immunotherapy and inhibition of Th2 immune responses: a sufficient strategy? *Curr. Opin. Immunol.* **14**, 644–51 (2002).
 156. Incorvaia, C., Masieri, S., Berto, P., Scurati, S. & Frati, F. Specific immunotherapy by the sublingual route for respiratory allergy. *Allergy Asthma. Clin. Immunol.* **6**, 29 (2010).
 157. Niederberger, V. & Valenta, R. Recombinant allergens for immunotherapy. Where do we stand? *Curr. Opin. Allergy Clin. Immunol.* **4**, 549–554 (2004).
 158. Wensch, M. *et al.* Serum IgE, tumor epidermal growth factor receptor expression, and inherited polymorphisms associated with glioma survival. *Cancer Res.* **66**, 4531–41 (2006).

159. Fu, S. L. *et al.* Immunoglobulin E antibodies from pancreatic cancer patients mediate antibody-dependent cell-mediated cytotoxicity against pancreatic cancer cells. *Clin. Exp. Immunol.* **153**, 401–9 (2008).
160. Karagiannis, S. N. *et al.* IgE-antibody-dependent immunotherapy of solid tumors: cytotoxic and phagocytic mechanisms of eradication of ovarian cancer cells. *J. Immunol.* **179**, 2832–43 (2007).
161. Karagiannis, S. N. *et al.* Activity of human monocytes in IgE antibody-dependent surveillance and killing of ovarian tumor cells. *Eur. J. Immunol.* **33**, 1030–40 (2003).
162. Miller, C. W., Krishnaswamy, N., Johnston, C. & Krishnaswamy, G. Severe asthma and the omalizumab option. *Clin. Mol. Allergy* **6**, 4 (2008).
163. Pelaia, G., Renda, T., Romeo, P., Busceti, M. T. & Maselli, R. Omalizumab in the treatment of severe asthma: efficacy and current problems. *Ther. Adv. Respir. Dis.* **2**, 409–21 (2008).
164. Putney, S. D. & Burke, P. A. Improving protein therapeutics with sustained-release formulations. *Nat. Biotechnol.* **16**, 153–7 (1998).
165. Oyewumi, M. O., Kumar, A. & Cui, Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. *Expert Rev. Vaccines* **9**, 1095–1107 (2010).
166. Kramer, P. A. Albumin microspheres as vehicles for achieving specificity in drug delivery. *J. Pharm. Sci.* **63**, 1646–1647 (1974).
167. Singh, M. N., Hemant, K. S. Y., Ram, M. & Shivakumar, H. G. Microencapsulation: A promising technique for controlled drug delivery. *Research in Pharmaceutical Sciences* **5**, 65–77 (2010).
168. Davis, S. S. & Illum, L. The targeting of drugs using polymeric microspheres. *Br. Polym. J.* **15**, 160–164 (1983).
169. Eniola, A. O. & Hammer, D. A. Artificial polymeric cells for targeted drug delivery. in *Journal of Controlled Release* **87**, 15–22 (2003).

170. Foster, N., Clark, M. A., Jepson, M. A. & Hirst, B. H. Ulex europaeus 1 lectin targets microspheres to mouse Peyer's patch M-cells in vivo. *Vaccine* **16**, 536–541 (1998).
171. Carreno-Gómez, B., Woodley, J. F. & Florence, A. T. Studies on the uptake of tomato lectin nanoparticles in everted gut sacs. in *International Journal of Pharmaceutics* **183**, 7–11 (1999).
172. Stella, B. *et al.* Design of folic acid-conjugated nanoparticles for drug targeting. *J. Pharm. Sci.* **89**, 1452–1464 (2000).
173. Rice-Ficht, A. C., Arenas-Gamboa, A. M., Kahl-McDonagh, M. M. & Ficht, T. A. Polymeric particles in vaccine delivery. *Current Opinion in Microbiology* **13**, 106–112 (2010).
174. Thomasin, C., Corradin, G., Men, Y., Merkle, H. P. & Gander, B. Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: Importance of polymer degradation and antigen release for immune response. in *Journal of Controlled Release* **41**, 131–145 (1996).
175. Slütter, B. *et al.* Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen. *J. Control. Release* **143**, 207–214 (2010).
176. Mallapragada, S. K. & Narasimhan, B. Immunomodulatory biomaterials. *International Journal of Pharmaceutics* **364**, 265–271 (2008).
177. Año, G. *et al.* A new oral vaccine candidate based on the microencapsulation by spray-drying of inactivated *Vibrio cholerae*. *Vaccine* **29**, 5758–5764 (2011).
178. Taranejoo, S., Janmaleki, M., Rafienia, M., Kamali, M. & Mansouri, M. Chitosan microparticles loaded with exotoxin A subunit antigen for intranasal vaccination against *Pseudomonas aeruginosa*: An in vitro study. *Carbohydr. Polym.* **83**, 1854–1861 (2011).
179. Garlapati, S. *et al.* Immunization with PCEP microparticles containing pertussis toxoid, CpG ODN and a synthetic innate defense regulator peptide induces

- protective immunity against pertussis. *Vaccine* **29**, 6540–6548 (2011).
180. Sharif, S., Wheeler, A. W. & O'Hagan, D. T. Biodegradable microparticles as a delivery system for the allergens of *Dermatophagoides pteronyssinus* (house dust mite): I. Preparation and characterization of microparticles. *Int. J. Pharm.* **119**, 239–246 (1995).
 181. Saint-Lu, N. *et al.* Targeting the allergen to oral dendritic cells with mucoadhesive chitosan particles enhances tolerance induction. *Allergy Eur. J. Allergy Clin. Immunol.* **64**, 1003–1013 (2009).
 182. Wu, J. & Horuzsko, A. Expression and function of immunoglobulin-like transcripts on tolerogenic dendritic cells. *Hum. Immunol.* **70**, 353–356 (2009).
 183. Shields, R. L. *et al.* High Resolution Mapping of the Binding Site on Human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and Design of IgG1 Variants with Improved Binding to the FcγR. *J. Biol. Chem.* **276**, 6591–6604 (2001).
 184. Bruhns, P. *et al.* Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses. *Blood* **113**, 3716–3726 (2016).
 185. Mimoto, F. *et al.* Engineered antibody Fc variant with selectively enhanced FcγRIIb binding over both FcγRIIa(R131) and FcγRIIa(H131). *Protein Eng. Des. Sel.* **26**, 589–98 (2013).
 186. Lazar, G. a *et al.* Engineered antibody Fc variants with enhanced effector function. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 4005–10 (2006).
 187. Richards, J. O. *et al.* Optimization of antibody binding to FcγRIIa enhances macrophage phagocytosis of tumor cells. *Mol. Cancer Ther.* **7**, 2517–2527 (2008).
 188. Woof, J. M. & Burton, D. R. Human antibody-Fc receptor interactions illuminated by crystal structures. *Nat. Rev. Immunol.* **4**, 89–99 (2004).
 189. Kiyoshi, M. *et al.* Structural basis for binding of human IgG1 to its high-affinity human receptor FcγRI. *Nat. Commun.* **6**, 1–11 (2015).

190. Hemsley, A. *et al.* A simple method for site-directed mutagenesis using the polymerase chain reaction. *Nucleic Acids Res.* **17**, 6545–6551 (1989).
191. Arkin, M. in *Brenner's Encyclopedia of Genetics* **2**, 46–50 (Elsevier, 2001).
192. Tirat, A., Freuler, F., Stettler, T., Mayr, L. M. & Leder, L. Evaluation of two novel tag-based labelling technologies for site-specific modification of proteins. *Int. J. Biol. Macromol.* **39**, 66–76 (2006).
193. Walper, S. A., Turner, K. B. & Medintz, I. L. Enzymatic bioconjugation of nanoparticles: Developing specificity and control. *Curr. Opin. Biotechnol.* **34**, 232–241 (2015).
194. Koutz, P. *et al.* Structural comparison of the *Pichia pastoris* alcohol oxidase genes. *Yeast* **5**, 167–177 (1989).
195. Tschopp, J. F., Brust, P. F., Cregg, J. M., Stillman, C. A. & Gingeras, T. R. Expression of the *lacZ* gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res.* **15**, 3859–3876 (1987).
196. Ellis, S. B. *et al.* Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. *Mol. Cell. Biol.* **5**, 1111–21 (1985).
197. Wei, Y. *et al.* Glyco-engineering of human IgG1-Fc through combined yeast expression and in vitro chemoenzymatic glycosylation. *Biochemistry* **47**, 10294–10304 (2008).
198. Kamiya, T., Sugio, S., Yamanouchi, K. & Kagitani, Y. Secretion of active Fc fragments of immunoglobulin E directed by the yeast invertase signal sequence in mammalian cells. *Tohoku J Exp Med* **180**, 297–308 (1996).
199. Li, H. *et al.* Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. *Nat. Biotechnol.* **24**, 210–5 (2006).
200. Wang, D. D. *et al.* Expression, purification and characterization of a human single-chain Fv antibody fragment fused with the Fc of an IgG1 targeting a rabies antigen in *Pichia pastoris*. *Protein Expr. Purif.* **86**, 75–81 (2012).

201. Cregg, J. M., Vedvick, T. S. & Raschke, W. C. Recent Advances in the Expression of Foreign Genes in *Pichia pastoris*. *Bio/Technology* **11**, 905–910 (1993).
202. Scorer, C. A., Buckholz, R. G., Clare, J. J. & Romanos, M. A. The intracellular production and secretion of HIV-1 envelope protein in the methylotrophic yeast *Pichia pastoris*. *Gene* **136**, 111–119 (1993).
203. Romanos, M. a, Scorer, C. a & Clare, J. J. Foreign gene expression in yeast: a review. *Yeast* **8**, 423–488 (1992).
204. Thomas, P. & Smart, T. G. HEK293 cell line: A vehicle for the expression of recombinant proteins. *J. Pharmacol. Toxicol. Methods* **51**, 187–200 (2005).
205. Kaufman, W. L. *et al.* Homogeneity and persistence of transgene expression by omitting antibiotic selection in cell line isolation. *Nucleic Acids Res.* **36**, (2008).
206. Duffy, M. F. *et al.* Activation and clustering of a *Plasmodium falciparum* var gene are affected by subtelomeric sequences. *FEBS J.* **284**, 237–257 (2017).
207. Parr, T. *et al.* Expression of calpastatin isoforms in muscle and functionality of multiple calpastatin promoters. *Arch. Biochem. Biophys.* **427**, 8–15 (2004).
208. Rutherford, C. *et al.* Phosphorylation-independent internalisation and desensitisation of the human sphingosine-1-phosphate receptor S1P3. *Cell. Signal.* **17**, 997–1009 (2005).
209. Kiseljak, D. *et al.* The use of filler DNA for improved transfection and reduced DNA needs in transient gene expression with CHO and HEK cells. *BMC Proc.* **5 Suppl 8**, P33 (2011).
210. Meyer, S. *et al.* Multi-Host Expression System for Recombinant Production of Challenging Proteins. *PLoS One* **8**, 1–9 (2013).
211. Rajendra, Y., Kiseljak, D., Baldi, L., Hacker, D. L. & Wurm, F. M. Influence of glutamine on transient and stable recombinant protein production in CHO and HEK- 293 cells. *BMC Proc.* **5**, P35 (2011).
212. Kramer, O. H., Zhu, P., Ostendorff, H.P., Golebiewski, M., Tiefenbach, J., Peters,

- M.A.Brill, B., Groner, B., Bach, I., Heinzl, T., Göttlicher, M. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *EMBO J.* **22**, 3411–3420 (2003).
213. Allen, M. J. *et al.* Identification of novel small molecule enhancers of protein production by cultured mammalian cells. *Biotechnol. Bioeng.* **100**, 1193–1204 (2008).
 214. Struhl, K. Histone acetylation and transcriptional regulatory mechanisms. *Genes and Development* **12**, 599–606 (1998).
 215. Cinatl, J. *et al.* Sodium valproate inhibits in vivo growth of human neuroblastoma cells. *Anti-cancer drugs* **8**, 958–63 (1997).
 216. Göttlicher, M. *et al.* Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* **20**, 6969–6978 (2002).
 217. Wulhfard, S., Baldi, L., Hacker, D. L. & Wurm, F. Valproic acid enhances recombinant mRNA and protein levels in transiently transfected Chinese hamster ovary cells. *J. Biotechnol.* **148**, 128–132 (2010).
 218. Backliwal, G. *et al.* Valproic acid: A viable alternative to sodium butyrate for enhancing protein expression in mammalian cell cultures. *Biotechnol. Bioeng.* **101**, 182–189 (2008).
 219. Chaga, G., Hopp, J. & Nelson, P. Immobilized metal ion affinity chromatography on Cobalt - carboxymethylaspartate – agarose Superflow, as demonstrated by one-step purification of lactate dehydrogenase from chicken breast muscle. *Biotechnol. Appl. Biochem.* **24**, 19–24 (1999).
 220. Kurokawa, T. *et al.* Expression of human immunoglobulin E e chain cDNA in *E. coli*. *Nucleic Acids Res.* **11**, 3077–3085 (1983).
 221. Röthlisberger, D., Honegger, A. & Plückthun, A. Domain interactions in the Fab fragment: A comparative evaluation of the single-chain Fv and Fab format engineered with variable domains of different stability. *J. Mol. Biol.* **347**, 773–789 (2005).

222. Wan, T. *et al.* The crystal structure of IgE Fc reveals an asymmetrically bent conformation. *Nat. Immunol.* **3**, 681–6 (2002).
223. Basu, M. *et al.* Purification and characterization of human recombinant IgE-Fc fragments that bind to the human high affinity IgE receptor. *J. Biol. Chem.* **268**, 13118–13127 (1993).
224. Holdom, M. D. *et al.* Conformational changes in IgE contribute to its uniquely slow dissociation rate from receptor FcεRI. *Nat. Struct. Mol. Biol.* **18**, 571–6 (2011).
225. Hendershot, L., Bole, D., Kohler, G. & Kearney, J. F. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. *J. Cell Biol.* **104**, 761–767 (1987).
226. Young, R. J. *et al.* Secretion of recombinant human IgE-Fc by mammalian cells and biological activity of glycosylation site mutants. *Protein Eng. Des. Sel.* **8**, 193–199 (1995).
227. Colosimo, a. *et al.* Review Transfer and Expression of Foreign Genes in Mammalian Cells. *Biotechniques* **29**, 314–331 (2000).
228. Durocher, Y., Perret, S. & Kamen, A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res.* **30**, E9 (2002).
229. Imai, S., Nishikawa, J. & Takada, K. Cell-to-Cell Contact as an Efficient Mode of Epstein-Barr Virus Infection of Diverse Human Epithelial Cells. *J. Virol.* **72**, 4371–4378 (1998).
230. Van Craenenbroeck, K., Vanhoenacker, P. & Haegeman, G. Episomal vectors for gene expression in mammalian cells. *Eur. J. Biochem.* **267**, 5665–5678 (2000).
231. Gorman, C. M., Gies, D., McCray, G. & Huang, M. The human cytomegalovirus major immediate early promoter can be trans-activated by adenovirus early proteins. *Virology* **171**, 377–385 (1989).

232. Massie, B. *et al.* Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. *J. Virol.* **72**, 2289–96 (1998).
233. Massie, B. *et al.* New adenovirus vectors for protein production and gene transfer. *Cytotechnology* **28**, 53–64 (1998).
234. Boussif, O. *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7297–301 (1995).
235. Schlaeger, E. J. & Christensen, K. Transient gene expression in mammalian cells grown in serum-free suspension culture. *Cytotechnology* **30**, 71–83 (1999).
236. Godbey, W. T., Wu, K. K. & Mikos, A. G. Poly(ethylenimine) and its role in gene delivery. *J. Control. Release* **60**, 149–160 (1999).
237. Hacker, D. L., De Jesus, M. & Wurm, F. M. 25 years of recombinant proteins from reactor-grown cells - Where do we go from here? *Biotechnol. Adv.* **27**, 1023–1027 (2009).
238. Zhu, J. Mammalian cell protein expression for biopharmaceutical production. *Biotechnol. Adv.* **30**, 1158–1170 (2012).
239. Reisman, D. & Sugden, B. trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. *Mol. Cell. Biol.* **6**, 3838–3846 (1986).
240. Sugden, B. & Warren, N. A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. *J. Virol.* **63**, 2644–9 (1989).
241. Gahn, T. a & Sugden, B. An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene. *J. Virol.* **69**, 2633–2636 (1995).
242. Mackey, D. & Sugden, B. Applications of oriP plasmids and their mode of

- replication. *Methods Enzymol.* **306**, 308–328 (1999).
243. Ambinder, R. F., Mullen, M. a, Chang, Y. N., Hayward, G. S. & Hayward, S. D. Functional domains of Epstein-Barr virus nuclear antigen EBNA-1. *J. Virol.* **65**, 1466–1478 (1991).
 244. Längle-Rouault, F. *et al.* Up to 100-fold increase of apparent gene expression in the presence of Epstein-Barr virus oriP sequences and EBNA1: implications of the nuclear import of plasmids. *J. Virol.* **72**, 6181–5 (1998).
 245. Godbey, W. T., Wu, K. K. & Mikos, a G. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 5177–5181 (1999).
 246. Ferrari, S. *et al.* ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther.* **4**, 1100–1106 (1997).
 247. Bragonzi, A. *et al.* Comparison between cationic polymers and lipids in mediating systemic gene delivery to the lungs. *Gene Ther.* **6**, 1995–2004 (1999).
 248. Goula, D. *et al.* Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther.* **5**, 1291–1295 (1998).
 249. Goula, D. *et al.* Rapid crossing of the pulmonary endothelial barrier by polyethylenimine/DNA complexes. *Gene Ther.* **7**, 499–504 (2000).
 250. Pham, P. L. *et al.* Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: Peptone additives improve cell growth and transfection efficiency. *Biotechnol. Bioeng.* **84**, 332–342 (2003).
 251. Tom, R., Bisson, L. & Durocher, Y. Transfection of HEK293-EBNA1 cells in suspension with linear PEI for production of recombinant proteins. *Cold Spring Harb. Protoc.* **3**, (2008).
 252. Dunlap, D. D., Maggi, A., Soria, M. R. & Monaco, L. Nanoscopic structure of DNA condensed for gene delivery. *Nucleic Acids Res.* **25**, 3095–3101 (1997).

253. Suh, J., Paik, H.-J. & Hwang, B. K. Ionization of Poly(ethylenimine) and Poly(allylamine) at Various pH's. *Bioorganic Chemistry* **22**, 318–327 (1994).
254. Akinc, A., Thomas, M., Klibanov, A. M. & Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.* **7**, 657–663 (2005).
255. Demeneix, B. & Behr, J. P. Polyethylenimine (PEI). *Advances in Genetics* **53**, 215–230 (2005).
256. Stern, B., Olsen, L. C., Tröbse, C., Ravneberg, H. & Pryme, I. F. Improving mammalian cell factories : The selection of signal peptide has a major impact on recombinant protein synthesis and secretion in mammalian cells. *Trends Cell Mol. Biol.* **2**, 1–17 (2007).
257. Adlersberg, J. B. The immunoglobulin hinge (interdomain) region. *Ric. Clin. Lab.* **6**, 191–205 (1976).
258. Carrington, J. C. & Dougherty, W. G. A viral cleavage site cassette: identification of amino acid sequences required for tobacco etch virus polyprotein processing. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 3391–5 (1988).
259. Forsberg, G. *et al.* An evaluation of different enzymatic cleavage methods for recombinant fusion proteins, applied on des(1-3)insulin-like growth factor I. *J. Protein Chem.* **11**, 201–211 (1992).
260. Stevens, R. C. Design of high-throughput of protein production for structural biology. *Structure* **8**, R177–R185 (2000).
261. Parks, T. D., Leuther, K. K., Howard, E. D., Johnston, S. A. & Dougherty, W. G. Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. *Analytical Biochemistry* **216**, 413–417 (1994).
262. Waugh, D. TEV Protease FAQ. *Macromol. Crystallogr. Lab.* 1–7 (2010).
263. Klint, J. K. *et al.* Production of Recombinant Disulfide-Rich Venom Peptides for Structural and Functional Analysis via Expression in the Periplasm of E. coli. *PLoS One* **8**, (2013).

264. Munro, S. & Pelham, H. R. B. An hsp70-like protein in the ER: Identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**, 291–300 (1986).
265. Gething, M. J., McCammon, K. & Sambrook, J. Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. *Cell* **46**, 939–950 (1986).
266. Lee, Y. K., Brewer, J. W., Hellman, R. & Hendershot, L. M. BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly. *Mol. Biol. Cell* **10**, 2209–2219 (1999).
267. Feige, M. J. *et al.* An Unfolded CH1 Domain Controls the Assembly and Secretion of IgG Antibodies. *Mol. Cell* **34**, 569–579 (2009).
268. Alberini, C. M., Bet, P., Milstein, C. & Sitia, R. Secretion of immunoglobulin M assembly intermediates in the presence of reducing agents. *Nature* **347**, 485–7 (1990).
269. Rojanasakul, Y., Wang, L. Y., Malanga, C. J., Ma, J. K. & Liaw, J. Targeted gene delivery to alveolar macrophages via Fc receptor-mediated endocytosis. *Pharmaceutical research* **11**, 1731–1736 (1994).
270. Cruz, L. J. *et al.* Targeting nanosystems to human DCs via Fc Receptor as an Effective Strategy to Deliver Antigen for Immunotherapy. *Mol. Pharm.* **8**, 104–116 (2011).
271. Rezaeipoor, R. *et al.* Fc-directed antibody conjugation of magnetic nanoparticles for enhanced molecular targeting. *J. Innov. Opt. Health Sci.* **2**, 387–396 (2009).
272. Kumar, S., Aaron, J. & Sokolov, K. Directional conjugation of antibodies to nanoparticles for synthesis of multiplexed optical contrast agents with both delivery and targeting moieties. *Nat. Protoc.* **3**, 314–320 (2008).
273. John, R. *et al.* In vivo magnetomotive optical molecular imaging using targeted

- magnetic nanoprobe. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 8085–8090 (2010).
274. Hogarth, P. M. & Pietersz, G. a. Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. *Nat. Rev. Drug Discov.* **11**, 311–31 (2012).
275. Falcone, F. H., Knol, E. F. & Gibbs, B. F. The role of basophils in the pathogenesis of allergic disease. *Clinical and Experimental Allergy* **41**, 939–947 (2011).
276. Harvima, I. T. & Nilsson, G. Mast cells as regulators of skin inflammation and immunity. *Acta dermato-venereologica* **91**, 644–650 (2011).
277. Harvima, I. T. *et al.* Molecular targets on mast cells and basophils for novel therapies. *J. Allergy Clin. Immunol.* **134**, 530–544 (2014).
278. Jönsson, F. & Daëron, M. Mast cells and company. *Front. Immunol.* **3**, 1–18 (2012).
279. Putney, S. D. Encapsulation of proteins for improved delivery. *Curr. Opin. Chem. Biol.* **2**, 548–552 (1998).
280. Marco, M. Di *et al.* Overview of the main methods used to combine proteins with nanosystems: Absorption, bioconjugation, and encapsulation. *International Journal of Nanomedicine* **5**, 37–49 (2010).
281. Saviranta, P., Haavisto, T., Rappu, P., Karp, M. & Lövgren, T. In vitro enzymatic biotinylation of recombinant Fab fragments through a peptide acceptor tail. *Bioconjug. Chem.* **9**, 725–735 (1998).
282. Cull, M. G. & Schatz, P. J. Biotinylation of proteins in vivo and in vitro using small peptide tags. *Methods Enzymol.* **Volume 326**, 430–440 (2000).
283. Li, Y. & Sousa, R. Expression and purification of E. coli BirA biotin ligase for in vitro biotinylation. *Protein Expr. Purif.* **82**, 162–167 (2012).
284. Kay, B. K., Thai, S. & Volgina, V. V. High-throughput biotinylation of proteins. *Methods Mol. Biol.* **498**, 185–198 (2009).
285. Sung, K., Maloney, M. T., Yang, J. & Wu, C. A novel method for producing

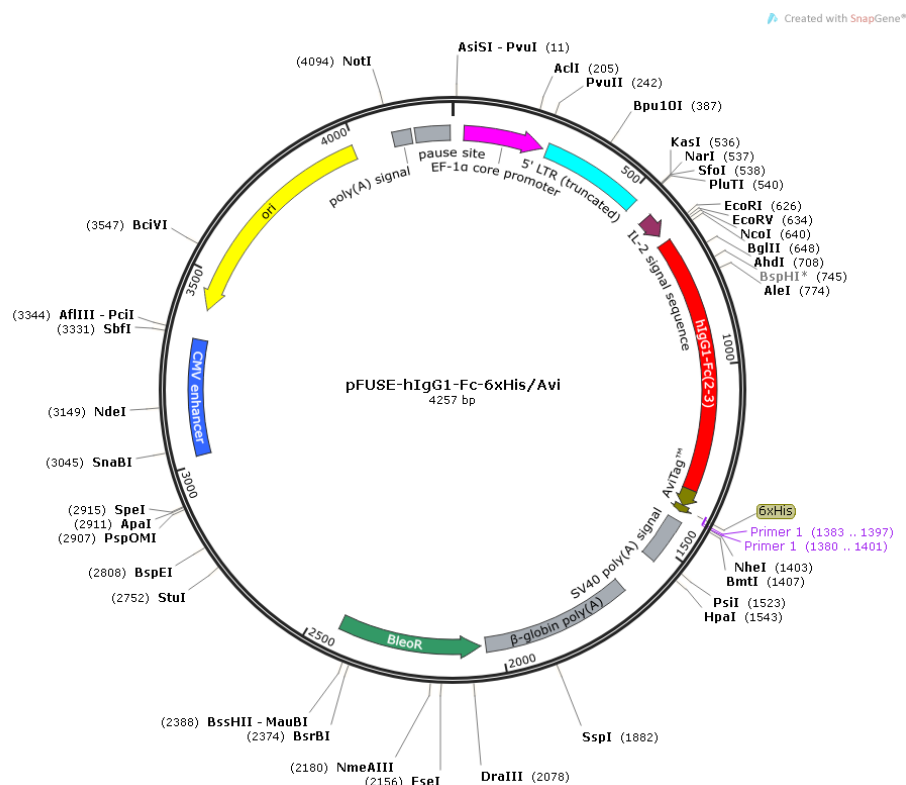
- mono-biotinylated, biologically active neurotrophic factors: An essential reagent for single molecule study of axonal transport. *J. Neurosci. Methods* **200**, 121–128 (2011).
286. Borkowski, T. A., Jouvin, M.-H., Lin, S.-Y. & Kinet, J.-P. Minimal Requirements for IgE-Mediated Regulation of Surface Fc RI. *J. Immunol.* **167**, 1290–1296 (2001).
 287. Wang, X. *et al.* Optimisation and use of humanised RBL NF-AT-GFP and NF-AT-DsRed reporter cell lines suitable for high-throughput scale detection of allergic sensitisation in array format and identification of the ECM-integrin interaction as critical factor. *Mol. Biotechnol.* **56**, 136–146 (2014).
 288. Knol, E. F. Requirements for effective IgE cross-linking on mast cells and basophils. *Mol. Nutr. Food Res.* **50**, 620–4 (2006).
 289. Pierini, L., Holowka, D. & Baird, B. FcεRI-mediated association of 6-μm beads with RBL-2H3 mast cells results in exclusion of signaling proteins from the forming phagosome and abrogation of normal downstream signaling. *J. Cell Biol.* **134**, 1427–1439 (1996).
 290. Pacheco, P. M., LE, B., White, D. & Sulchek, T. Tunable Complement Activation by Particles with Variable Size and Fc Density. *Nano Life* **3**, 1341001 (2013).
 291. Huang, Y.-F., Liu, H., Xiong, X., Chen, Y. & Tan, W. Nanoparticle-mediated IgE-receptor aggregation and signaling in RBL mast cells. *J. Am. Chem. Soc.* **131**, 17328–34 (2009).
 292. Joralemon, M. J., Smith, N. L., Holowka, D., Baird, B. & Wooley, K. L. Antigen-decorated shell cross-linked nanoparticles: Synthesis, characterization, and antibody interactions. *Bioconjug. Chem.* **16**, 1246–1256 (2005).
 293. Alsaleh, N. B., Persaud, I. & Brown, J. M. Silver nanoparticle-directed mast cell degranulation is mediated through calcium and PI3K signaling independent of the high affinity IgE receptor. *PLoS One* **11**, 1–25 (2016).
 294. Ortega, V. A., Ede, J. D., Boyle, D., Stafford, J. L. & Goss, G. G. Polymer-Coated

- Metal-Oxide Nanoparticles Inhibit IgE Receptor Binding, Cellular Signaling, and Degranulation in a Mast Cell-like Cell Line. *Adv. Sci.* **2**, 1–12 (2015).
295. Eshhar, Z., Ofarim, M. & Waks, T. Generation of hybridomas secreting murine reagenic antibodies of anti-DNP specificity. *J. Immunol.* **124**, 775–80 (1980).
 296. Chen, E. Y. *et al.* A Mixture of Anatase and Rutile TiO₂ Nanoparticles induces Histamine Secretion in Mast Cells. *Part. Fibre Toxicol.* **9**, 2 (2012).
 297. Pacheco, P., White, D. & Sulchek, T. Effects of Microparticle Size and Fc Density on Macrophage Phagocytosis. *PLoS One* **8**, 1–9 (2013).
 298. Gibbs, B. F., Papenfuss, K. & Falcone, F. H. A comparison of procedures for purifying human basophils to near homogeneity. *Inflamm. Res.* **57**, 11–12 (2008).
 299. Falcone, F. H., Haas, H. & Gibbs, B. F. The human basophil: a new appreciation of its role in immune responses. *Blood* **96**, 4028–38 (2000).
 300. Gibbs, B. F., Papenfuss, K. & Falcone, F. H. A rapid two-step procedure for the purification of human peripheral blood basophils to near homogeneity. *Clin. Exp. Allergy* **38**, 480–485 (2008).
 301. Nakamura, R. *et al.* A convenient and sensitive allergy test: IgE crosslinking-induced luciferase expression in cultured mast cells. *Allergy Eur. J. Allergy Clin. Immunol.* **65**, 1266–1273 (2010).
 302. Stuchbury, G. & Münch, G. Optimizing the generation of stable neuronal cell lines via pre-transfection restriction enzyme digestion of plasmid DNA. *Cytotechnology* **62**, 189–194 (2010).
 303. Passante, E. & Frankish, N. The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell. *Inflamm. Res.* **58**, 737–45 (2009).
 304. Larsen, J. N., Broge, L. & Jacobi, H. Allergy immunotherapy: The future of allergy treatment. *Drug Discov. Today* **21**, 26–37 (2016).
 305. Zheng, K., Bantog, C. & Bayer, R. The impact of glycosylation on monoclonal

- antibody conformation and stability. *MAbs* **3**, 568–576 (2011).
306. Krapp, S., Mimura, Y., Jefferis, R., Huber, R. & Sonderrmann, P. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *J. Mol. Biol.* **325**, 979–989 (2003).
 307. Harrison, P. T. & Allen, J. M. High affinity IgG binding by FcγRI (CD64) is modulated by two distinct IgSF domains and the transmembrane domain of the receptor. *Protein Eng.* **11**, 225–232 (1998).
 308. Krahn, N. *et al.* Inhibition of glycosylation on a camelid antibody uniquely affects its FcγRI binding activity. *Eur. J. Pharm. Sci.* **96**, 428–439 (2017).
 309. Needham, D. & Rudoll, T. L. Increased Microvascular Permeability Contributes to Preferential Accumulation of Stealth1 Liposomes in Tumor Tissue². *Cancer Res.* **53**, 3765–3770 (1993).
 310. Monfardini, C. & Veronese, F. M. *Stabilization of substances in circulation. Bioconjugate Chemistry* **9**, (1998).
 311. Mumper, R. J., Cui, Z. & Oyewumi, M. O. Nanotemplate Engineering of Cell Specific Nanoparticles. *J. Dispers. Sci. Technol.* **24**, 569–588 (2003).
 312. Gibbs, B. F., R  thling, A., Zillikens, D., Huber, M. & Haas, H. Initial Fc  RI-mediated signal strength plays a key role in regulating basophil signaling and deactivation. *J. Allergy Clin. Immunol.* **118**, 1060–1067 (2006).
 313. Huber, M. Activation/Inhibition of mast cells by supra-optimal antigen concentrations. *Cell Commun. Signal.* **11**, 7 (2013).
 314. Gimborn, K., Lessmann, E., Kuppig, S., Krystal, G. & Huber, M. SHIP Down-Regulates Fc R1-Induced Degranulation at Supraoptimal IgE or Antigen Levels. *J. Immunol.* **174**, 507–516 (2005).
 315. Huang, Z.-Y., Hunter, S., Kim, M.-K., Indik, Z. K. & Schreiber, A. D. The effect of phosphatases SHP-1 and SHIP-1 on signaling by the ITIM- and ITAM-containing Fcγ receptors FcγRIIB and FcγRIIA. *J. Leukoc. Biol.* **73**, 823–829 (2003).

316. Ott, V. L. & Cambier, J. C. Activating and inhibitory signaling in mast cells: New opportunities for therapeutic intervention? *J. Allergy Clin. Immunol.* **106**, 429–440 (2000).

Appendices



Appendix 1 : A map of the pFUSE-hlgG1-Fc-6xHis/Avi plasmid

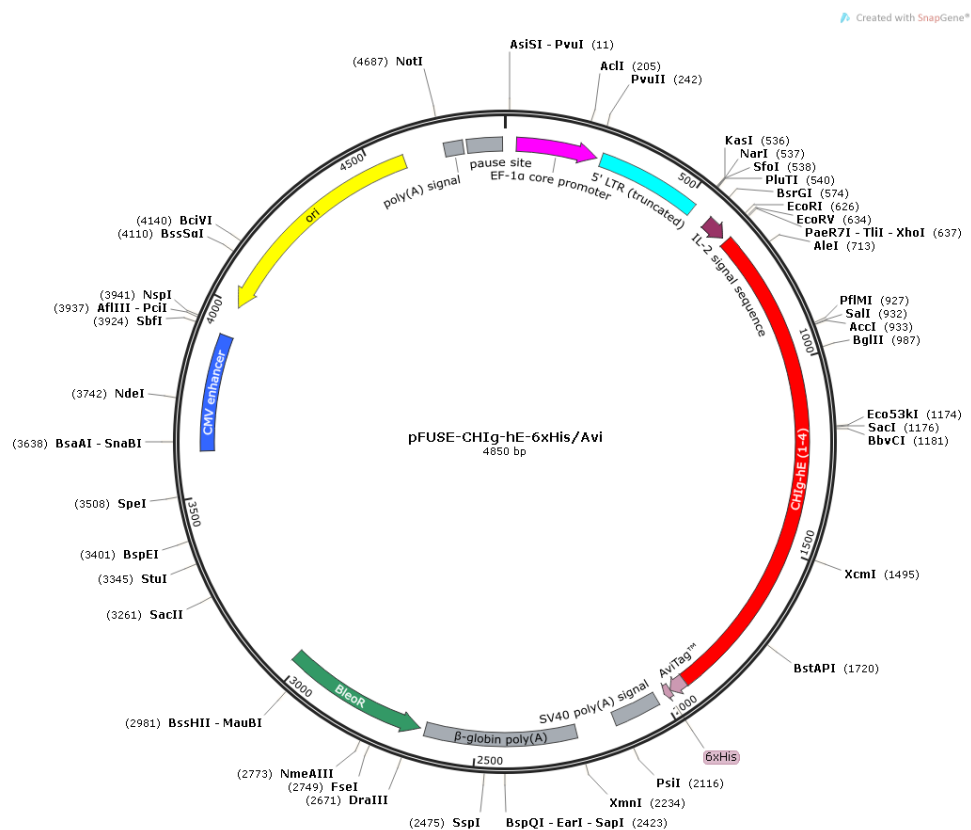
Keys:

EF-1α core promoter	β-globin poly(A)	5' LTR (truncated)	5'	BleoR
IL-2 signal sequence	CMV enhancer	hlgG1-Fc(2-3)	Ori	
AviTag/6xHis tags	IgG Fc mutations sites	SV40 poly(A) signal		

Plasmid sequence :

GGATCTGCGATCGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCGG
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Appendix 2: A map of the pFUSE-CHlg-hE-6xHis/Avi plasmid

Keys:

EF-1α core promoter	β-globin poly(A)	5' LTR (truncated) 5'	BleoR
IL-2 signal sequence	CMV enhancer	CHlg-hE Fc (1,2,3 and 4)	Ori
Avi /6xHis tags	SV40 poly(A) signal		

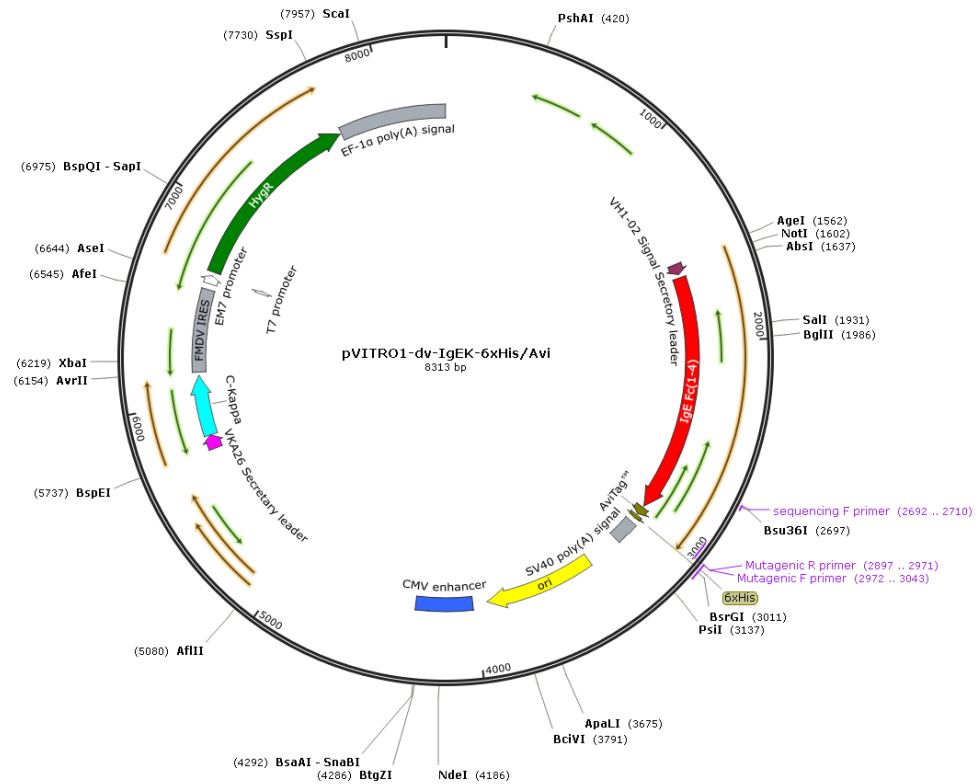
Plasmid sequence:

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Appendix 3: A map of the pVITRO1-dv-IgEK-6xHis/Avi plasmid

Keys:

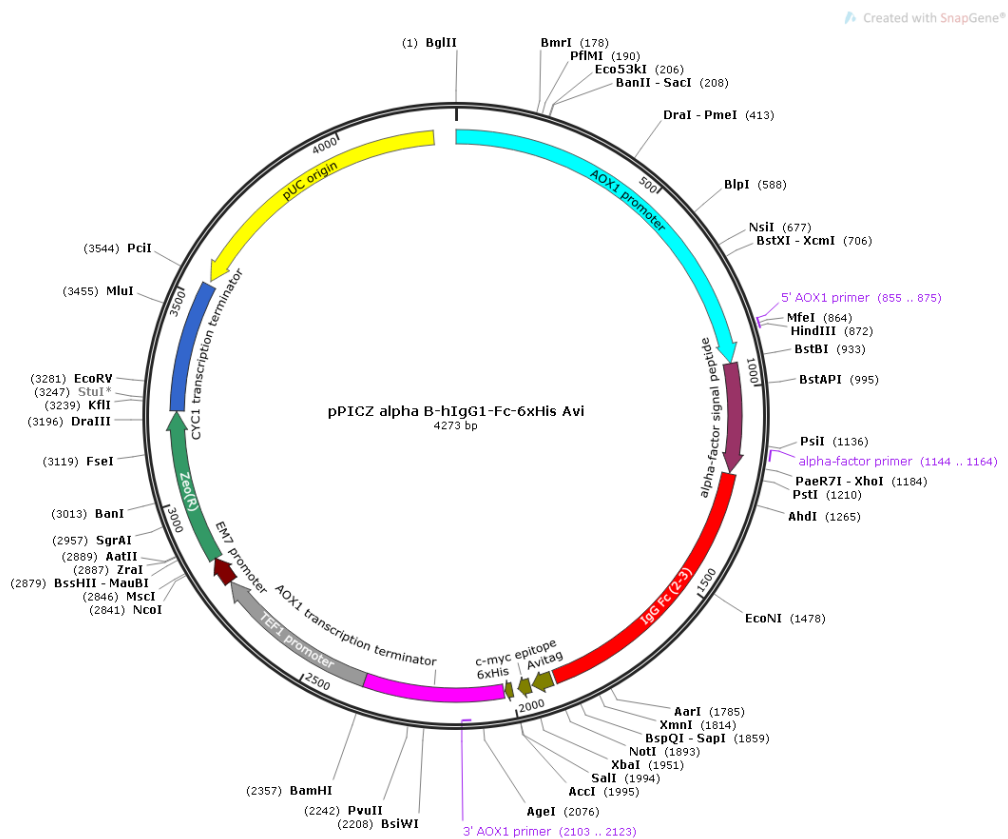
VH1-02 Signal Secretary leader	FMDV IRES	IgE Fc (1-4)	HygR
VKA26 Secretary leader	CMV enhancer	C-Kappa	Ori
Avi /6xHis tags	SV40 poly(A) signal	EF-1α poly(A) signal	

Plasmid sequence:

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Appendix 4: A map of the pPICZ alpha B-hlgG1-Fc-6xHis /Avi plasmid

Keys:

alpha-factor signal peptide	TEF1 promoter	IgG Fc (2-3)	Zeo R
AOX1 transcription terminator	CYC1 transcription terminator	AOX1 promoter	
PUC origin	Avi /c-myc /6xHis tags	EM7 promoter	IgG Fc mutations sites

Plasmid sequence:

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Appendix 5: A map of the pPICZ alpha B-CHlg-hE-6xHis/ Avi plasmid

Keys:

alpha-factor signal peptide

TEF1 promoter

IgE Fc (1-4)

Zeo R

AOX1 transcription terminator

CYC1 transcription terminator

AOX1 promoter

PUC origin

Avi /C-myc /6xHis tags

EM7 promoter

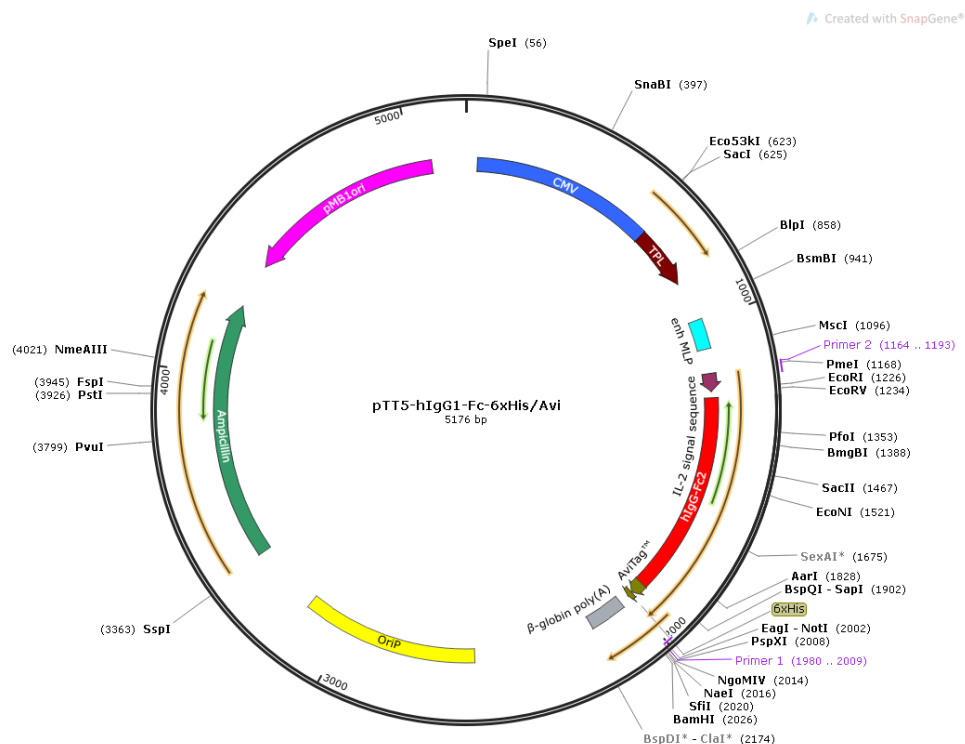
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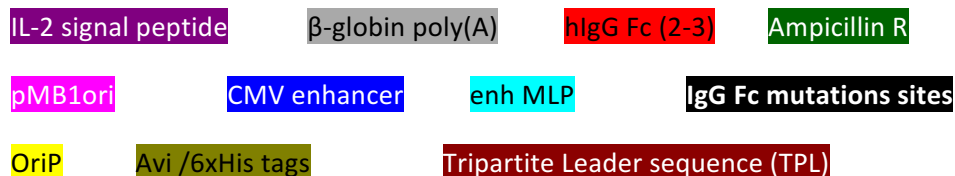
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Appendix 6: A map of the pTT5-hlgG1-Fc-6xHis/Avi plasmid

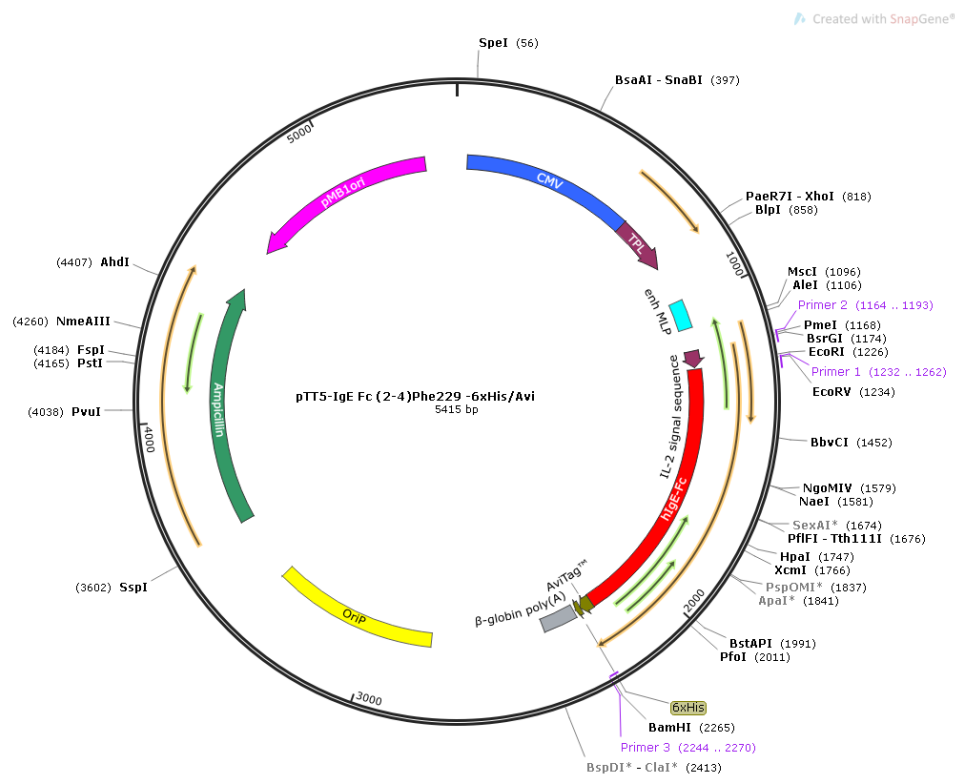
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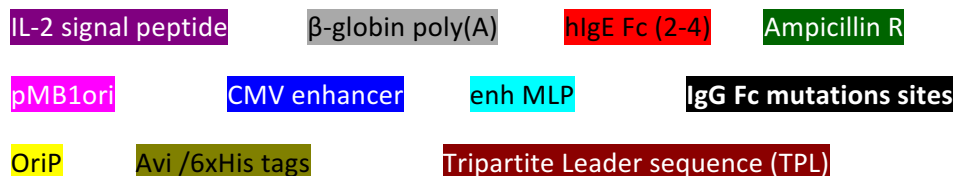
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Appendix 7: A map of the pTT5-IgE Fc (2-4)Phe229 -6xHis/Avi plasmid

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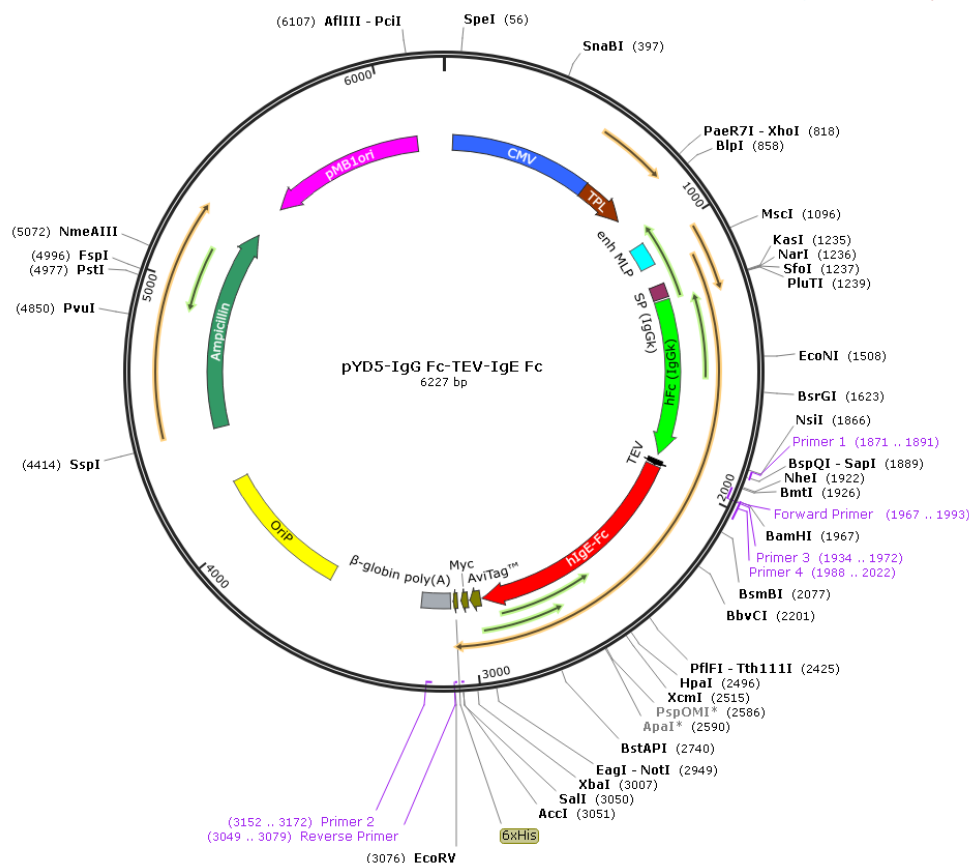


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ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGC



Appendix 8: A map of the pYD5-IgG Fc-TEV-IgE Fc plasmid

Keys :

IgGK signal peptide	β-globin poly(A)	hIgE Fc (2-4)	hFc(IgGK)
Ampicillin R	pMB1ori	CMV enhancer	enh MLP
TEV cleavage site	Avi /6xHis tags	Tripartite Leader sequence (TPL)	OriP

Plasmid sequence :

GTACATTTATATTGGCTCATGTCCAATATGACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGT
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