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Use of Humanised Reporter Cell Lines for High-Throughput Detection of Allergic Sensitization

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As you set out for Ithaka

hope your road is a long one,

full of adventure, full of discovery.

Laistrygonians, Cyclops,

angry Poseidon—don’t be afraid of them:

you’ll never find things like that on your way

as long as you keep your thoughts raised high,

as long as a rare excitement stirs your spirit and your body.

- Ithaka, C. P. Cavafy

Abstract

The prevalence of allergic diseases is growing rapidly in Europe. Today, it is estimated that more than 150 million individuals living in EU suffer from chronic allergic diseases, half of whom are underdiagnosed or poorly managed due to lack of awareness and shortage of medical specialists. Moreover, many patients do not report their symptoms or are not correctly diagnosed, while approximately 45% of the patients have never received an allergy diagnosis.

In vivo tests used for allergy diagnosis such as skin tests (e.g. SPT) and oral food challenge (OFC) may be uncomfortable for the patients, can pose a risk for anaphylaxis, and their results may be affected by the use of medications targeting the histamine receptor. On the other hand, *in vitro* tests are able to overcome most of these limitations. However, various *in vitro* methods used to detect allergen specific IgE (sIgE) in patients' serum, such as the ImmunoCAP ISAC, do not always provide clinically relevant results. sIgE may not always be capable to crosslink the high-affinity receptor (FcεRI) in the presence of cognate antigen, an important mechanism that triggers mast cell and basophil degranulation and release of mediators responsible for most of the well-known allergy symptoms. In this project, we tested a new high-throughput *in vitro* method for allergy diagnosis using rat basophilic leukaemia (RBL) cell lines. After stable transfection of RBL cells expressing human FcεRIα with either FcεRIɣH or a nuclear factor of activated T cells (NFAT)-responsive intracellular fluorescence gene (DsRed), two reporter systems were generated: RBL NFAT-DsRed FCER1G and RBL SX-38 NFAT-DsRed-Express2, respectively. Transfection of RBL NFAT-DsRed with $FcεRIɣ_H$ increased $FcεRIα_H$ chain expression levels, thus enhancing cell sensitivity compared to its parental cell line (NFAT-DsRed), albeit still not to a level comparable with RS-ATL8 expressing the highest level of $FcεRIα_H$ receptors/cell. Attempts to generate a more sensitive reporter system (the fluorescent equivalent of the RS-ATL8 luciferase reporter) were unsuccessful, as RBL SX-38 NFAT-DsRed-Express2 showed low levels of red fluorescence protein (RFP) expression after stimulation. Alternatively, using the RBL 703/21- NFAT-DsRed cells, generated previously in our lab, which express satisfactory levels of RFP after stimulation, we demonstrated a proof-of-concept high-throughput reporter system with a potential to detect clinically relevant allergic sensitization. After assessing cell adhesion, printed-protein stability and cell activation onto three different polymer-treated surfaces, we showed that N-hydroxysuccinimide (NHS)-ester coated slides treated with fibronectin are a feasible proposition for further development of an allergy diagnosis system combining humanised RBL cells with printed allergens.

Declaration

I declare that all the work presented in this dissertation is my own, except where otherwise stated.

Marina Kalli
September 2020

Publications, Conferences

Publications

1. Kalli M, Blok A, Jiang L, Starr N, Alcocer MJC, Falcone FH. Development of a protein microarray-based diagnostic chip mimicking the skin prick test for allergy diagnosis. Sci Rep. 2020 Oct 23;10(1):18208. https://doi: 10.1038/s41598-020-75226-y.

2. Kalli, M., Alcocer, M. J. C., Blok, A. J., & Falcone, F. H. (2020). Use of Humanized Fluorescent Reporter Cell Line RBL NFAT-DsRed for the Detection of Allergen-Specific IgE in Patient Sera Using Allergen Microarrays. https://doi.org/10.1007/978-1-0716-0696-4_12.

3. Ali, E. A., Kalli, M., Wan, D., Nakamura, R., Onion, D., Alanine, D. G. W. Falcone, F. H. (2019). Characterization of human FcεRIα chain expression and gene copy number in humanized rat basophilic leukaemia (RBL) reporter cell lines. PLoS ONE, 14(8). https://doi.org/10.1371/journal.pone.0221034.
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4. de Melo TT, Mendes MM, Alves CC, Carvalho GB, Fernandes VC, Pimenta DLF, de Moraes Mourão M, Gai F, Kalli M, Coelho A, de Azambuja Ribeiro RIM, Falcone FH, Pereira RADS, Fonseca CT. The Schistosoma mansoni cyclophilin A epitope 107-121 induces a protective immune response against schistosomiasis. Mol Immunol. 2019 Jul;111:172-181. doi: 10.1016/j.molimm.2019.04.021Erratum in: Mol Immunol. 2019 Oct;114:171. PMID: 31063938.

Conference Oral Presentations

1. World Allergy Congress, WAC 2019, Lyon, France. Allergy Diagnosis Session, 12th December 2019: "Protein microarrays: The future of allergy diagnosis? Optimization of coating and printing of allergen arrays used with fluorescent humanised basophil reporter cell line NFAT-DsRed RBL".
2. International Symposium On Molecular Allergology, ISMA 2019, Amsterdam, The Netherlands. Session 8 – New Diagnostic Developments, 30th November 2019: “Development Of High-Throughput Technology To Diagnose AllergyOptimised Surface Coating And Characterisation Of Protein Microarrays Using ToF-SIMS To Detect Fluorescent RBL Cell Activation”.

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At last, I would to thank my dear family for their continuous encouragement and unconditional love. I would like to dedicate this Thesis to my dear mother (Pelagia), my kind siblings (Meropi, Konstantinos), my little nephew (Dimitris or Mitsos) for being my happy thoughts, my sweet granny Eugenia (who I lost only a month before submission of this Thesis), for always being proud of my achievements and my lovely partner in life (Dimitris) who is always there for me and supports my decisions. Lastly, to my dad (Lazaros) who although not being here anymore I know he would have been very proud and has always been next to me.

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List of abbreviations

Abbreviation	Expanded name
APC	antigen-presenting cell
BAT	basophil activation test
Bet v 1,2	birch pollen (Betula verrucosa)
bFGF	basic fibroblast growth factor
BM	basement membrane
BSA	bovine serum albumin
Ca^{2+}	calcium
$CaCl_2$	calcium Chloride
CO2	carbon dioxide
CO	collagen
CRD	component resolved diagnostics
CSR	class switch recombination
CRAC	calcium release-activated calcium
DAG	diacylglycerol
DMEM	dulbecco's modified Eagle's medium
D2O	deuterium dioxide
ECM	extra cellulose matrix
EMTU	epithelial mesenchymal trophic unit
ER	endoplasmic reticulum
ERK1/ERK2	extracellular-signal-regulated protein kinase
FCS	fetal calf serum
FcR	Fc receptor
FN	fibronectin
GADS	GRB2-related adaptor protein
GMP	granulocyte-monocyte progenitor
GRB2	growth-factor-receptor-bound protein
HSC	hematopoietic stem cells
LAT	linker for activation of T cells
LPR	late phase reaction
LT	leukotriene
Ig's	immunoglobulin
IgE	immunoglobulin E (erythema)
EMTU	epithelial–mesenchymal trophic unit
IL-	interleukin
Ionophore	calcium ionophore A23187
IP3	inositol 1, 4, 5-trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
MAPKS	mitogen-activated protein kinases
MC	mast cell

MEM	minimum essential medium
MHC class II	major histocompatibility complex (MHC) class II
ml	millilitre
NaCl	sodium chloride
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor-κB
NGF	nerve growth factor
NLS	nuclear-localization signal
OFC	oral food challenge
PAF	platelet activating factor
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PGD2	prostaglandin D2
Phl p 1,2,6	phleum pollen (phleum pratense)
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PMA	phorbol myristate acetate
PRR	pattern recognition receptor
RAST	radioallegosorbent immunoassay test
RBL	rat basophilic leukaemia
SCF	stem cell factor
SLP-76 kDa	Src homology 2 domain containing leukocyte protein of 76
SOC	store-operated channel
SPT	skin prick test
STIM1	stromal interaction molecule 1
Syk	spleen tyrosine kinase
TCRs	T-cell antigen receptor
TGFβ	transforming growth factor beta
TM	transmembrane domain
TNF	tumour necrosis factor
µg	microgram
µl	microlitre

Chapter 1: Introduction

1.1. Allergy and allergic diseases

The term allergy was first introduced by Clemens von Pirquet in 1906 in the Münchener Medizinische Wochenschrift as “specifically altered reactivity of an organism” (1). In 1960, the British immunologists Gell and Coombs proposed a classification scheme for allergic reactions, which later became a reference for defining the pathogenesis of allergic drug reactions. Based on the classification system drug hypersensitivity reactions are divided in four distinct types based on the pathophysiological mechanisms involved, Type I: immediate hypersensitivity or anaphylaxis, Type II: antibody-mediated cytotoxic reactions, Type III: immune complex-mediated reactions, Type IV: delayed hypersensitivity (2). Based on Gell and Coombs conception, these disorders are mediated by a particular class of immunoglobulins, the immunoglobulin E (IgE). Typically, IgE antibodies bind to the high affinity receptor, FcεRI, on the surface of mast cells and basophils. Bridging of adjacent cell-bound IgE molecules by a matching bivalent or multivalent allergen (3) induces receptor crosslinking triggering cell degranulation and release of mediators such as histamine (causing an immediate reaction), leukotrienes (resulting in the more delayed symptoms) and other mediators that cause the signs and symptoms of a Type I hypersensitivity reaction (4). Today, the World Allergy Organization (WAO) has defined allergy as a hypersensitivity reaction initiated by immunologic mechanisms (5).

The immune response in allergy begins with allergic sensitization. In the airway lumen, allergens are taken up by dendritic cells (DCs) or enter tissues through disrupted epithelium (6, 7) or some allergen with protease activity after cleaving epithelial cell tight junctions (8, 9) are captivated by submucosal dendritic cells (10, 11). Interleukin-33 (IL-33) or else named “alarmin” acting through the ST2 (IL1RL1) receptor, functions as an alarm signal which can initiate an allergic response (12). Together with other pro-Th2 cytokines released by epithelial cells (IL-25, IL-1α, thymic stromal lymphopoietin (TSLP) and GM-CSF) activate DCs to drive Th2 responses. IL-33 also drives activation of group 2 innate lymphoid cells (ILC2) which produce large amounts of IL-5 and IL-13 (13-15). Activated dendritic cells then migrate to regional lymph nodes or to sites in the local mucosa. There, they process and present allergen-derived peptides in the context of major histocompatibility complex (MHC) class II molecules to naïve CD4+ T cells (16, 17). In the presence of interleukin 4 (IL-4) (released earlier in the process from a range of cells including basophils, mast cells, eosinophils, natural killer T cells), naïve T cells differentiate into T helper 2 (Th2) cell subtype (27). IL-4 and IL-13 cytokines produced by Th2 cells and the ligation of appropriate co-stimulatory molecules (CD40 with CD40 ligand, and CD80 or CD86 with CD28) induce B cells to undergo immunoglobulin class-switch recombination (CSR) in which gene fragments that encode the immunoglobulin heavy chain are rearranged as such that antibodies of the IgE class are produced (27-31).

IgE production by plasma cells occurs mostly in the bone marrow, spleen and lymph nodes (18-20). IgE development has also been detected in the gut (21-23). However, allergic sensitization can occur in all parts of the skin, respiratory tract (nose, lungs), conjunctivae and the gastrointestinal tract. This suggests that IgE antibodies, which diffuse locally, first enter the lymphatic vessels, then enter the blood and following they are distributed systemically. Once entering the interstitial fluid, they bind to the high-affinity receptor (FcεRI) on mast cells residing on tissues, thereby inducing cell sensitization. Subsequent exposure to the same allergen triggers a series of immunological events that lead to the characteristic allergy symptoms (32).

Allergic inflammation is classified in three phases; early-phase reactions, late-phase reactions and chronic inflammation. Early-phase reactions occur within seconds to minutes and late-phase reactions prevail within few hours. The chronic allergic inflammation is a continuous inflammation occurring after repeated exposure to allergens (33). An early-phase response or Type I immediate hypersensitivity reaction occurs within minutes of allergen exposure and is characterized by immediate release of mediators by mast cells (MCs) locally at the affected sites (34). On the surface of mast cells of sensitized individuals allergen-specific IgE are tightly bound to the high-affinity IgE receptors (FcεRI). Crosslinking of FcεRI-bound IgE by bivalent or multivalent allergen results in aggregation of FcεRI, triggering complex intracellular signalling events leading to the secretion of biologically active products stored in the cytoplasmic granules, lipid derived

mediators and newly synthesized cytokines, chemokines, growth factors and other products (35). Preformed mediators present in mast cell granules are secreted when the membrane of the mast cells' cytoplasmic granules fuses with the plasma membrane in a process called degranulation (or, to distinguish it from "piecemeal" degranulation, it is also called anaphylactic degranulation or compound exocytosis) (36), releasing the granule content in the extracellular environment. Prestored mediators released from the granules include biogenic amines (histamine, serotonine) (37), several serine and other proteases (tryptase-α, -βI, -βII, -βIII, -γ, -δ, chymase-1, cathepsin G, granzyme B, and carboxypeptidase A3) (38-48), lysosomal enzymes (β-glucuronidase, β-hexosaminidase, arylsulfatase) (37), cytokines (TNF (49), bFGF (29), IL-4 (50), and SCF (51), (52) and proteoglycans (heparin) (53, 54), chondroitin sulfates (53)). These prestored mediators are encapsulated in an anionic gel matrix composed of chondroitin sulfates and heparin. Activated mast cells also release arachidonic acid metabolites (primarily) prostaglandin (PG) D2, platelet activating factor (PAF) and cysteinyl leukotrienes (LTCs) (55-57). The resulting signs and symptoms of preformed and lipid-derived mediators depend on the site of reaction. Their action on local nerves can induce vasodilation and erythema of the skin or conjunctiva. Increased vascular permeability results in tissue swelling and tear formation in the eyes. Bronchial smooth muscle contraction produces airflow obstruction and wheezing, while increased mucus secretion results in aggravated airflow blockage in the lower airways and runny nose. Stimulation of nociceptors of sensory nerves such as C-

group unmyelinated nerve fibres and type III group Aδ myelinated fibres of the nose, skin and airway that result in sneezing, coughing or itching (32). The local release of such mediators results in early-phase reactions whereas the rapid and systemic release of those from activated mast cells and basophils is associated with the pathology of anaphylaxis (58). The late phase response is mediated by inflammatory cell influx, predominately T lymphocytes, basophils and eosinophils (59).

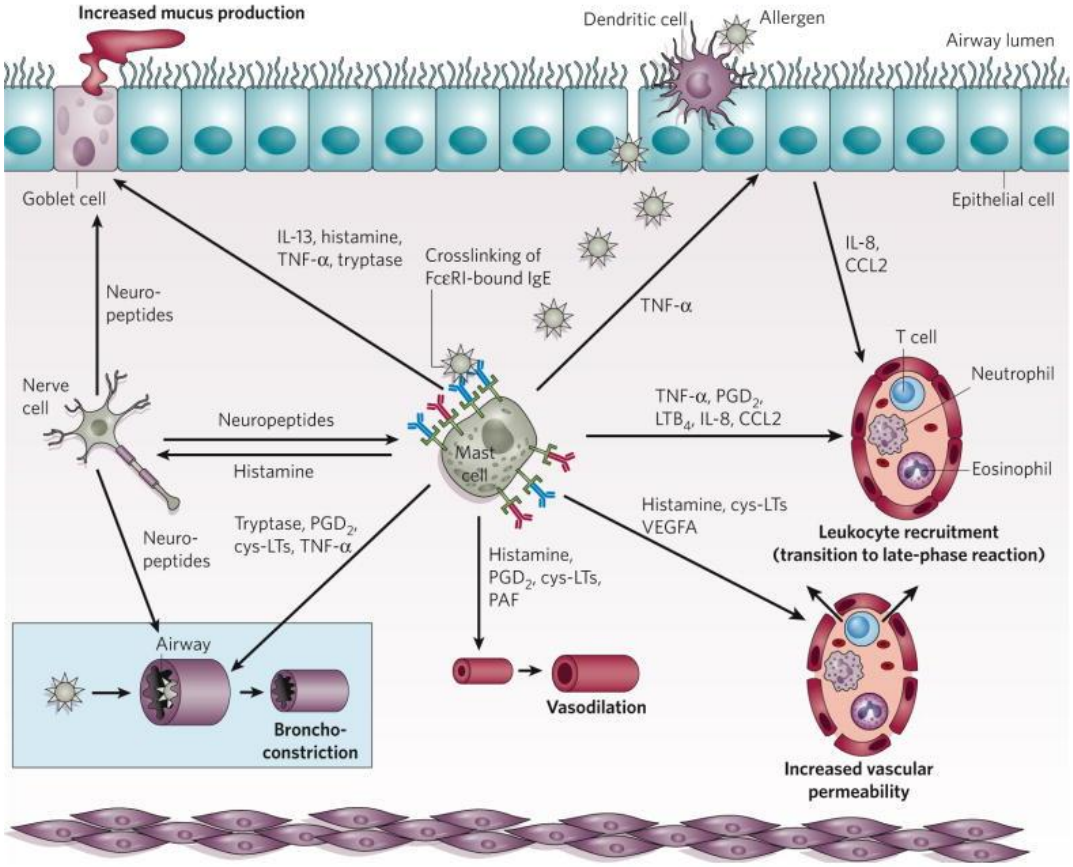
In humans, mast cells deriving from the skin and human airways have been shown to express IL-6, IL-8, TNF-α, IL-4, IL-5 and IL-13 cytokines (60), (61), (62), (63), (64) These cytokines expressed by human mast cells may contribute to the late phase allergic reaction by attracting inflammatory cells to the site of allergic response. (65) .However, unlike IL-4, IL-13 does not act on T cells (66), (67). IL-13, has several important roles in the allergic reaction such as induction of IgE synthesis by B cells and VCAM-1 expression on endothelial cells (68), (69). This suggests a pivotal role of mast cells in upregulating allergic inflammation (65).

Similarly, T cells after recognizing allergen-derived peptides release products such as IL-4, IL-13, IL-5 and TGB-β, that are also implicated in late-phase reactions (28). In chronic asthma, IL-5 released from Th2 cells promotes the survival of infiltrated eosinophils. Eosinophils directly affect airway narrowing via the release of basic proteins and lipid mediators and indirectly result in airway remodelling by releasing IL-4, IL-13 and TGF- β (70) (**Figure 1**). On the other hand, some mast cell

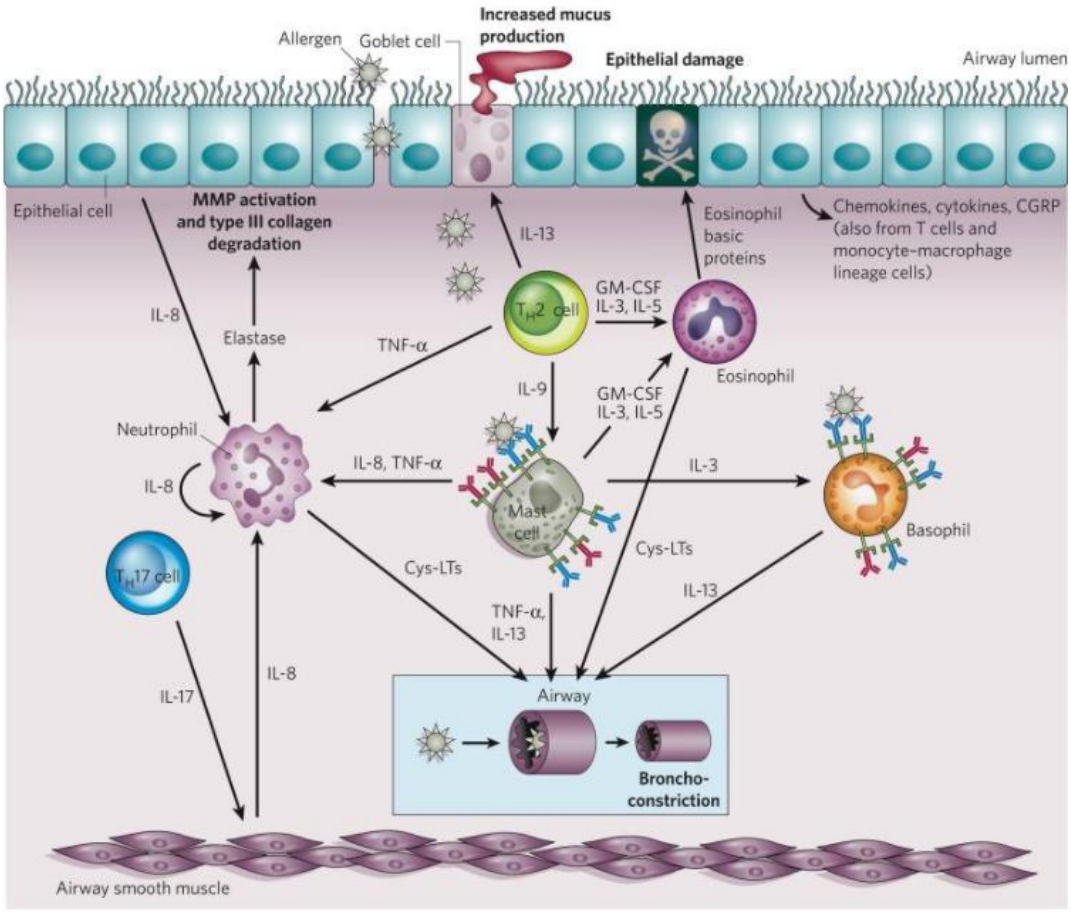
mediators including IL-10, TGF-β, IL-4 and histamine can potentially have anti-inflammatory activity (71).

In human skin, leukocytes such as eosinophils are attracted, and CD4+ T cells and basophils infiltrate the site. A similar pattern of cell accumulation is observed in asthmatic and nasal reactions, whilst basophils are not prominent in the lower airways (28). A late-phase reaction typically develops after 2-6 h and often reaches a peak 6-9 h after exposure to the allergen. However, it is not fully understood why not all sensitized subjects develop a late-phase reaction (72). Chronic inflammation is the result of a continuous or repetitive allergen exposure, with many innate and adaptive immune cells deriving from the blood at sites of allergen encounter. The persistent inflammation results in alterations in the structural cells at the affected sites, sometimes also affecting the function of the affected organs. However, there is no clear understanding how under the effect of continuous and/or several exposures to allergen, local tissue inflammation changes from early- and then from late-phase reactions to chronic allergic inflammation (32). For example, in patients with chronic asthma, inflammation affects all layers of the airway wall resulting in epithelial cell alterations such as increased number of mucus-producing goblet cells. Epithelial cells also produce an increased number of cytokines and chemokines. Increased epithelial cell proliferation results in thickening of the epithelium and increased lamina reticularis also known as subepithelial fibrosis, a condition often observed in patients with severe to moderate asthma (73). Inflammation of the submucosa is characterized by intensification of vascularity

and increased presence of eosinophils and mast cells. Eosinophils due to their immunomodulatory effects are involved in airway remodelling. Eosinophil-derived cytokines induce deposition of extracellular matrix proteins (such as fibronectin, and type I, III and V collagen) in the subepithelial basement membrane (BM). Moreover, eosinophils produce TGFβ1 which induces proliferation of fibroblasts and myofibroblast maturation. Both eosinophils and mast cells produce angiogenic factors (e.g. VEGF), promoting angiogenesis in the submucosa (74). In addition, the increased thickness of the muscular layer of the airways results in bronchoconstriction and more severe narrowing of the airway lumen (32). Another characteristic of chronic allergic inflammation of the airways is the tissue remodelling regulated by communication between the damaged epithelium and the underlying mesenchymal cells known as the epithelial mesenchymal trophic unit (EMTU) (75). In addition to asthma, tissue remodelling due to chronic allergic inflammation, also occurs in atopic dermatitis and allergic rhinitis. Characteristics of tissue remodelling are long-term alterations of the structure of the affected sites such as increased vascularity and impaired barrier function of the affected epithelia. In a number of patients suffering from allergic rhinitis, structural changes involve the development of nasal polyps, whereas upper airway impeded barrier function may result in chronic sinus infection (76). In atopic dermatitis skin barrier dysfunction results in increased risk of infections (77).



Early phase of allergen-induced airway inflammation



Late phase of allergen-induced airway inflammation

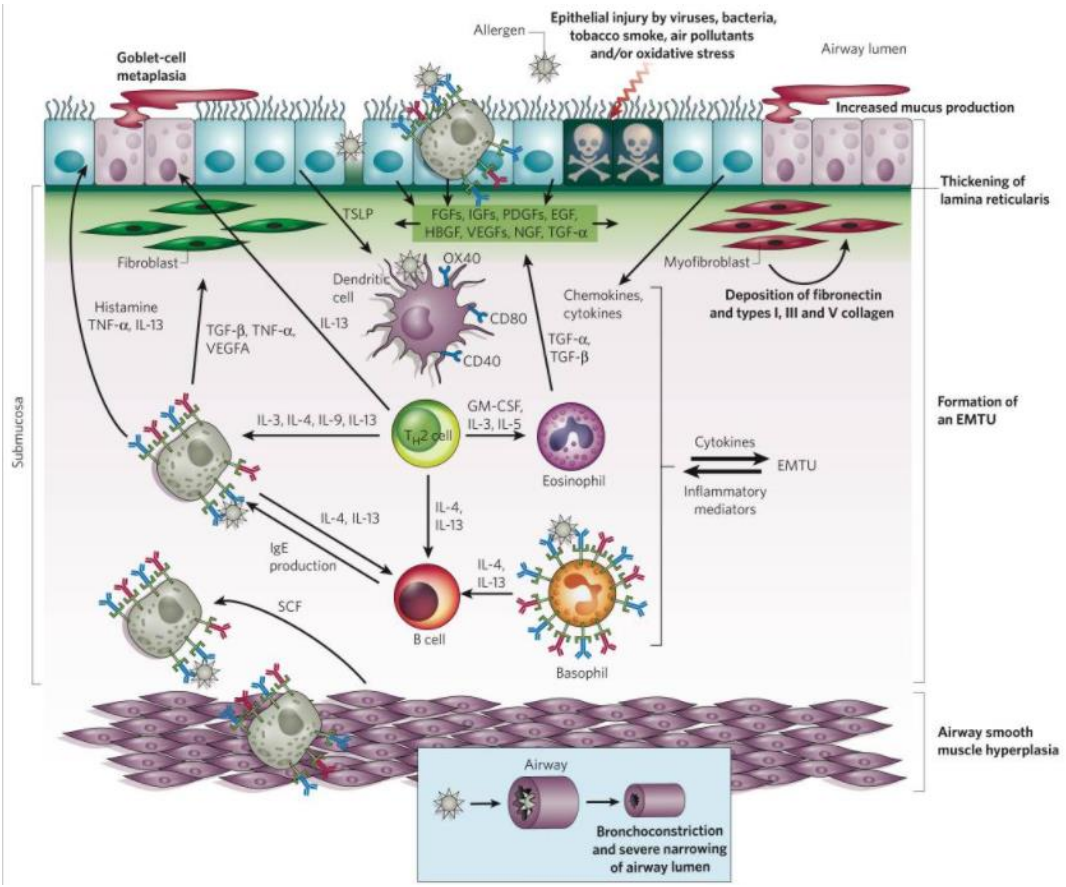


Figure 1. Phases of Type I immediate hypersensitivity reactions. (Adapted from Galli S. J. *et al*. (2008) (32).

Allergic diseases such as allergic rhinitis (hay fever), atopic dermatitis (also known as eczema), urticaria, food, drug, insect sting and bite hypersensitivity and in some cases fatal systemic allergic reaction, termed anaphylaxis, affect more than 25 % of the population in developed countries. These diseases are increasing in prevalence and they markedly add to the burden of healthcare costs (28, 78), therefore accurate identification of the culprit allergen is critical for targeted prescription of therapeutic interventions (79). Allergies also result in severe social

and economic impact, including costs of health care but also lowering of quality of life of affected individuals and their relatives (80). Allergies can develop in almost every organ. However, they are most frequently initiated in the skin and mucous membranes which consist the first line of defence between the human body and the external environment (81). The signs and symptoms vary from mild to life-threatening, depending on the nature of allergen (raw or cooked food), the type of allergen and the route of exposure. In case of food allergies, systemic symptoms include nausea, abdominal pain, diarrhoea, dyspnoea, wheezing, urticaria, angioedema, hypotension, and shock (58). In allergic rhinitis, also called hay fever, common coldlike symptoms are observed such as nasal congestion, rhinorrhoea, repeated sneezing, and nasal itching as well as watery, itchy, red eyes, headaches and general feeling of discomfort (82). However, in rare cases, allergy can lead to lifethreatening allergic reaction, called anaphylaxis. It is a systemic reaction involving multiple organ systems, which usually develops only a few minutes after exposure to a culprit allergen (83). Most allergens are proteins (some are carbohydrates or lipids) and several are proteases such as the house-dust-mite allergen, Der p 1. Proteases can compromise epithelial barrier function (84) or hydrolyse substrates mediating the development of Th2-cell responses (85). Exogenous proteases (from mites and moulds) augment IgE production to allergens and promote infiltration of eosinophils, basophils, neutrophils, monocytes, and lymphocytes, thus inducing allergic inflammation, even in the absence of IgE (86). Allergens can be found in a variety of sources, such as grass

and tree pollen, dust mites, animal dander, food, insect bites, stings and medicines. However, the majority of those allergens are commonly found in the environment and are generally harmless to non-susceptible individuals (53). 9 The complete aetiology of allergic diseases is not yet fully understood (87). It is a complex interplay between environmental and genetic factors. Among others, presumable parameters include the environmental pollution and irritants (gas, smoke, etc.), changes in diet, stress and the "hygiene hypothesis" leading to reduced exposure to infections and to other pathogenic and non-pathogenic microorganisms with increased use of antibiotics (32, 87, 88). Genetic predisposition counts for a large number of individuals who show elevated immunoglobulin E (IgE) responses to otherwise harmless antigens (allergens) commonly described as atopy (89).

1.2. Allergy mechanism

1.2.1. Immunoglobulin E (IgE)

In 1921, Prausnitz and Küstner were the first to demonstrate the existence of a blood factor able to impose sensitivity to allergens (90). Around 1966, Kimishige and Teruko Ishizaka and later Gunnar Johansson and Hans Bennich described it as a new class of antibody carrying reaginic activity. In 1968, after general agreement it was named immunoglobulin E (IgE) protein (91). IgE is one of the five classes of immunoglobulins (IgM, IgG, IgD, IgA, IgE). In addition to its function in the pathogenesis of allergic diseases, it also provides immunity to parasites, tumours and also protection against venoms (92). It is the least abundant antibody isotype in plasma (~100 ng/mL), compared to other classes of immunoglobulins such as

IgG (5-10 mg/mL) and has the shortest half-life (~2 days) (24). In non-susceptible individuals, IgE levels in cord blood are low (<2 kIU/L; <4.8 mg/L), gradually increasing during childhood, reaching a peak at 10-15 years of age, while decreasing during adulthood. Total IgE levels are also determined by the host's genotype, race, environmental factors and the immune status (93).

Elevated IgE levels are also detected in other disorders, including parasitic infections (e.g schistosomiasis, ascariasis), nonparasitic infections (e.g CMV, HIV), hematologic malignancies (e.g Hodgkin lymphoma and IgE myeloma), inflammatory diseases (e.g Kawasaki disease and Kimura disease), cutaneous diseases (e.g Netherton syndrome), cystic fibrosis, nephrotic syndrome and primary immunodeficiency diseases (e.g hyper-IgE syndrome, immune dysregulation, Omenn syndrome, enteropathy). Increased IgE levels are also seen after hematopoietic stem cell transplantation, particularly in male smokers and in alcoholism (94).

IgE antibodies are tetramers, composed of two heavy (ε) and two light chains (κ or λ) linked by disulfide bonds. At the N-termini of the heavy (VH) and light (VL) chains, variable (V) sequences create unique antigen-specific binding sites (Fab region). The C-terminal end of the ε-heavy chains consists of 4 Cε domains, encoded by the Cε1 to Cε4 exons located close the 3' end of the immunoglobulin's heavy chain locus. The Fc domains Cε2-4 consist the binding sites to its receptors, FcεRI and CD23. Unlike Fcɣ and Fcμ receptors, Fcε does not activate complement. The Cε3 domain mediates contact with the high-affinity IgE receptor α-chain (95). Cε2

region in IgE and IgM molecules replace the ‘‘hinge’’ region present near the centre of the molecule in other immunoglobulins such as IgG (96), (97). Based on its receptor bound configuration, this provides the required flexibility to the molecule so there can be a sharp turn in the Cε2-3 region, with the Cε2 domains folding back over Cε3-4 (98). IgE antibodies are more heavily glycosylated than other antibody isotypes, containing 7 N-linked glycosylation consensus sequences (N-X-S/T) on each heavy chain, one (at N394 in Cε3) that is necessary for binding to the high-affinity IgE receptor, FcεRI. The heavy glycosylation of IgE antibodies results in the molecule’s high affinity for galectins, lectin-type proteins capable of interacting with both free and cell-bound IgE.

Although IgG antibodies have a half-life of about 3 weeks, IgE antibodies, as mentioned before, have a shorter half-life in plasma (∼2-3 days) (24). However, receptor-bound IgE can remain bound to mast cells in tissues for weeks or even months. This in turn can have significant clinical implications. For example, mast cell–fixed IgE in donor organ can induce peanut reactions in non-sensitised organ transplant recipients (99).

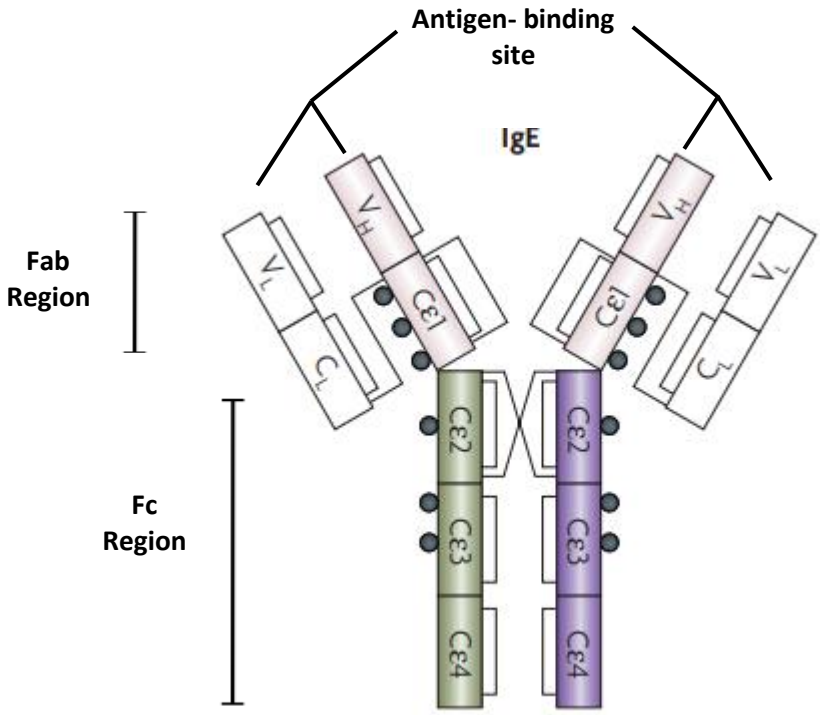


Figure 2. Structure of IgE molecule. Figure adapted from Gould *et al*., 2008 (31). Schematic representation of the domain structure of human IgE. V: variable region, C: constant region, H, Cε1-4: Heavy chain, L: light chain

Antigens express on their surface two types of epitopes, called sequential or conformational. Antibody binding to sequential linear epitopes exists in denatured proteins as well as in small peptide fragments. On the other hand, in conformational epitopes, the binding of antibodies relies on proper arrangement of the three-dimensional shape or tertiary structure of an antigen. As such, a discontinuous stretch of amino acids is brought together during protein folding to form an antibody binding site (100). In airborne allergens, most epitopes are conformational as these proteins are not subjected to transformation processes and digestion similar to food (25). Nonetheless, conformational epitopes are also

present in food allergens if sensitization prevails to intact or partially digested allergens (26).

1.2.2. IgE receptors

The biological functions of IgE antibodies are facilitated by their interactions with specific antigens and two structurally different receptors, the FcεRI and FcεRII/CD23 expressed on various effector cells (101).

1.2.2.1 FcεRI, the high-affinity IgE receptor

FcεRI is defined as the high affinity receptor for IgE. It is expressed in abundance (200,000-600,000 molecules/cell) on mast cells and basophils and at much lower levels on platelets, eosinophils, monocytes Langerhans and dendritic cells, and is therefore more difficult to detect. For example, monocytes from normal (nonatopic) individuals express ~3000 surface FcεRI molecules per cell (102). The IgE high-affinity receptor (FcεRI) is expressed as a tetrameric complex ($\alpha\beta\gamma_2$) on basophils and mast cells (**Figure 3**), and as a trimeric complex ($\alpha\gamma_2$) on eosinophils, platelets, monocytes, peripheral blood dendritic cells (DCs) and cutaneous Langerhans cells at levels ranging between 10- to 100-fold lower than the tetrameric one (99, 103-105). FcεRI shares a number of common characteristics with other Fc receptors (106, 107). The immunoglobulin-binding chain of FcγRIIa shares similar homology with the αsubunit from FcεRI (108-110). FcγRIIa from macrophages share identical γ-chains with FcεRI present on mast cells (111). The α-chain is a member of the immunoglobulin superfamily and contains two

extracellular immunoglobulin domains to which the Fc region (Cε3 domain) of IgE binds. It includes a transmembrane domain and a short cytoplasmic tail (101). It is also N-glycosylated at seven different sites, a process highly important for efficient interaction between the α-chain and the folding machinery present in the endoplasmic reticulum (ER) (112). The β- and γsubunits are not involved in ligand binding. The β-subunit contains 4 transmembrane-spanning segments, with both N- and C- terminal ends present on the cytosolic side of the plasma membrane. The ɣ-chains express a transmembrane disulfide-linked domain. Both β- and ɣ-chains possess intracellular immunoreceptor tyrosine-based activation motifs (ITAMs), 18 amino acid tyrosinecontaining sequences acting as binding sites for Src homology 2 (SH2) domaincontaining proteins when phosphorylated, including the signalling protein tyrosine kinase SYK (spleen tyrosine kinase) mediating FcεRI signalling. IgE antibodies stabilize FcεRI at the cell surface thus, regulate the density of their high-affinity receptor (113-118). FcεRI has a high affinity for IgE, (dissociation constant [Kd] 10- 9 mol/L), therefore under physiologic conditions, once IgE binds to it remains strongly attached until internalization (119). However, in immediate hypersensitivity reactions crosslinking of IgE/ FcεRI complex by a matching allergen triggers a cascade of signalling events, resulting in mediator release and gene transcription (120). Crosslinking of neighbouring FcεRI receptors result in aggregation and phosphorylation of β- and ɣ-chain ITAMs present in the cytosol by Lyn tyrosine kinase. These ITAM tyrosines provide binding sites for the SH2- containing SYK protein tyrosine kinase. Once SYK is activated, it

phosphorylates tyrosines in adapter molecules such as LAT1/2, SLP-76, and Grb2, leading to the formation of a supramolecular signalling complex expressed on plasma membrane. Downstream signalling events induce increase in cytosolic calcium, transcription of genes (IL-4, TNF, and IL-6), synthesis of prostaglandins and leukotrienes and granule fusion with the plasma membrane leading to release of preformed mediators such as histamine, proteases and proteoglycans. In turn, these mediators induce vasodilation, plasma extravasation, mucus production, tissue oedema, and smooth muscle constriction (99). IgE-dependent cell-surface density of FcεRI is a strong factor of signalling threshold. For example, omalizumab, a monoclonal anti-IgE antibody that binds to the Fc domain of circulating IgE, indirectly results in downregulation of FcεRI by preventing IgE binding to the high affinity receptor, possibly accounts for the majority of the clinical benefits of IgE obstruction (121). SYK downregulation is likely responsible for reduced sensitivity of FcεRI signaling, after repeated activation events (122). Similarly, parallel signalling of IgG antibodies with the same allergens as FcεRI, can also impair the strength of IgE signal. Interaction of IgG antibodies with the inhibitory IgG receptor (FcgRIIb), initiates negative signalling pathways emerging from receptor-linked protein tyrosine phosphatases and inositol phosphatases. Reduced allergen sensitivity occurring after subcutaneous immunotherapy in aeroallergen-sensitive patients and food-allergy patients completing oral immunotherapy might result from the strong inhibitory IgG responses generated by these therapies (123, 124).

1.2.2.2 FcεRII/CD23, the low-affinity IgE receptor

The FcεRII/CD23 or else the low-affinity receptor, strongly binds IgE with an association constant (KA) of 10^8 mol^{-1} (125). Two isoforms of the protein are encoded by RNA splice variants, the CD23a expressed mainly on B cells and CD23b expressed on monocytes, Langerhans cells, follicular dendritic cells, macrophages, eosinophils, and gastrointestinal and respiratory epithelial cells. CD23, unlike FcεRI and the other FcRs, is not a member of Ig superfamily. It is related to the C-type lectin-like domain superfamily, which includes several carbohydrate pattern recognition receptors (PRRs) and adhesion molecules (126). CD23 is a type II transmembrane protein (N-terminus intracellular) with an IgE-binding C-type (calcium-dependent) lectin domain at the distal, C-terminal end of the extracellular sequence. It attaches to IgE-binding domains with alpha-helical coiled-coil stalks terminating in IgE-binding C-type lectin heads which mediate multimerization, and only oligomeric CD23 binds IgE (127). Apart from serving as a receptor for IgE, it also binds a second ligand in humans the CD21 receptor expressed on B-cells (128). Like FcεRI, IgE regulates CD23 surface levels. Cleavage of vulnerable sites proximal to C-type lectin heads by proteases (Der p 1 protease of dust mites and disintegrin and metalloproteinases (ADAMs) proteases, especially ADAM10) is hindered by IgE binding to the receptor (129). On intestinal epithelial cells, CD23 plays an important role in transferring food antigen–IgE complexes from the gut lumen into the mucosa. In antigen-presenting cells (APCs), it facilitates the processing and presentation of allergen–IgE complexes to T-cells. Binding of CD23 on IgE-secreting

B cells inhibits IgE synthesis. Overexpression of CD23 in transgenic mice resulted in suppression of IgE responses, whereas CD23-deficient mice showed higher specific IgE titres after immunization (99).

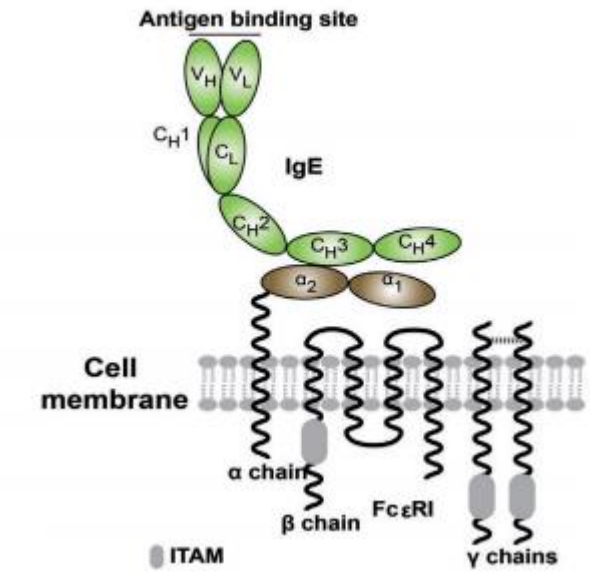


Figure 3. Tetrameric structure of the FcεRI complex on basophils and mast cells. Figure used from Hellman, L. T *et al*., 2017 (130).

1.2.2.3 Regulation of human FcεRI cell-surface expression

While rodents only express the tetrameric $\alpha\beta\gamma_2$ structure of FcεRI on basophils and mast cells, humans also express the trimeric form $\alpha\gamma_2$ on monocytes, eosinophils, platelets, peripheral blood dendritic cells and Langerhans cells (101). Human platelets and neutrophils also express the FcεRI, however with unclear subunit composition (131, 132). Binding of IgE to cell-surface FcεRI initiates a number of events leading to allergic reactions, thus the amount of FcεRI receptors expressed on the cell surface regulates the number of allergen-specific IgE (sIgE) and

therefore allergens that gain access to effector cells to start an allergic reaction (133). FcεRI expression is modulated by specific receptor components and the availability of IgE which affects receptor loss from the cell surface, however, it is not until there is a substantial decrease in occupied receptors, outnumbered by unoccupied, that FcεRI expression begins to fall (134). Occupation of FcεRI by IgE also prevents endocytosis of the receptor (135) suggesting that a linear relationship exists with increasing FcεRI expression as the level of serum IgE increase (113). On mast cells, the level of surface expression of FcεRI could be upregulated (~32-fold) after in vitro incubation with IgE or injection of IgE in vivo. IgE-dependent FcεRI upregulation could also be observed on human mast cells and basophils and mouse basophils (101). FcεRI upregulation results only from monomeric IgE as dimeric or higher oligomeric IgE inhibit upregulation (134). Oppositely, elimination of IgE during culture of human basophils resulted in downregulation of FcεRI expression (134). Consequently, therapeutic approaches targeted the regulation of FcεRI expression by serum IgE such as the anti-IgE monoclonal antibody (omalizumab) to treat allergic asthma (136).

Non-covalent interactions of the various subunits of FcεRI maintain the structural integrity of the receptor (137). Several factors affect FcεRI expression on the cell surface such as synthesis of FcεRI α-, β- and γ-chains and free IgE levels (138). The human α-chain when expressed alone is retained in the endoplasmic reticulum (ER) as a dilysine motif positioned at the cytoplasmic C terminus of the FcεRI α-chain, acts as a retrieval signal effectively directing localization of this subunit to the ER

(139). In the ER it undergoes signal peptide cleavage and N-glycosylation with endo-H sensitive mannose rich sugars. Co-translational assembly with β- and γ-chains promote its insertion in the ER, signal peptide cleavage, N-glycosylation and export from the ER (137). Disulfide bond formation and core glycosylation occur in the lumen of the endoplasmic reticulum (ER) which are necessary to achieve proper folding (112, 140). In humans, association only with γ-chain is sufficient to mask the ER retention signals, allowing the $\alpha\beta\gamma_2$ and/or $\alpha\gamma_2$ complex to be exported to the Golgi compartment for terminal glycosylation and from there, to the plasma membrane. In the absence of γ-chain, the immature and core glycosylated α-chain accumulates in the endoplasmic reticulum (101, 140, 141). However, in rodents assembly of α-chain with both β and γ chains is obligatory for masking ER retention signals (142). Co-expression of the β-chain is necessary for cell-surface expression of FcεRI, suggesting the existence of additional ER retention signal(s) in murine α-chain that are masked after assembly with the FcεRI β-chain (143). The full-length FcεRI β-chain acts as an amplifier for γ-chain signaling and possibly stabilizes the FcεRI complex on the cell surface (144) FcεRIα expression in human mast cells and antigen-presenting cells (APCs) is induced by the T helper 2 (Th2)-cell-derived cytokine interleukin-4 (IL-4) (141, 145, 146). On cultured human mast cells, the presence of IL-4 increased surface expression of FcεRI and stimulated activation signals in the cells resulting in histamine and β-hexosaminidase release (147). Similarly, IL-4 promoted FcεRI α-chain mRNA expression in human eosinophils (148) indicating the presence of an analogous machinery in the production of

FcεRIα mRNA between eosinophils and mast cells (147). FcεRIα mRNA expression is also increased in human bronchial and tracheal smooth muscle (B/TSM) cells after incubation with IL-4 (149). In addition, increased intracellular FcεRIα expression was induced in human CD1a+ LC/DC cells cultured in the presence of IL-4 (146). Consequently, the presence of the Th2 cytokine IL-4 is crucial for FcεRI expression by human cells suggesting a new crucial role of IL-4 besides stimulation of IgE production by B cells (133).

1.2.3. Mast cells and basophils

1.2.3.1. Mast cells

Mast cells are tissue-based inflammatory cells deriving from haematopoietic stem cells. They circulate as immature progenitors and migrate into vascularized tissues, where they complete their maturation (131-134). Mature mast cells are normally found close to epithelia, blood vessels, nerves, in the airways and gastrointestinal tract, as well as near smooth muscle cells and mucus-producing glands (150-153). Mast cells are ovoid or irregularly elongated in tissues, up to 20 µm diameter with an ovate nucleus containing in the cytoplasm metachromatic granules. The presence of sulfated proteoglycans such as heparin and chondroitin sulfates in the granules results in the metachromatic granule staining (154).

In humans, two main types of mast cells have been identified according to the secretory protease content designated as MC_T or MC_{TC} based on the presence of tryptase with or without chymase. MC_{TC} cells contain tryptase and chymase as well as carboxypeptidase and cathepsin G, whereas MC_T cells contain only tryptase.

Thus, tryptase staining is used to identify all mast cells in tissues and consists the principal method of visualizing mast cells. While MC_{TC} cells predominate in skin and the respiratory and gastrointestinal submucosa, cells of the MC_T subset are more abundant at mucosal surfaces (155). Mast cells show unique growth requirements as they remain differentiated and viable in c-kit ligand, also known as stem cell factor (SCF) (151). Depending on their tissue source, state of differentiation, and conditions of culture, they express a variety of surface receptors (154). Human resting mast cells express the high-affinity IgE receptor (FcεRI) and FcγRIIb (CD32) and the high-affinity receptor for IgG (FcγRI, CD64) in the presence of interferon (IFN)-γ (156). MC_{TC} cells which are the exclusive mast cell type in skin, express C3a and C5a receptors, and can be activated by these anaphylatoxins (C3a and C5a) leading to degranulation and production of bioactive lipids, cytokines, and chemokines (157). Similarly, different chemokines are expressed depending on the mast cell type, such as CCR3, CXCR1, CXCR3, and CXCR4 that were found to be highly expressed by human lung mast cells (LMCs). Likewise, chemokines CCR1 and CCR4 were found to be expressed by cord blood-derived mast cells and LMCs from patients with allergic asthma (158) . MCs also express IL-3R, IL-4R, IL-5R, IL-9R, IL-10R, GM-CSFR, IFNγR, and toll-like receptors (TLRs) (156). In tissues, mast cell survival, maturation and biologic expression is influenced by cytokines such as IL-4, IL-5, and IFN-γ (159-163). In human mast cells, IL-4 has been reported to promote both maturation and apoptosis in the presence of IL-6 (164). IL-5 induces mast-cell proliferation in the presence of SCF, whereas IFN-γ exposure upregulates

FcγRI on the mast cell membrane (162, 163). Mast cells express the FcεRI receptor ($\alpha\beta\gamma_2$), aggregation of which results to mast cell activation, granule exocytosis, and mediator release. Activation of mast cells can also result by nerve growth factor (NGF) through TRKA (165), and IgG through FcγRI (166). Mast cells are also activated by TLR ligands (167- 170). Mast cells upon activation release mediators that are divided in three categories: preformed mediators, newly synthesized lipid mediators, and cytokines. The preformed mediators are densely packed within secretory granules. After cell activation, the granule content is released in the extracellular environment within minutes. The main granule constituents are histamine, serine proteases, carboxypeptidase A, and proteoglycans (heparin and chondroitin sulfate E) (171). Approximately 2 to 5 pg of histamine is present in individual human mast cells (172). Histamine in granules is present in ionic association with acidic residues of the glycosaminoglycan side chains of heparin and chondroitin sulfate E. Dissociation from these glycosaminoglycans in extracellular fluids is performed by exchanging with sodium ions (173). Histamine actions are mediated through three distinct receptors. The most important histamine effects are mediated through the H1 receptor and they include smooth muscle contraction, increased vascular permeability, pruritus, prostaglandin generation, reduction in atrioventricular node conduction time resulting in tachycardia, stimulation of vagal reflexes, and increased cyclic guanosine monophosphate. The effects mediated by the H2 receptor include gastric acid secretion, oesophageal contraction, enhanced lower airway mucus secretion,

increased cyclic adenosine monophosphate, inhibition of basophils but not mast cells, histamine release, inhibition of neutrophil activation, and activation of suppressor T cells. However, several effects are mediated through combined effect of H1/H2-receptors such as hypotension, flushing, and headache, vasodilation-related tachycardia and catecholamine secretion. The third histamine receptor, located in neural tissue, acts by inducing a negative feedback on histamine synthesis (174). Heparin and chondroitin sulfate proteoglycans are thought to mediate storage of preformed molecules that dissociate from these proteoglycans in physiologic buffer solution (93). Most of the protein in mast cell granules is composed of four neutral proteases: chymase, tryptase, carboxypeptidase, and cathepsin G. Tryptase tetramers are stabilized after interaction with heparin (175). *In vitro* tryptase can cleave C3 and C3a (176), activate fibroblasts (177), and promote accumulation of inflammatory cells (178). Prostaglandin D2 (PGD2), the major cyclooxygenase product, and the lipoxygenase product leukotriene (LT) C4 are the main lipid mediators synthesized by mast cells. The active metabolites LTD4 and LTE4 derive from extracellular peptidolytic processing of LTC4. Skin mast cells produce more PGD2 than LTC4, compared to lung mast cells. PGD2 and LTC4, LTD4, and LTE4 are bronchoconstrictors (179-182) while LTC4, LTD4, and LTE4 also enhance vascular permeability (183).

Human mast cells secrete and synthesize various cytokines. A major cytokine stored and synthesized after human mast cell activation is TNF-α (184-187). TNF-α induces upregulation of endothelial and epithelial adhesion molecules (188, 189),

stimulates bronchial responsiveness (190) and enhances antitumor effects (191, 192). In 1992, Bradding P. *et al*. (193) and others (194), (195) supported that purified human lung mast cells and mast cells located in the conjunctival submucosa have the capability to synthesize IL-4. Controversially, Ochi H. *et al*.(2000) and other groups, did not confirm IL-4 production by human mast cells (196), (197). This indicates that several mast cell released cytokines including IL-6 and IL-13, are susceptible to degradation by released by mast cell proteases which also likely degrade the IL-4 they release (198), (199). IL-3, GM-CSF, and IL-5, are critical cytokines for eosinophil development and survival (200). Human mast cells also produce IL-6 (201), IL-8 (202) and IL-16 (203). They also produce chemokines such as macrophage inflammatory protein (MIP) 1α (204), and the chemokines lymphotactin which may contribute to the recruitment of lymphocytes to areas of allergic inflammation 205).

1.2.3.2. Basophils

Basophils, similar to mast cells, express the FcεRI receptor, secrete Th2 cytokines and release histamine upon activation but account for a distinct lineage expressing unique features (206). A characteristic feature of basophils is the rapid production of the regulatory cytokines IL-4 (207, 208-211). Morphologically, basophils are 5–8 μm in diameter, contain a segmented nucleus and can be identified by staining with basic dyes (206), such as toluidine blue or Alcian blue 93). Compared to mast cells they contain fewer but larger granules (212). Basophils express a variety of

cytokine receptors (e.g. IL-3R (213), IL-5R (214), and GM-CSFR (213) chemokine receptors such as CCR3 (215), complement receptors (CD11b, CD11c, CD35, and CD88 (216), prostaglandin receptors (CRTH2) (217), immunoglobulin Fc receptors (FcεRI (113) and FcγRIIA and FcγRIIB (218, 219), and TLRs (220, 221).

Basophils similar to mast cells, neutrophils, eosinophils, and monocytes are thought to develop from CD34+ progenitors found in the bone marrow as well as in peripheral and cord blood (206). They differentiate and mature in the bone marrow, and are released as mature cells in the peripheral blood where they constitute <1% of peripheral blood leukocytes (222). Basophils have a short half-life of few days (223) whereas mast cells are long-lived cells that can survive for several months (93). IL-3 is the dominant cytokine for successful growth and differentiation of basophils (224, 225), whereas mast cells develop under the presence of stem cell factor (SCF) (226). Similar to IL-3, a number of growth factors exist for basophils such as interleukin (IL)-5 (227), the granulocyte-macrophage colony-stimulating factor (GM-CSF) (228), transforming growth factor-b (TGF-b), and nerve growth factor (NGF) (229). Mature basophils compared to mast cells exclusively express on their surface IL-3 a chain (CD123, found on basophils and other cells but not on mast cells), while mast cells selectively express SCF receptor (c-kit/CD117, weakly or not expressed on mature basophils) (206).

The mast cells which are found in the tissues, close to small blood vessels and postcapillary venules are important effector cells in the early phase of IgE-mediated allergic reactions (218), whereas basophils, eosinophils, and Th2

lymphocytes are attracted to the site of inflammation during allergic late-phase reactions (LPRs). Lin *et al*. and co-workers (230) tried to elucidate whether basophils might be responsible for acute allergic and anaphylactic reactions. After performing an emergency department-based anaphylaxis study, they observed in almost 50% of the patients administered to the emergency department lack of total tryptase elevations (which is produced by mast cells and not basophils (231) while they had increased plasma histamine levels suggesting basophil involvement. However, Simons *et al*. reported that several factors might be implicated in total tryptase levels and a more sensitive mast cell activation marker for anaphylaxis such as mature β-tryptase is needed (232).

As basophils express on their surface a complete FcεRI ($\alpha\beta\gamma_2$), crosslinking of IgE bound to FcεRI by corresponding antigens (allergens) results in basophil activation. Tissue influx and basophil activation can also result from the action of hematopoietic cytokines such as IL-3, IL-5, and GM-CSF. In addition, these cytokines induce mediator release after IgE-dependent stimulation or activation due to platelet-activating factor (PAF), C5a, and C3a (206). Basophil activation also results from TLR2 and TLR4 expressed on basophils, and activation results in IL-4 and IL-13 secretion in response to IgE and IgE-independent responses (220, 233). Similarly, IL-33, a member of the IL-1 superfamily, induces basophil activation through the ST2 receptor promoting IL-4 and IL-13 expression and resulting in IgE mediated degranulation (234, 235).

Priming basophils with IL-3, IL-5, GM-CSF, and the neurotrophic cytokine NGF can almost equally stimulate IgE-mediated release of histamine and LTC4, with IL-3 being the most potent (IL-3>NGF>IL-S>GMCSF) (236). The continuous production of LTC4 and cytokines from basophils possibly plays an important role in chronic allergic inflammatory diseases, such as asthma. Notably, IL-3 in conjunction with C5a, induces mediator release independently of IgE-receptor cross-linking. Thus, basophils may play a role in non-allergic inflammatory diseases exacerbated by bacterial infections or IgG immune complexes (206). Interestingly, C5a can induce histamine release after binding to the C5a receptor, CD88 (216). On the contrary, most human mast cell subtypes except for skin mast cells do not express CD88 and do not respond to C5a (216). Mature human mast cells also do not express receptors for IL-3 and GM-CSF (237). These findings suggest that basophils compared to mast cells are more prone to the effects of pro-inflammatory factors and immunomodulatory cytokines, likely more abundant in LPRs and other (non-allergic) inflammatory diseases (206).

1.2.4. IgE-mediated mast cell and basophil activation

As described earlier (1.2.3) both mast cell and basophil precursors are generated in the bone marrow. However, although immature basophils differentiate and mature in the bone marrow, immature mast cells circulate in the blood to tissues where they mature and reside (150-153). Mast cell activation and mediator release

is central to the pathogenesis of allergic diseases including allergic rhinitis, allergic asthma and anaphylaxis (238).

Mast cell products can both initiate an immediate hypersensitivity reaction (or else early-phase) and contribute to a late-phase reaction (238). The immediate phase reaction occurs within minutes of FcεRI crosslinking (32). Pre-formed mediators present in the granules and newly synthesized mediators released during this phase include histamine, serotonin, proteases such as tryptase, chymase and/or carboxypeptidase A3 and proteoglycans (heparin and/or chondroitin sulfates), as well as

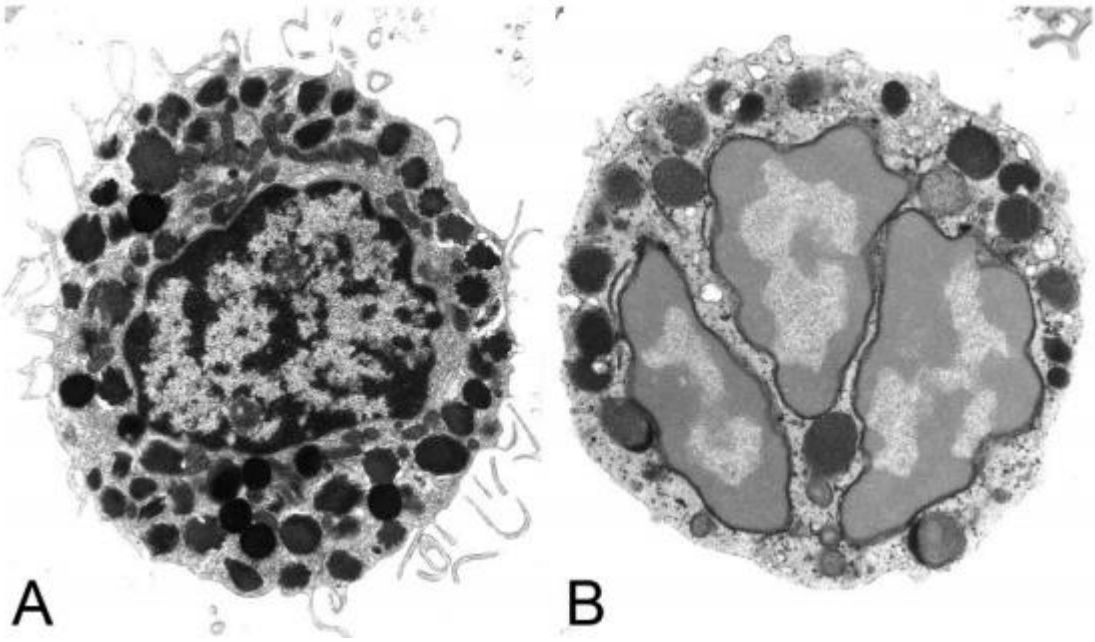


Figure 4. Ultrastructure of human mast cells (HMCs) (A) and human basophils (HBs) (B). Human lung mast cells (HLMCs) and human basophils isolated from the peripheral blood show electron-dense secretory granules. (A) x 10,000; (B) x 17,000. Figure used from Dvorak, A. M. *et al*., 2005 (36).

newly formed lipid-derived mediators, for example, PGD2, LTB4, LTC4, LTD4 and LTE4 and certain cytokines are released by mast cells within minutes of antigen exposure (238).

Late-phase reactions peak 6 to 12 hours following exposure to a matching allergen (239) and are associated with cytokine and chemokine production (240) released in part from eosinophils, neutrophils and basophils that have entered the inflammatory site following the immediate reaction (241). Mast cells are also involved in chronic allergic inflammation, a classical example of which is the chronic asthma (242).

In sensitized individuals, mast cells and basophils already express allergen-specific IgE bound to their surface high-affinity IgE receptors (FcεRI). Crosslinking of the IgE–FcεRI complex on mast cell and basophils by a matching allergen, triggers intracellular signaling events and cell activation. However, several studies have shown that IgE alone, the so-called “highly cytokinergic IgE”, can induce FcεRI aggregation without crosslinking by antigen resulting in mast cell activation and survival (243). Highly cytokinergic IgEs do not promote DNA synthesis but render

mast cells resistant to apoptosis induced by growth factor deprivation. Therefore, the ability of IgE to promote mast cell survival and FcεRI expression may result in amplified allergic reactions (244-246).

These molecules, although shown to be monomeric in solution they form clusters on the cell membrane capable to crosslink FcεRI and activate mast cells (133).

In activated mast cells and basophils, preformed mediators present in cytoplasmic granules including histamine, serine proteases (tryptase and chymase), carboxypeptidase A, and proteoglycans (**Figure 4**) fuse with the plasma membrane in a process known as anaphylactic degranulation (**Figure 5**) (36).

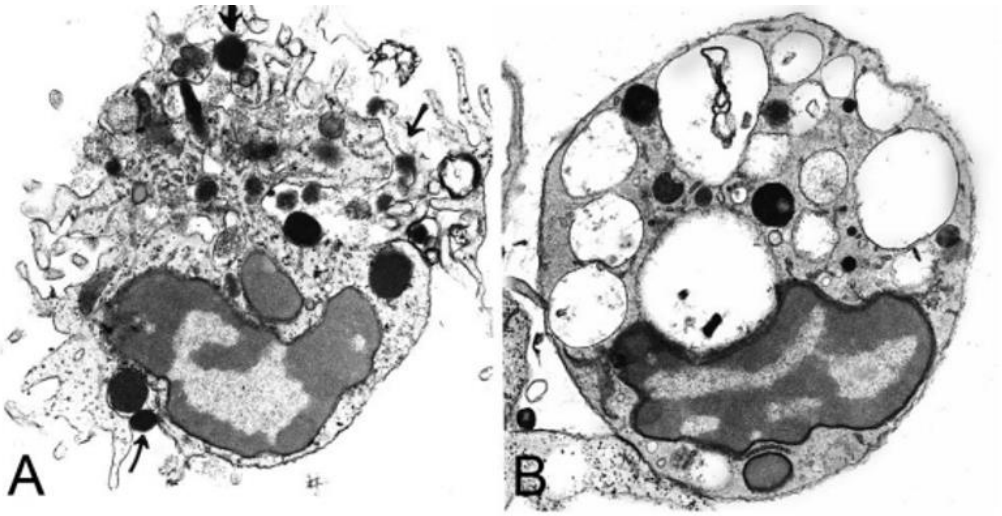


Figure 5. Secretory patterns from HBs (A, B). Images show peripheral blood basophils stimulated with formyl methionyl leucyl phenylalanine (FMLP). After 20 seconds, secretory granules fuse with the cell membrane (arrows) with subsequent amplification of surface architecture, characteristic of anaphylactic degradation (AND) (A). (B) Empty granules and smooth surface architecture as observed in piecemeal degranulation (PMD). (A) x 12,500; (B) x 17,000. Figure used from Dvorak, A. M. *et al*., 2005 (36).

The local release of preformed mediators such as histamine and chemotactic factors contribute to the acute signs and symptoms associated with early-phase reactions. Histamine can initiate all or most of the pathologic processes involved in allergic asthma, rhinitis, urticarial and anaphylaxis. These signs and symptoms usually include vasodilation (effect of mediators on local nerves and producing erythema (reddening) of the skin), increased vascular permeability in nearby blood vessels (tissue swelling), smooth muscle contraction (airflow obstruction and wheezing), and increased mucus secretion (airflow obstruction in the lower airways and a runny nose). Histamine can also stimulate release of substance P from sensory nerve fibres, leading to histamine-induced vasodilation and secretion of neurokinin A and calcitonin gene-related peptide, contributing to the wheal and flare reactions in skin reaction (247). Some of the secreted mediators (e.g. histamine, PGD2 and LTC4) also promote recruitment of inflammatory cells to the local tissues, resulting in the development of late-phase reaction (151, 248).

Initially, histamine was considered the dominant mediator of anaphylaxis. Now it is known that other mediators are also involved such as neutral proteases, PGD2, Cysteinyl leukotrienes (CysLTs), Platelet-activating factor (PAF), cytokines and chemokines (IL-4, TNF-a, (249). Anaphylaxis is a severe life-threatening acute reaction. Symptoms such as air blockage due to smooth muscle contraction in the respiratory tract and the formation of oedema provoke asphyxiation and cardiovascular collapse that can lead to death (250).

The late-phase reactions have many features in common with early-phase reactions. However, late-phase reactions typically develop after 2-6 h, peak 6-12 h after allergen challenge and fully resolve in 1-2 days. They are the result of innate and adaptive immune cells liberated from the circulation and the production of inflammatory mediators by tissue-resident cells. The innate immune cells include eosinophils, neutrophils, monocytes, basophils and mast cells. Tissue-resident or recruited T-cells after been activated by allergen peptide recognition also secrete inflammatory mediators. Overall, the clinical features of late-phase reactions are the result of both resident cells and recruited leukocytes, in particular basophils. For example, in human skin late-phase reactions involve oedema, pain, warmth and erythema and in the lungs airway obstruction and increased mucus secretion. Reflecting the action of activated Th2 cells, eosinophils, basophils and other leukocytes (251).

Basophils apart from their action as effector cells that migrate to the sites of inflammation after Th2 cytokine responses, they also play a key role in the development of Th2 cytokine-mediated inflammation. They consist a potent source of IL-4 production promoting $CD4^+$ Th2 cell-mediated inflammation (252).

1.2.5. FcεRI signal transduction

Crosslinking of FcεRI-bound IgE complex by a matching allergen stimulates a cascade of intracellular signaling events leading to effector functions. FcεRI signaling is separated in two pathways the primary and complementary pathway that modulate FcεRI-induced degranulation. The effector signaling events initiate

from the β- and γ-chains of FcεRI which contain immunoreceptor tyrosine-based activation motifs (ITAMs).

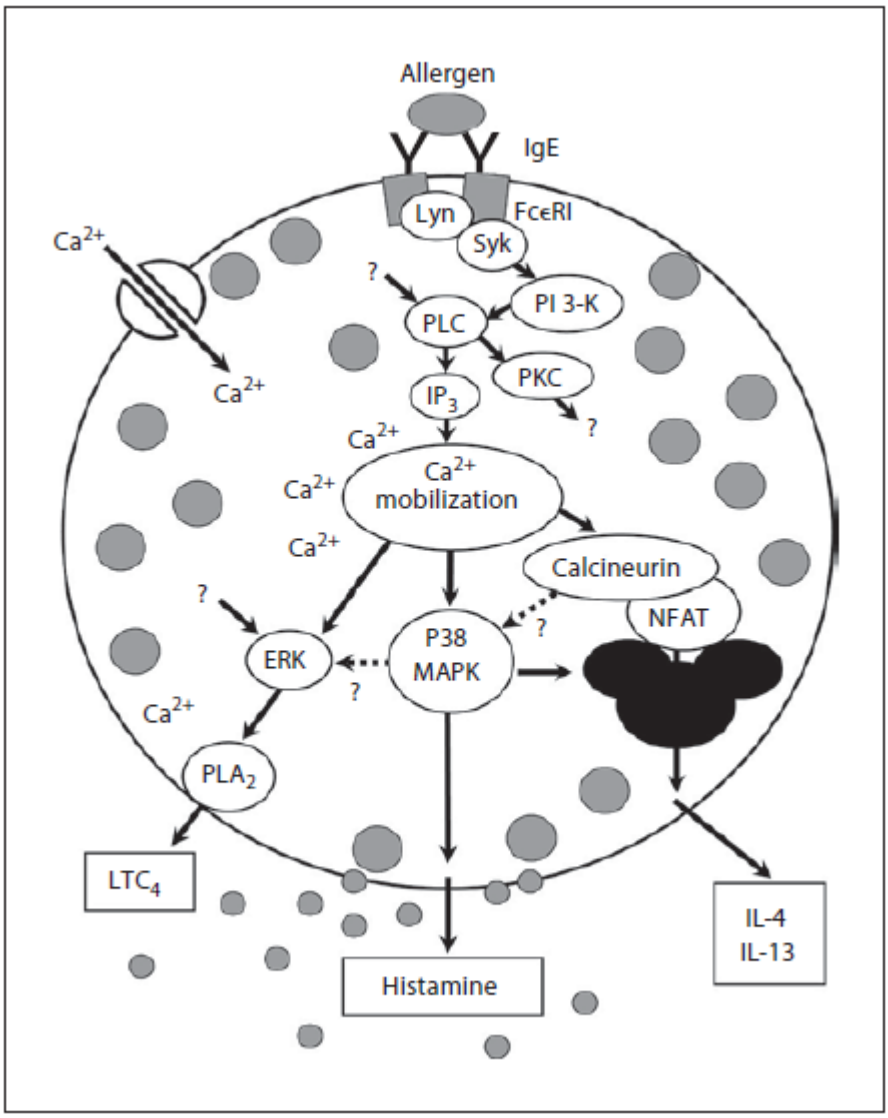


Figure 6. Schematic representation of basophil activation caused by IgE receptor triggering. Figure used from Kleine-Tebbe, J. *et al*, 2006 (253).

Aggregation of FcεRI results in phosphorylation of the tyrosine residues within ITAMs. Phosphorylated ITAMs bind the SH2 domains of the SRC family kinases LYN and FYN, as well as SYK (254). LYN and FYN are associated with FcεRIβ, and SYK is

associated with FcεRIγ. The primary pathway develops from LYN which is activated and phosphorylates ITAM residues in β- and γ-chains, promoting SYK association with γ-chains and SYK activation. SYK phosphorylates other proteins such as LAT (linker for activation of T cells), which then stimulates Grb2-related adaptor protein (GADS), SLP-76 (Lymphocyte cytosolic protein 2), Vav (guanine nucleotide exchange factors (GEFs) and PLCγ (phospholipase Cγ). PLCγ is phosphorylated and activated through PI3K (phosphoinositide 3-kinase)-mediated membrane recruitment of BTK (Bruton's tyrosine kinase). From phosphatidylinositol 4,5-bisphosphate (PIP2), activated PLCγ then produces DAG (diacylglycerol), which activates PKCs (protein kinases C), and IP3 (inositol-1,4,5-trisphosphate), resulting in Ca^{2+} release from the ER (endoplasmic reticulum). Ca^{2+} store depletion activates STIM1 (stromal interaction molecule 1) which triggers opening of the store-operated channel (SOC) leading to influx of extracellular Ca^{2+} and CRAC (calcium-release-activated current). The Ca^{2+} influx is mainly mediated by the transcription factor -nuclear factor of activated T-cells (NFAT). In resting cells, NFAT is kept phosphorylated by serine/threonine kinases. Multiple phosphorylation masks NFAT's nuclear localisation sequence (NLS) preventing it from entering the nucleus. Increased levels of cytosolic Ca^{2+} result in binding of Ca^{2+} to calmodulin, which in turn activates the NFAT phosphatase, calcineurin which is capable to unmask the NLS of NFAT. After NFAT is dephosphorylated, it enters the nucleus, where it initiates the transcription of cytokine genes such as IL-4 (255).

Collectively, PKC activation and Ca^{2+} mobilization lead to degranulation. The complementary pathway originates after FYN and SYK activation inducing a signaling complex with VAV, GRB2, GAB2 (GRB2-associated binding protein 2) and PI3K. PI3K activation leads to PDK1 (3-phosphoinositide-dependent protein kinase 1) activation which in turn stimulates PKCδ, ultimately leading to degranulation. PLD (phospholipase D), LYN or FYN activates sphingosine kinases (SPHKs) resulting in extracellular Ca^{2+} influx. GRB2 and SOS (son-of-sevenless homologue) promote RAS activation, which subsequently leads to activation of the ERK (extracellular-signal-related kinase) MAPK (mitogen-activated protein kinase) cascade and eicosanoid production via PLA2. These kinases are crucial signaling mediators between the cytosol and the nucleus and regulate the expression of several transcription factors such as the nuclear factor-κB (NFκB) (255).

1.3. Allergy diagnosis

History of allergy diagnosis

In 1880, the first skin test for allergies was attempted by Dr. Charles H. Blackley who was the first to deliver scientific evidence on the causative role of pollen on hay fever, by directly administering fresh or dried pollen to his skin, eyes, nose and tongue. This resulted in the typical symptoms of rhinitis. Later, in 1921, Carl Prausnitz injected intradermally various dilutions of Heinz Küstner's serum, his collaborator in the Hygienic Institute in Breslau who was allergic to fish and the following day, injected those places and control places in his skin with fish extract. With this experiment they proved that fish allergy could be momentarily

transmitted by administering serum from an allergic individual to healthy, normal people. Those discoveries set the ground for the most widely applied methods in allergy diagnosis, namely the skin prick test (SPT) (**Figure 7**) (256).

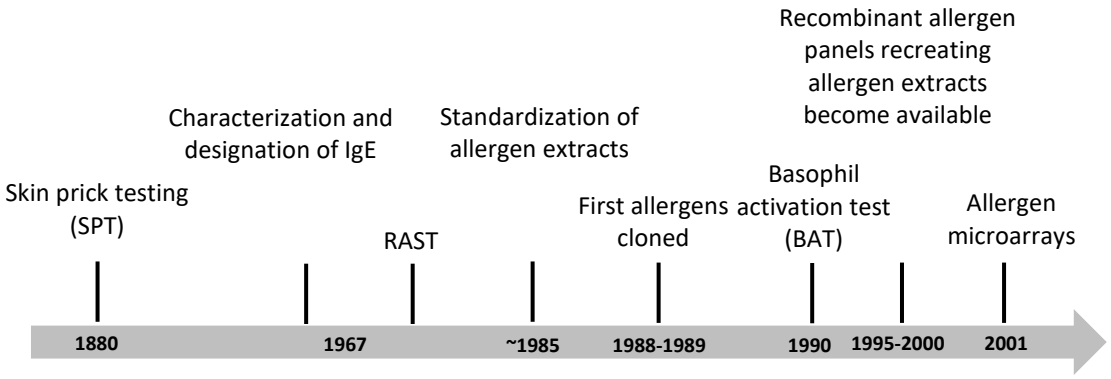


Figure 7. Timeline showing the evolution of allergy diagnosis. Figure adapted from Harwanegg, C. *et al*, 2003 (257).

Prausnitz and Küstner, also proved experimentally the involvement of antibodies in Type I allergic reactions. Almost 50 years later, Ishizaka and Johansson & Bennich research groups, contributed in the characterization and designation of IgE as the antibody responsible for initiation of allergic reactions. In 1967, Wide, Johansson and Bennich proposed the first radioallergosorbent assay (RAST), a solid-phase sandwich radioimmunoassay (RIA) (258). The RAST allowed doctors for the first time to perform in vitro allergy diagnosis by measuring allergen specific IgE (259). Beginning of the 20th century, attempts to further elucidate allergic diseases led to the development of allergen extracts. However, since extracts consist of heterogeneous mixtures of proteins, glycoproteins, glycolipids and polysaccharides from a given allergenic source, their standardization was difficult

to perform. The quality of an extract is influenced by the source material (e.g. the plants, varieties, climatic and soil growth conditions) and the production process. In addition, the allergen content of extracts varies between different manufacturers and batches (260).

In the 1980s, the World Health Organization and the Allergen Standardization Sub-committee of the International Union of Immunological Societies (WHO/IUIS), in an attempt to improve standardization of allergen extracts, introduced for the first time reference preparations as reference standards for five allergenic preparations (261-264). In the early 1990s, the "Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification", known under the acronym CREATE was proposed by the European Union. The aim of the project was to determine reference standards on purified recombinant allergens as well as development and validation of methods used for the quantification of allergenic extracts. To achieve this, purified natural allergens were used as the gold standard to assess the properties of recombinant proteins (265). Between 1988 and 1989, the first allergens were cloned. Numerous allergen sequences became available, setting the ground for variant recombinant proteins similar to complex natural allergen sources (266). The use of recombinant allergens offers several advantages compared to allergen extracts as extract-based diagnosis is capable to identify the allergen-source as a whole, but cannot distinguish the culprit molecules. Therefore, recombinant allergens can discriminate between patients who are sensitized to unrelated allergen sources and eliminate cross-reactivity

between different allergen sources (267). The abundance of recombinant allergens resulted in the development of novel diagnostic tests able to monitor patient's IgE reactivity to numerous different molecules. Therefore, only 20 years ago, the first protein microarray was developed for the diagnosis of allergy or autoimmune diseases (268).

Current methods

As discussed earlier, accurate diagnosis of allergic disease and accurate detection of the culprit allergens helps to determine appropriate treatment options. Currently, Type I allergy diagnosis includes evaluation of patients' symptoms and clinical history, followed by detection of allergen-specific IgE. The most common *in vivo* and *in vitro* tests used as well as future diagnostic developments are described below.

1.3.1. In vivo allergy tests

In vivo allergy tests are usually carried out to the patients. They are performed by trained personnel in specialized facilities where resuscitation can be carried out as the risk of anaphylactic reaction is high, especially with food or respiratory allergen challenge tests. The most common are the skin prick test (SPT) and the oral food challenge (OFC).

1.3.1.1. Skin prick testing

The skin prick test (SPT) relies on the application of a very small amount of allergen extract into the epidermis using standardized methods (a disposable fine needle or

lancet device, which is replaced for each test allergen) to secure comparability and reproducibility of the results. SPT is considered a safe procedure, with minimal discomfort. Adverse events can occur but rarely (269). Between 1990 and 2001, no instances of near-fatal or life-threatening reactions to inhalant prick or puncture tests were recorded (270).

Analysis of the SPT is based on measurement of wheal and flare reaction resulting from the release of histamine on the skin, however SPT results may be affected by the use of medications targeting histamine receptors (271). Furthermore, the site of testing plays an important role as the wrist and lower back are usually less reactive. Histamine dihydrochloride or codeine and saline are used as a positive and negative control, respectively. The diameter of the resulting weal is recorded in two dimensions after 10 minutes for the positive control and after 15 minutes for the tested allergen. Positive is considered a test which has a mean of the two weal diameters at least 3 mm greater than the negative control. Positive and negative controls are crucial for the interpretation of test results. (272). The SPT is used to confirm sensitization to a specific allergen. Interpretation of its clinical relevance must be performed in parallel with the medical history and clinical symptoms (273). Depending on the allergen used and the tested population the clinical relevance of SPT results vary. For example, in some individuals sensitization to house dust mite occurs in the absence of clinical relevance (274). For the diagnosis of food allergies, most of the commercial food extracts used are not standardized and the diagnostic efficiency is unknown (275). Moreover, in the case

of food allergies, it has been shown that negative SPT results are reliable to exclude immediate food hypersensitivity but a positive test is only suggestive even in cases of patients where the prevalence of food hypersensitivity is high (276).

1.3.1.2. Oral food challenge

The oral food challenges are employed to make an accurate diagnosis of immediate and occasionally delayed adverse reactions to foods. Parameters such as the type of challenge, foods to be tested, and dosing schedule should be determined based on the patient's history and age (277). The OFC should be designed and carried out by experienced health care personnel in specialized facilities where the challenge can be closely monitored to avoid potential life-threatening reactions (278). The double-blind placebo-controlled food challenge (DBPCFC) is considered the current "gold standard" for the diagnosis of food allergy. In this test, patients are randomly administered colourless and odourless capsules which contain allergen extracts or placebo. Before each dose, the patients are examined clinically, including inspection of the skin, lung auscultation, abdominal auscultation, blood pressure, heart rate, and oxygen saturation measurement (279). However, the DBPCFC can put the patient in potential danger of anaphylaxis, it is costly, only a limited number of allergen can be tested at a time and time-consuming as the patient must be monitored for several days (280). A negative OFC test results in introduction of the food in the diet, whereas a positive test results in food avoidance (281). OFCs due to high chance to induce life-threatening anaphylaxis are only recommended when other diagnostic tests give unclear results (282).

1.3.2. In vitro allergy tests

In vitro tests are usually performed using patients' serum. They offer several advantages over *in vivo* tests. There is no direct risk for anaphylaxis as patients are not exposed to the tested allergen. In addition, the patients' medication does not interfere with the tests' outcome. In vitro tests compared to in vivo tests, are also capable to screen higher number of allergens at a time. Sera can be stored and posted to a different laboratory if necessary, while methods involving cells, such as the basophil activation tests discussed later, need to be performed within a short time window of about 4-8 hours.

1.3.2.1. Allergen-specific IgE

Initially, allergen-specific IgE evaluation was based on the detection and quantification of IgE antibodies in patients' serum using crude natural extracts. However, as mentioned in subchapter 1.3 (History of allergen diagnosis), different research groups reported varying allergen composition and potency of natural extracts (283). Alongside the molecular characterization of allergens, new methods to measure allergen-specific IgE have been developed. Fluorescence enzyme immunoassay (FEIA) using cellulose as solid phase, took the place of RAST (radioallergosorbent test), which used radio-labelled anti-IgE antibodies. Meanwhile, allergen molecules purified by natural sources or recombinant allergens allowed screening of IgE reactivity at a molecular level, setting the grounds for the concept of component-resolved diagnostics (CRD) (266). In CRD,

well-defined recombinant allergens that can be genetically engineered are used to examine the individual patient's IgE reactivity profile to identify the disease eliciting molecules. Recombinant allergens can be produced identically avoiding genetic, biological or batch-to-batch variation of the protein. Furthermore, by using recombinant allergens it is possible to measure humoral, cellular and biological responses to specific molecules (266). Molecular biology techniques such as the sequence analysis of allergens revealed that structurally related allergens exist not only to allergens belonging to the same family source but also to taxonomically distant species (284). Thus, those cross-reactive molecules are not easily differentiated by the patient's immune system (285).

Allergen-specific IgE can be determined using singleplex (one assay per sample) or multiplex analysis (multiple assays per sample). In singleplex tests, allergen extracts or allergen components are used. For accurate diagnosis, the clinician selects the allergens to be tested based on the patient's clinical history, the allergen source and the availability of single allergen. On multiplex analysis a microarray technology is performed, allowing screening of multiple allergen components to determine the existence of sIgE. One example of the multiplex technology is the ImmunoCAP® ISAC (Phadia AB, Uppsala, Sweden), an Immuno Solid-phase Allergen Chip, developed to determine IgE and IgG from 112 different allergens (286). Detection thresholds for sIgE have been used as an indicator of sensitization. Traditionally, cut-off limits for sIgE quantification was 0.35 kUA/L. However, after improvements in analytical sensitivity, in daily practice a lower limit

of quantification (LoQ) of 0.1 kUA/L is used for singleplex autoanalyzers. Nevertheless, a debate on the clinical relevance of different in vitro sIgE tests had been ongoing for more than 20 years. Alternatively, the Receiver Operating Characteristic (ROC) analysis curve was proposed allowing establishment of cut-off points using graphical illustration of diagnostic sensitivity (true positives) and specificity (true negatives) (287). Comparison between the multiplex ISAC 112 with singleplex analysis, using a variety of grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5b, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12, and nPhl p 4), showed statistically significant correlations between all allergens (r= 0.70 to 0.96). However, notable discrepancies were observed, between the two methods, for some allergens. Those differences could be attributed to the distinct surface technology which influences allergen binding and the overall test sensitivity (288). Despite the source of the allergens, extracts or molecules and the method used, singleplex or multiplex, detection of sIgE indicates allergic sensitization (289). Until now, only an experienced clinician can determine the presence of clinically relevant IgE after the presence of a positive sIgE test and a positive patient clinical history. In many cases, multiplex result analysis can be challenging for the physicians and interpretation of sIgE should be performed in agreement with the clinical history (290).

1.3.2.2. Basophil activation test (BAT)

The BAT is a flow cytometry-based assay that involves measuring the expression of activation markers on the surface of basophils that are upregulated upon cross-linking of IgE bound to the high-affinity IgE receptor (FcεRI) after stimulation with

allergen or anti-IgE. These surface markers can be labelled using fluorescently-labelled monoclonal antibodies (253). The BAT has the capability to replicate Type I hypersensitivity reactions *in vitro*, similar to those developed *in vivo* after exposure of allergic individuals to allergens (291). In food allergic patients a good correlation was observed between the BAT results and the oral food challenge (OFC). Similar, in hymenoptera venom allergic patients, up-regulation of basophil activation markers was reported in vitro after yellow jacket or honey bee venom stimulation and *ex vivo* following a positive sting challenge (292). Following stimulation with allergen, the expression of different proteins is up-regulated on the surface of basophils (293), namely CD63 (255) and CD203c (294, 295). The CD63 antigen or else know as lysosomal-associated membrane glycoprotein-3 (LAMP-3) is a 53 kDa integral membrane glycoprotein (296, 297). In resting basophils, CD63 is present on the membrane of intracellular secretory granules, while upon stimulation by FcεRI, the granules fuse with the plasma membrane and CD63 is expressed on the surface of degranulated basophils (296). CD63 surface expression has also been induced on other cell types such as platelets with the CD63 antigen being present in their intracellular granules (296, 298, 299). Cloning of CD63 antigen revealed that it contains four putative hydrophobic transmembrane regions (298). This indicated that the CD63 antigen is anchored in the granule membrane and fusion of the granules with the plasma membrane results in expression of CD63 on the cell surface (253). CD63 is located in the same secretory lysosome as histamine and may be a more convenient marker for

degranulation (300). Human basophils, including basophils from other species, secrete histamine via two mechanisms the piecemeal degranulation or anaphylactic degranulation. In piecemeal degranulation small distinct vesicles containing histamine germinate from the main histamine granules and are transported to the plasma membrane for fusion, releasing histamine to the extracellular space. On the contrary, in anaphylactic degranulation the main granules fuse directly with the plasma membrane, releasing the entire contents of the granules (histamine and matrix proteins) to the extracellular environment (301). CD63 is a direct marker of anaphylactic degranulation through regulated exocytosis resulting from allergen-mediated activation of mast cells and basophils (300). CD203c an ecto-nucleotide pyrophosphatase/phosphodiesterase, hydrolytically removing 5'-nucleotides successively from the 3'-hydroxy-termini of oligonucleotides (302). It is expressed at low levels on the surface of resting basophils and mast cells while upon activation its expression rapidly increases (303). Two distinct groups of basophil activation markers are formed simultaneously: one including CD63, CD107a and CD107b and another including CD203c, CD13 and CD164 (304). However, CD63 and CD203c markers are the most commonly used (302). As mentioned before, the clinical impact of the basophil activation test (BAT) relies on the distinct ability of basophils to degranulate upon crosslinking of the IgE- FcεRI complex by allergen exposure (305). During degranulation translocation of CD63 to the cell membrane can be measured by flow cytometry (284). Thus, BAT test can be performed in all research and routine

laboratories equipped with a flow cytometer (300). Treatment with antihistamines does not interfere with BAT (306, 307), but systemic steroids (306) and cyclosporin A (308) should be avoided. The basophil activation test can be performed using whole blood or isolated peripheral blood mononuclear cells (PBMCs). PBMC isolation is usually performed using density gradient separation and additional negative selection using magnetic particles allows enrichment for basophils (309). However, an increased risk of cell loss is present from the centrifugation steps as excessive handling of the cells may result in background activation (293). Alternatively, flow cytometry and fluorescent staining techniques allow investigation of basophils in whole blood without further cell manipulation (310). Whole blood BAT should ideally be performed within 4 h of blood collection to avoid risking cell viability and functionality (311, 312), as basophil reactivity considerably decreases over time (293). The timing of blood sampling might be important as diurnal variation in the reactivity to CD203c exists (313). However, this is yet to be confirmed for CD63 (300). Normally BAT, tests are carried out using whole blood. Protective elements found in plasma are separated from cells to optimize activation through cell-bound sIgE (305). Interleukin-3 (IL-3), promotes allergen-specific upregulation of CD63 (314), but non-specifically upregulates CD203c (315). It also intensifies kinetics, reactance, and sensitivity of blood basophils to FcεRI-mediated activation independently of extracellular calcium (316). However, before assessing CD63 surface expression on basophils an extra basophil-selective marker or the analysis of poor basophils is required as CD63 is

not selective to basophils. For example, anti-IgE can be used as specific marker (253). Another limitation is that in the normal population, about 10-20 % of donors have basophils with little or no responsiveness to crosslinking of the IgE receptor (317, 318). A possible explanation to this has been the selective decrease in Syk expression in the non-releasing basophils (319, 320). Culture of basophils with IL-3 causes a partial recovery of secretion in a subgroup or the low or non-responders (320, 321).

1.3.2.3. Rat Basophilic Leukaemia (RBL) cell lines

Besides, SPT and BAT, another cell-based diagnostic method to assess cell sensitization has been developed. The humanized rat basophilic leukaemia (RBL) cell lines can be sensitized with serum from allergic individuals. Therefore, RBL cells are capable to detect allergen-specific IgE with high sensitivity providing in parallel a biological readout once activated, without the need of specialized equipment. As described in 1.2.2 IgE receptors, human basophils express the tetrameric highaffinity receptor ($\alpha\beta\gamma_2$) FcεRI. Similarly, RBL cells express the rat tetrameric receptor FcεRI with the limitation that human IgE does not bind the rat α-chain (101). Therefore, these cells are not suitable to test human serum. Alternatively, several groups have suggested the use of humanized RBL cells expressing either the human α-chain (FcεRI α) or the human β- and ɣ- chains as shown in **Table 1**. (322, 323). Briefly, in 1992, Gilfillan *et al*. demonstrated that the human FcεRI α subunit could substitute for the rat FcεRI α subunit during signal transduction

(324). Later, Taudou *et al*. (1993), generated the RBL T8 clone by transfecting the α_H chain cDNA into RBL 2H3 cells (325). However, the T8 clone showed low αH surface expression, on average only 4100 receptors per cell.

Table 1. Overview of Humanized RBL cell lines. Table adapted from Falcone F. H. *et al*. (2015) (322) and Ali E. A. *et al*. (2019) (323).

Name of cell line/clone	Transgenes	α_H/Cell	Detection	Application test
SX-38	αγ	450,000	5-Hydroxytryptamine Beta-hex	sIgE detection Allergen characterization
RBL-30/25	α	n.d.	Beta-hex (50 % D2O)	Allergen standardization
RBL-2H3.E5.D12.8	α	n.d.	Beta-hex	sIgE detection
I5/3/C	α	1800	[3H] 5-HT	sIgE detection
H 212lC	α	3200		
H 7/1/A	α	2500		
RBL-hEIa-2B12	α	n.d.	Beta-hex	sIgE detection
RBL 48	α	25,000	Histamine, AA	sIgE detection
RBL T8	α	4100	Calcium influx	IgE dependent activation
RBL NFAT DsRed	α	16,000	DsRed (fluorescence)	sIgE detection
RS ATL-8	αγ	500,000	Luciferase	sIgE detection; allergenicity assessment
NPY-mRFP	α	n.d.	mRFP (Fluorescence)	sIgE detection; allergenicity assessment

Takagi and co-authors assessed IgE levels using human sera after transfecting α_H into RBLhEIa-2B12 cells. Wiegand *et al*. (1996) (326), developed the triple transfectant RBL SX-38 cell lines, expressing the human α, β, and γ chain. Dibbern and co-workers, assessed the sensitivity of RBL SX-38 cells after using sera from peanut allergic patients. They reported incubation of cells with sera only induced

cell degranulation that could be reduced after removing cytotoxic components present in the human serum (327). Another triple transfectant cell line named RBL 30/25, was generated by Vogel et al. (2005). RBL 30/25 cells although it was found that they were expressing only the αH mRNA, they responded well after stimulation with an optimum allergen concentration (100 ng/mL) using between 1:20 and 1:40 serum dilutions (328). Eventually, chimeric RBL cell lines were used only for standardization of allergens as a number of limitations, such as failing to provide reproducible results when using sera from different patients, hampered their use as a diagnostic tool. In 2010, Nakamura R. and co-workers developed a novel chimeric cell line named RS-ATL8, for use in the EXiLE test (IgE crosslinking (x)-induced luciferase expression) (329). The EXiLE system was generated after stably transfecting RBL SX-38 cells with a nuclear factor of activated T cells (NFAT)-responsive luciferase reporter gene. Traditionally, activation of RBL cells relies on measurement of released mediators from activated cells such as histamine or betahexosaminidase. However, since the beta-hexosaminidase has low sensitivity the need for enhancing agents like deuterated water or N-ethyl-5'-carboxamidoadenosine (NECA) can influence the final readings due to low signalto-noise ratio (330, 331). On the contrary, the EXiLE system using RS-ATL8 cells, is based on expression of the NFAT-driven firefly luciferase reporter gene upon activation as described on 1.2.5. FcεRI signal transduction. The high sensitivity of the system allows the detection of lower concentrations of allergens and use of higher dilutions of serum preventing serum cytotoxicity compared to conventional

non-reporter RBL cell lines.

1.3.2.4. Protein microarrays

The development of microarray technology aimed to monitor in parallel gene expression in genomes. The wide application of microarrays to basic research and for diagnostic applications resulted from the ability to measure simultaneously several parameters, the need for small volumes of biological samples and their high detection sensitivity using fluorescent probes (e.g. DNA, RNA, proteins). Initially, protein microarrays were successfully used to study protein-ligand interactions (332). Almost twenty years ago, protein or peptide arrays were applied for the diagnosis of allergy or autoimmune diseases by using purified allergens (268, 333). Haab *et al*. (2001), developed spotted-array-based quantification of 115 antigenantibody pairs (334). MacBeath and Schreiber *et al*. (2000), introduced spotted protein arrays to detect protein-small molecule interactions (335). The principle of allergen microarrays is applied as follows. Purified or recombinant allergen molecules are printed onto a solid surface (e.g. activated glass slides) in the form of circular spots or dots using an automated microarray spotter (printer). Nanolitre volumes of the allergen solution is applied in a contact (e.g. pin-and-ring or split pin) or non-contact technologies (e.g. piezo or capillary). Provided the coated surface chemistry on the glass slide and the concentration of printed proteins are optimized for their immobilization, affinity interaction molecules such as antibodies successfully recognize the later. Collectively, since only a few hundred picograms of protein covalently attach on the surface, an enormous

number of allergen arrays can be generated from a few micrograms of protein. On a standard microscope slide of (75 x 25 mm) up to 20.000 protein spots can be printed, exceeding the number of purified or recombinant allergens currently available. Therefore, they provide an adequate substrate for several independent assays to perform on a single slide. Until now, allergen extracts only revealed sensitivity to unspecified allergens. On the contrary, microarrayed recombinant allergens describe the patient's sensitization profile to individual allergen molecules. A major advantage of this technology is that a single test requires a small volume of serum to sufficiently determine the reactivity profile against multiple allergens. Given the printed capacity of a single surface, thousands of allergens or epitopes could be tested at a time. However, due to cross-reactivity among several allergens this proposition is not feasible yet. Also for clinical purposes, each allergen needs to be validated individually. Alternatively, the use of various fluorescent dyes to label different antibody classes can provide a more explicit diagnostic analysis (334, 336-338). Allergen microarrays are evolving to a valuable tool to analyse the development of Type I allergic reactions (257). Also, If they are applied to larger study groups, the array profiling in combination with the multivariate techniques can potentially improve the prognostic of allergic patients to milk proteins or others (336).

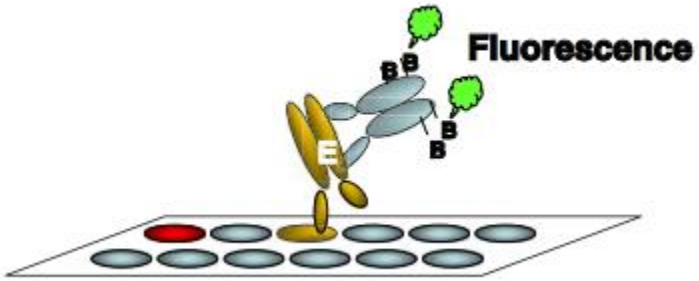


Figure 8. The basic components of standard protein array. Allergen-specific antibodies from patient's serum bind to immobilized protein molecules on the surface. Secondary antibodies and fluorescent markers allow the target molecule to be detected and quantified. Figure adapted from Renault, N. K. *et al*, 2006 (339).

1.4. Aims and objectives

As described in Chapter 1.3, several methods have been developed until today capable to measure sIgE levels but also assess the clinical relevance of those antigen-specific IgE. The cell activation reporting tests such as the BAT and SPT, although they provide a biological readout by indirectly reporting cellular activation in the form of mast cells in the case of SPT and directly reporting basophil activation in the BAT test, they still carry a number of limitations. For example, such tests can be costly, time-consuming and most often allow screening of few allergens at a time (1.3.1.1 SPT and 1.3.2.2 BAT). Similarly, the serological tests (ImmunoCAP, protein-arrays) that merely measure sIgE levels in patient's serum, they frequently only provide information on allergen sensitization, which is not always equivalent to clinical allergy. Thus, interpretation of those tests should be

accompanied with the patient's clinical history. Lastly, the humanized RBL cell lines although used in allergen standardization and in pharmacological or immunological inhibitor testing, they showed limited usefulness as a diagnostic tool for detection of sIgE in serum samples. A critical limitation as reported by Ladics, was the lack of consistency when using sera from different patients. This, collectively may be attributed to the low human α-chain expression levels, the low ratio of allergen-specific IgE to total IgE in some patient's sera, leading to insufficient cell sensitization after incubation with high serum dilutions and the toxicity of some sera (322). Efforts to overcome those limitations led to use of protein arrays with purified peripheral basophils (340). However, the complex setup of this method and the need for fresh peripheral blood basophils do not allow it to enter clinical practice. Alternatively, the ease of use and high sensitivity of the reporter RBL cells together with the high-throughput analysis of protein microarrays paved the way for a novel approach to diagnose allergy. Reporter RBL cell lines expressing intracellular fluorescent reporter genes such as DsRed and GFP, can be used together with highthroughput methods (microarrays) to screen simultaneously multiple parameters using only a small volume of patient serum.

Overall, our work has focused on the development of a highly sensitive reporter system capable to report cell activation after development of the optimum solid-phase microarray substrate. Therefore, the following objectives were addressed towards this aim:

1. Generate a highly sensitive reporter cell line comparable to RS-ATL8, after inserting the NFAT-DsRed fluorescence reporter gene into RBL SX-38 cells (mother cell line of RS-ATL8).
2. Increase the sensitivity of the NFAT-DsRed reporter cells, which consist an ideal candidate to use with microarrays, after inserting the FcεRI γ_H. Our next step was to assess the sensitivity of the newly synthesized cell line as well as expression of the FcεRI α_H compared to all other RBL 2H3 derived cell lines.
3. Assess the effectiveness of various solid-phase surfaces to support reporter RBL cell attachment and activation

Chapter 2: Construction of RBL SX-38 pNFAT-DsRed-Express2 Reporter System

2.1. Introduction

As described in 1.3.2.3 subchapter, numerous humanized reporter and non-reporter RBL cell lines have been generated by several research groups in an attempt to improve the diagnosis of allergic sensitization and detect the presence of allergen-specific IgE (sIgE) in serum from allergic individuals. The RBL cell lines used throughout this project were the RBL-2H3, RBL 703/21, RBL SX-38, RBL NFAT-DsRed and RS-ATL8. The RBL-2H3 cells which express only the rat FcεRI consisted the basis for the generation of all chimeric cell lines (341, 342). The RBL 703/21 cells (which was derived from RBL 30/25) (328) express only the FcεRIα_H and the triple human α-, β-, and γ-chain transfectant RBL SX-38 cell lines, created by Wiegand *et al*. (1996) (343). Activation of the latter and degranulation relied on measurement of released histamine or most commonly beta-hexosaminidase as a less expensive marker. However, the need for enhancing agents to increase cell sensitivity or the application of cumbersome protocols to measure released histamine limited the usefulness of chimeric RBL cell lines as a diagnostic tool. Later, the generation of the reporter cell lines RS-ATL8 and RBL NFAT-DsRed deriving from the RBL SX-38 and RBL 703/21 cells, respectively, gave birth to novel applications.

Earlier, the role of the individual human α-, β- and ɣ- chains on FcεRI receptor was discussed (1.2.2 IgE Receptor). More specifically, α_H is responsible for IgE binding, making it possibly the most important parameter for cell activation. Therefore,

higher FcεRIα_H levels in RBL cells determine the ability of those cell lines to efficiently bind human IgE in serum samples, thus cell sensitization. In a study performed from our group, the human FcεRIα chain expression levels in different RBL cell lines was quantified for the first time (323).

Table 2. Human FcεRIα chain expression was measured in different RBL Cell Lines after overnight sensitization with human IgE. Table adapted from Ali, E. A. *et al*, 2019 (323).

Name of cell line/clone	Transgenes	α_H/Cell
RS ATL-8	αγ	500,000
RBL SX-38	αγ	450,000
RBL 703/21	α	52,000
RBL NFAT DsRed	α	16,000

Since RS-ATL8 and RBL SX-38 cell lines, express higher levels of FcεRIα_H on their surfaces, one would conclude that these two cell lines are ideal candidates to use for the development of our diagnostic system of interest. However, although RS-ATL8 cells have approximately 500,000 FcεRIα_H molecules per cell similar to the maximal number of receptors described on human peripheral blood basophils (up to 600,000/cell) (88) and express a highly sensitive NFAT-dependent reporter gene, they are not suitable for use in an array format. The highly sensitive detection technology of the EXiLE system (≥15 pg/ml IgE, using 1:100 diluted human sera) (329) is based on luciferase activity measurement which requires cell lysis. This process though would disconnect the cell signal from the allergen spots on the microarrays (344). On the other hand, the RBL SX-38 cells, although expressing similar levels of FcεRIα_H to RS-ATL8, do not possess a reporter gene and their

activation is measured with the conventional methods mentioned earlier, leading to the same problem of spatial disconnection.

Aim and Objectives

In order to create a novel system to diagnose IgE-mediated allergic diseases, we aimed at the development of a highly sensitive cell line compatible with protein microarrays. To overcome previously described limitations, we proposed the introduction of the NFAT-DsRed-Express2 fluorescent reporter gene in RBL SX-38. In principle, this would enable the application of the new cell line on protein microarrays.

2.2. Materials and methods

2.2.1. Cell Culture

RBL-2H3 cells were obtained from the European Collection of Cell Cultures (ECACC), UK (Catalogue No.: 86061001). RBL 703/21 cells were kindly provided under a Material Transfer Agreement (MTA) by Stefan Vieths and Lothar Vogel at Paul-Ehrlich Institute (Langen, Germany) and were cultured with 1 mg/mL G418 sulphate (Invivogen, USA) for selection of HsFcεRIα expression. RBL SX-38 cells were obtained through an MTA and were kindly provided by Jean-Pierre Kinet (Beth Israel Deaconess Medical Centre, Boston, Massachusetts, USA). The RBL SX38 cells were cultured with 1mg/mL G418 sulphate for HsFcεRIγ expression. The RS-ATL8 cells were obtained from MTA, were kindly donated from Ryosuke Nakamura (National Institute of Health Science, Tokyo, Japan) and were grown under 1mg/mL G418 sulphate for selection of HsFcεRIα and 600µg/mL hygromycin

B (Invitrogen, UK) selection of luciferase reporter activity. RBL NFAT-DsRed cells were generated at the University of Nottingham by Franco H. Falcone and Marcos J. C. Alcocer (344). The RBL NFAT-DsRed cells were also cultured under 1mg/mL G418 sulphate for selection of HsFcεRIα and 20 µg/mL blasticidin S (Invivogen, USA) for NFATp-DsRed-Express2 expression. All RBL cell lines were cultured in Minimum Essential Medium Eagle (MEM) (Merck, UK) supplemented with 10 % v/v heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L–glutamine (RBL medium). Cell culture was carried out in class II microbiological safety cabinets, using sterile techniques. RBL cells were subcultured in polystyrene flasks treated for optimal attachment in $25cm^2$, $75cm^2$ or 125 cm^2 flasks surface area, containing 5, 10 and 25 ml RBL medium respectively, with vent caps (Corning, USA), in a humidified 37°C/5% CO_2 incubator (Galaxy S Plus CO_2 incubator, RS Biotech, UK). Each flask was refreshed every three days and passaging was carried out twice a week. Detachment of cells was performed by incubating 10 mL of medium with 2 mL trypsin-EDTA (Gibco, UK) for 15 minutes following two washes with Ca2+/Mg2+ free DPBS (Gibco, UK) and resuspended in fresh medium or detached using cell scrapers (TPP, Switzerland). Frozen stocks of cells were prepared as follows: the cells were centrifuged at 300xg for 5 min at room temperature and resuspended in RBL medium with 10 % v/v DMSO to obtain a cell concentration of 5×10^6 cells/mL. 1mL of cell suspension was transferred to each CryoTube vial (Nunc, Denmark) and then transferred to a Mr. Frosty™

(Nalgene) container filled with isopropanol and placed in a −80 °C for several hours or overnight. The next day a vial was defrosted to check the viability of the batch.

2.2.2. Bacterial Transformation

Competent DH5α E. coli cells (Invitrogen, UK) were prepared as described by Sambrook J. & Russell, D. W *et al*., (2006) (345). All plasmids were transformed by heat shock into competent DH5α bacterial cells by mixing 5 µL plasmid into 100 µL bacteria cells in a 1, 5 mL Eppendorf tube on ice for 30 minutes. Following, the tubes were transferred to a water bath set to 42˚C for 90 seconds and immediately after on ice for 2 minutes. Next, the cells were incubated for 1 hour at 37˚C with 1 mL LB medium to express the antibiotic resistance genes. For selection of transformed E. coli, sterile Petri dishes were covered with approximately 10 mL LB agar containing 50 µg/mL Ampicillin where bacteria cells were spread onto and incubated overnight at 37˚C. The next day, the single colonies formed were picked with a sterile inoculating loop and inoculated in a 50 mL Falcon tube with 5 mL LB per colony containing appropriate antibiotic. According to the purification protocol, 5 mL growth medium was used for a Plasmid Miniprep or larger volume of LB (25 mL) was used for a Plasmid Midi Kit (QIAGEN, Germany). The bacteria culture was kept in a shaker incubator overnight at 37 °C set at 250 rpm. The following day, glycerol stocks were prepared, before plasmid purification, by mixing 500 µL bacteria and 500 µL 60% v/v glycerol in 1.5mL tubes and kept in -80˚C until use. After plasmid purification, plasmid concentrations were measured

using NanoDrop 3000 spectrophotometer (Thermo Scientific, USA). All confirmatory DNA sequencing was carried out by Source Bioscience, Nottingham.

2.2.3. pNFAT-DsRed-Express2 Plasmid

The NFAT-DsRed-Express2 plasmid was generated at the University of Nottingham in the groups of Falcone and Alcocer. Construction of the NFAT-DsRed-Express2 plasmid has been described in detail by Wang *et al.*, (2013) (344). In brief, the NFAT enhancer was amplified from the pNFAT-hrGFP plasmid (Stratagene, US) and the DsRed-Express2 gene was amplified from the pDsRed-Express2-1 plasmid (Clontech, US). Following, the NFAT enhancer and DsRed-Express2 PCR products were sequentially ligated into pUB6/V5-His A (Invitrogen, UK) plasmid using T4 DNA ligase (Promega, UK) after digestion of vector with appropriate restriction enzymes. (Plasmid map in Appendix I-**Figure 31**.)

2.2.4. Transfections

2.2.4.1. Electroporation

Once RBL SX-38 cells reached 70 to 85% confluency, they were collected as described in subchapter 2.1 Cell Culture. For all transfections the SF Cell Line Optimization 4D-Nucleofector™ X Kit (Lonza, UK) was performed. Pre nucleofection the cell density was determined to $1x10^6$ cells/mL. Following, all steps were carried out in class II microbiological safety cabinet. The nucleofection solution was prepared as described by the manufacturer. Initially, for the transfection, as suggested by the manufacturer 2 µg plasmid DNA was electroporated in $2×10^6$ cells in 100 µL nucleofection solution in nucleocuvette vessels using a specific nucleofection program (DS-150). The pmaxGFP™ Vector, a

plasmid that constitutively expresses a green fluorescence protein was used as a positive control to assess transfection efficiency. After transfection, both cell suspensions were kept at room temperature for 10 minutes. Next, each nucleofection cuvette was rinsed with 500 µL antibiotic free MEM prewarmed to 37 °C. After rinsing with another 200 µL prewarmed medium, the final volume (700 µL) was transferred to a clear TC-treated 6-well plate (Costar, Corning, USA) containing 500 µL warm MEM medium without antibiotics. Transfected cells were kept in a humidified 37°C/5% CO2 incubator for 48 h. After 2 days, fresh medium containing 20 µg/mL blasticidin S was added.

2.2.4.2. Transfection (Optimized protocol)

Prior to transfection, RBL SX-38 cells were grown in Minimum Essential Medium Eagle (MEM) containing appropriate antibiotics until they reached a confluency level of 70-85%. Cells were then harvested using 1 ml trypsin-EDTA solution (Sigma, UK) for 15 minutes following two washes by Ca2+/Mg2+ free DPBS (Sigma, UK) and resuspended in fresh medium to a concentration of 4×10^6 cells/mL. All cells were transfected via electroporation using the Amaxa™ 4D-Nucleofector™ protocol for cell lines (Lonza, USA). In brief, for each transfection 2 µg linearized plasmid DNA was electroporated in 1×10^6 cells in 100 µL nucleofection solution in nucleocuvette vessels using a specific nucleofection program (DS-150). The pmaxGFP™ Vector, a plasmid that constitutively expresses a green fluorescent protein was used as a positive control to assess transfection efficiency. Post nucleofection, transfected cells were slowly resuspended with 700 µL warm RPMI-1640 medium (Sigma, UK)

low in Ca^{2+} and kept for 7 minutes at 37 °C. After 7 minutes, they were incubated in a 6-well plate containing 1 mL MEM without antibiotics at 37 °C for 24h. The next day, 1 mL MEM containing Penicillin/Streptomycin was added. Finally, 48h post transfection the medium was exchanged with 1 mL fresh MEM containing 20µg/mL blasticidin S.

2.2.4.3. Lipid transfcetion

On the day of transfection, 1.6 x 10^4 cells were seeded in each well of a transparent 96-well plate (Corning, Costar, USA) to a total volume of 100 µL/well, until reach 70-90% confluency until transfection. The next day, the transfection master mix and reagents were prepared according to manufacturer's protocol. In brief, 5 µL Opti-MEM® Medium were mixed with 0.15 µL and 0.3 µL Lipofectamine® 3000 Reagent (Thermo Fisher Scientific, USA), respectively. To prepare the DNA master mix 0.2 µg NFAT-DsRed-Express2 plasmid and subsequently 0.2 µg pmaxGFP™ Vector were mixed with 10 µL Opti-MEM® Medium and 0.4 µL P3000™ Reagent. Next, 5 µL of the diluted DNA were diluted with 5 µL diluted Lipofectamine® 3000 Reagent (1:1 ratio). After 5 minutes incubation at room temperature, the DNA-lipid complex was added to the cells. Following, cells were incubated for 2–4 days at 37 °C.

2.2.5. NFAT-DsRed-Express2 plasmid linearization

2.2.5.1. Molecular biology

Molecular biology work was performed using pyrogen-free DNA, RNA, DNase/RNase-free filter tips and PCR tubes (Starlab, UK) and molecular biology grade (DNase- and RNase-free) water (HyClone, UK).

2.2.5.2. pNFAT-DsRed-Express2 restriction digestion and purification

Prior to NFAT-DsRed-Express2 plasmid linearization, we performed plasmid sequence analysis to detect the presence of DsRed-Express2 gene (678bp) with primers specific for amplification of the target gene (Appendix I-**Table 9 & Figure 32**.). NFAT-DsRed-Express2 plasmid linearization was carried out by mixing 2.3 µL DNA Template, 1 µL BglII enzyme (New England Biolabs, USA), 10 µL NEBuffer 3.1 (New England Biolabs, USA) and 86.7 µL molecular biology-grade water to a final reaction volume of 100 µL (Appendix V, Table 6). Sample master mix was then kept at 37 °C for 6 hours. Linearized plasmids were purified using the QIAquick Purification Kit 62 (QIAGEN, USA) following the manufacturer's instructions. Plasmid concentrations were measured using a NanoDrop 3000 spectrophotometer (Thermo Scientific, USA).

2.2.5.3. Gel electrophoresis

Purified linearized plasmids were run on an agarose gel along with the uncut plasmid and 1Kb Quick-load purple DNA ladder (New England Biolabs, USA). The 1% w/v agarose gel was prepared by suspending 1 gr agarose powder in 100 mL 0.5x TBE and heating it in a microwave until fully dissolved, allowing it to cool down to 60 °C, mixing with ethidium bromide (Fisher, UK) to a final concentration of 500 ng/mL and allowing to set in a gel tray at room temperature, with appropriate combs to create wells for sample loading. The gel run at 100V for 60 minutes before

imaging under UV light using a GeneGenius Gel Imaging System (Syngene, UK) using a 400ms exposure for image capture.

2.2.5.4. Selection of clones

To obtain stably transfected RBL SX-38 NFAT-DsRed-Express2 clones, the transfected cells were treated with 20 µg/mL blasticidin S two days post transfection. After 4-weeks of antibiotic selection (2.3.5 subchapter) the stably transfected cells were sensitized with human serum from a donor with known allergy to grass pollen (see Appendix XIII, **Table 18**) (1/50 dilution in RBL medium, heat-inactivated at 56 °C for 5 minutes (see Appendix III, **Figure 34**) and stimulated with 1 µg/mL polyclonal goat anti-human-IgE antibody (Merck, UK) (see Appendix II, **Figure 33**) and 3 µg/mL ionomycin calcium salt (Merck, USA). The highest responding clones were sorted by MoFlo Astrios Cell Sorter (Beckman Coulter, USA). This equipment enabled sorting of single cells into each well of a 96-well plate with 50 µL pre-warmed medium. The sorted stable transfectants were then grown under selective pressure for another 4 weeks until they reached confluency. After one month, selected clones were activated with different concentrations of anti-human-IgE and fluorescence intensity was measured with an Infinite M200 plate reader (Tecan, Switzerland) using 530 nm excitation and 590 nm emission filters.

2.2.5.5. SDS-PAGE and Western Blotting

In order to assess NFAT-DsRed-Express2 expression in RBL SX-38 transfected cells, we determined the expression of DsRed protein in RBL cell lines (transfected with

or without the gene of interest) by Western Blotting. In brief, $1x10^6$ RBL SX-38 NFAT-DsRed-Express2 and RBL 703/21 NFAT-DsRedExpress2, were sensitized overnight with human serum from a donor with known allergy to grass pollen (1/50 dilution in RBL medium), in a 24-well plate (Thermo Scientific, USA) to a final volume 500 µL/well. The next day, cells were stimulated with 1 µg/mL goat anti-human IgE (Merck, USA) for 24, 48 and 72 hours. The cell pellet was collected as described in 2.2.1 Cell Culture and kept at – 40°C until use. After collection, the pellets were resuspended in 100 µL (2x) non-reducing sample buffer. Samples were heated at 100 °C for 5 minutes in 1.5 mL Eppendorf tubes.

SDS-PAGE

After sample preparation, 6 µL protein ladder (Precision Plus Kaleidoscope Prestained Protein Standard, Bio-Rad, UK) was added on the first lane and following 17 µL of cellular fraction samples were loaded into Mini-PROTEAN TGX Precast gels (Bio-Rad, USA), and run in a Mini-PROTEAN™ Tetra Cell (Bio-Rad, USA) at 110 V, according to the manufacturer's instructions.

Western Blot

Once the SDS-PAGE was completed, the membrane was transferred in the transfer buffer (Appendix VI, **Table 11**) for 15 minutes. Soaked filter papers in transfer buffer together with the precast gel and a 0.2 µm nitrocellulose membrane were stacked according to manufacturer's instruction using a Trans-Blot® Turbo™ Transfer System (Bio-Rad, USA). After blotting, the membrane was blocked with 10 mL of blocking solution, containing 5% non-fat milk and 1x TBS-T solution (Tris-buffered

saline (TBS) with 0.1% Tween 20, on a tube roller for 1 hour at room temperature. After the blocking, the membrane was washed once and incubated overnight at 4°C on the roller with 5 mL primary antibody solution containing 3% non-fat milk in 1x TBS-T solution and 5 µL mouse anti-RFP monoclonal antibody [clone 6G6] (Chromotek, USA) (primary antibody) at a 1:1000 dilution. The next day, before adding the secondary antibody the membrane was washed twice with 1x TBS-T for 5 minutes at a time. Next, the membrane was incubated for 1 hour with 10 mL secondary antibody solution containing 3% non-fat milk in 1x TBS-T solution and 5 µL goat anti-mouse IgG, HRP conjugated (secondary antibody) (Merck, USA) at a 1:2000 dilution. After 3-4 consecutive washes of the membrane with 1x TBS-T, to visualize the membrane a chemiluminescent substrate PierceTM ECL Western Blotting Substrate (Thermo Scientific, USA) was added which is peroxidase solution and luminol enhancer solution. Images were taken under high binning mode using a FUJI-LAS 4000 charge coupled device camera (Fujifilm, Japan).

2.3. Results

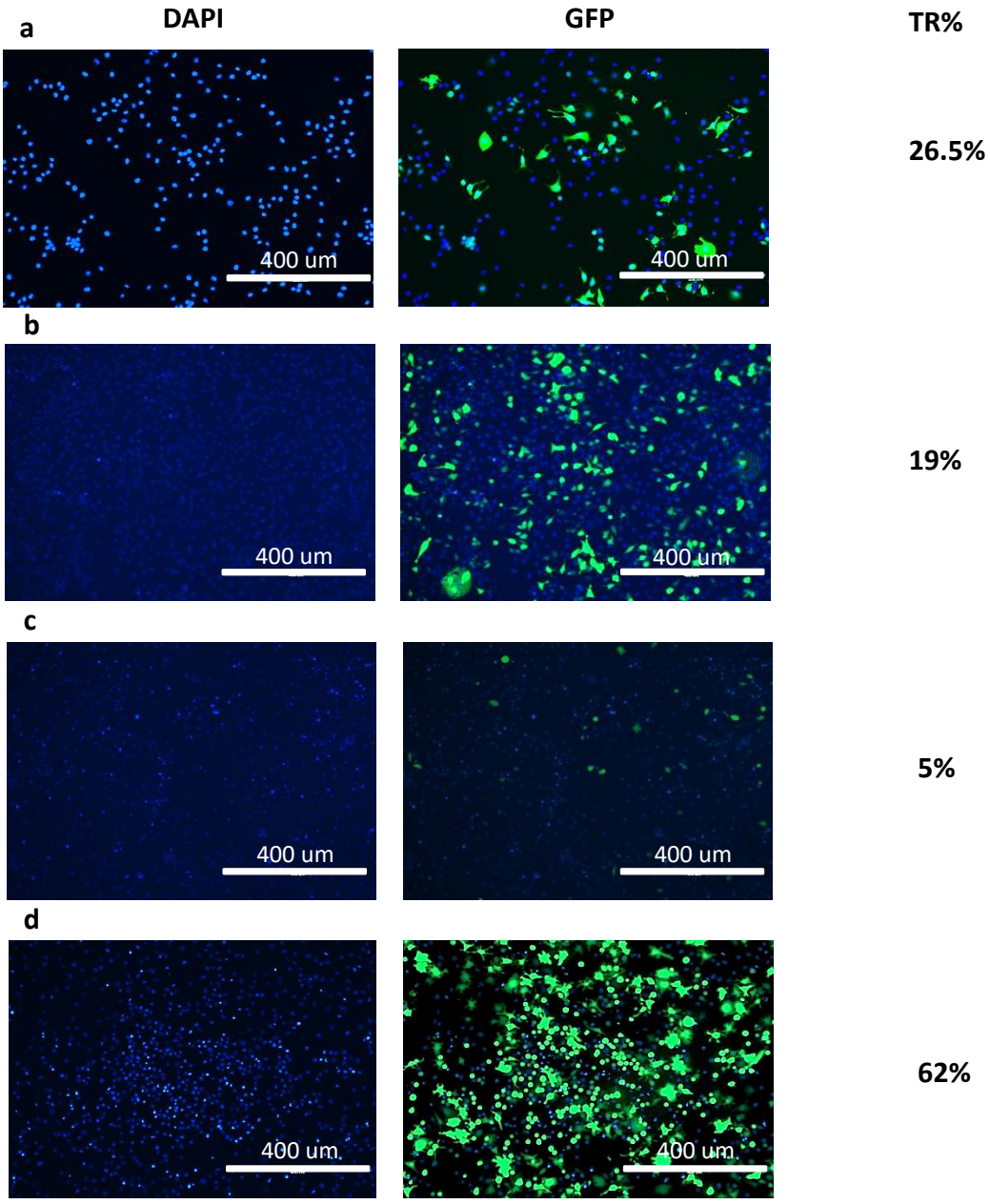
2.3.1. Transfections

RBL SX-38 cells were transfected with various concentrations of the NFAT-DsRed-Express2 plasmid and transfection efficiency was assessed 24 hours post transfection using the pmaxGFP™ Vector, which encodes the green fluorescent protein (GFP). The transfection efficiency was determined by the transfection rate: Transfection Rate (TR %) = No. of green cells/No. of total cells in the same area in each well. In each well, four different areas were measured and the average of

those four measurements resulted in the final transfection rate. As shown in **Figure 9** A a-d & **Figure 9** B, 2 µg pNFAT-DsRed-Express2 transfected in $1x10^6$ cells RBL SX-38 cells, resulted in the highest transfection rate. Hence, these cells were chosen for the final selection of RBL SX-38 NFAT-DsRed-Express2 stable transfectants.

A)

Electroporation



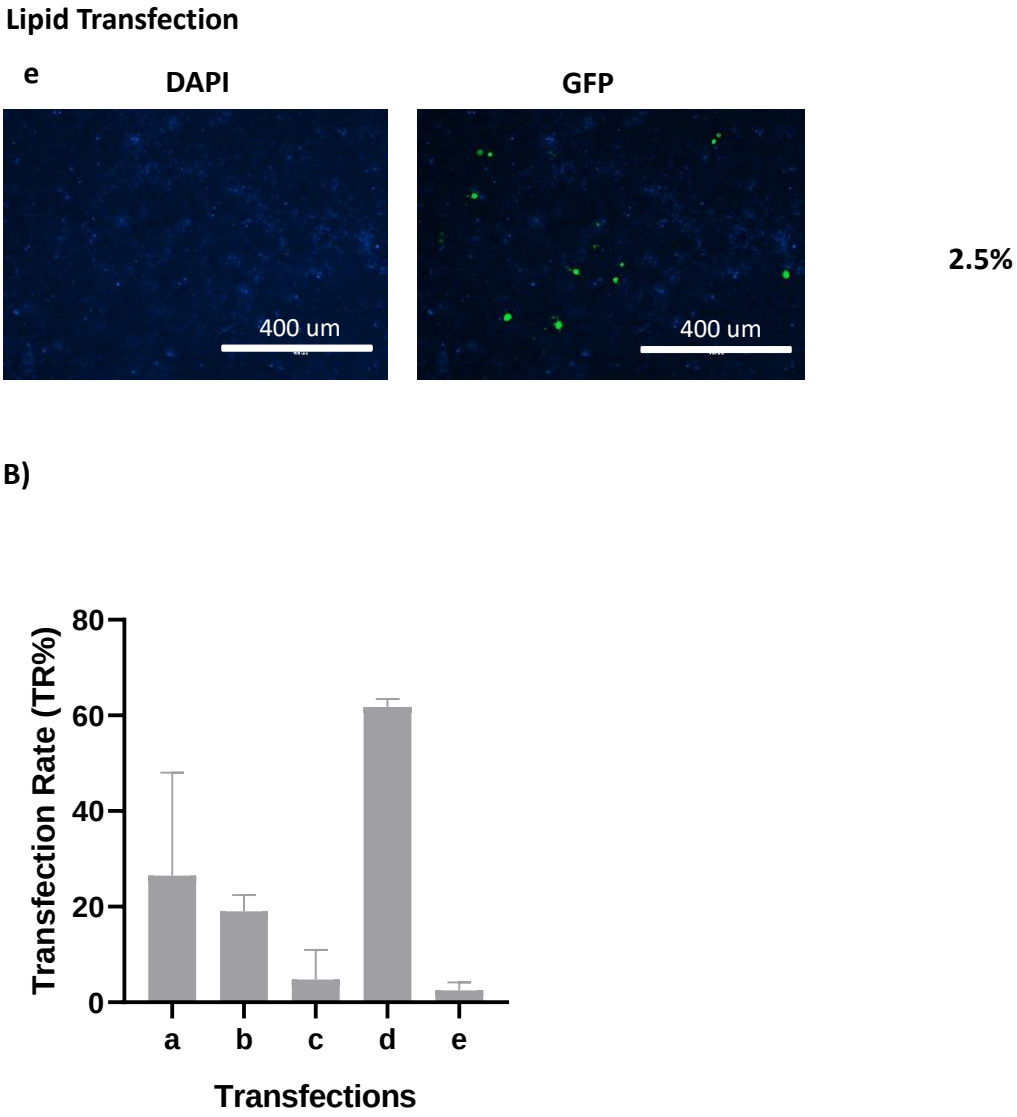


Figure 9. Transfection efficiency of pmaxGFP in RBL SX-38 cells. Representative images, 24 hours post transfection, of RBL SX-38 cells transfected with various concentrations of pmaxGFP plasmid, using two different transfection methods. **A**) Images of transfection with electroporation using a) 2 µg plasmid in $2x10^6$ cells (TR 26.5%), b) 1 µg plasmid in $2x10^6$ cells (TR 19%), c) 4 µg plasmid in $2x10^6$ cells (TR 5%) and d) 2 µg plasmid in $1x10^6$ cells (TR 62%). e) lipid transfection using 0.2 µg plasmid in 1.6 x 10^4 cells (TR 2.5%). Transfection rate was determined after quantitative analysis of the percentage of GFP cells (green) among the total DAPI cells (total population, blue). **B**) TR% of pmaxGFP plasmid in RBL SX-38 cells using different transfection methods and plasmid concentrations in the same order described in A. (Data derived from single experiments). Transfection rate (TR%) was determined as the average of four independent measurements. Statistical analysis was performed using Ordinary One-Way *ANOVA*.

2.3.2. NFAT-DsRed plasmid linearization

After several attempts to transfect the circular DNA plasmid, transfection efficiency was less than 50%. In order to increase RBL SX-38 transfection efficiency, the NFAT-DsRed-Express2 vector was linearized using the BglII restriction enzyme, after performing restriction digestion. As shown in **Figure 10**, linearization of NFAT-DsRed-Express2 circular plasmid DNA template generated linear DNA samples that were used in RBL SX-38 transfection experiments.

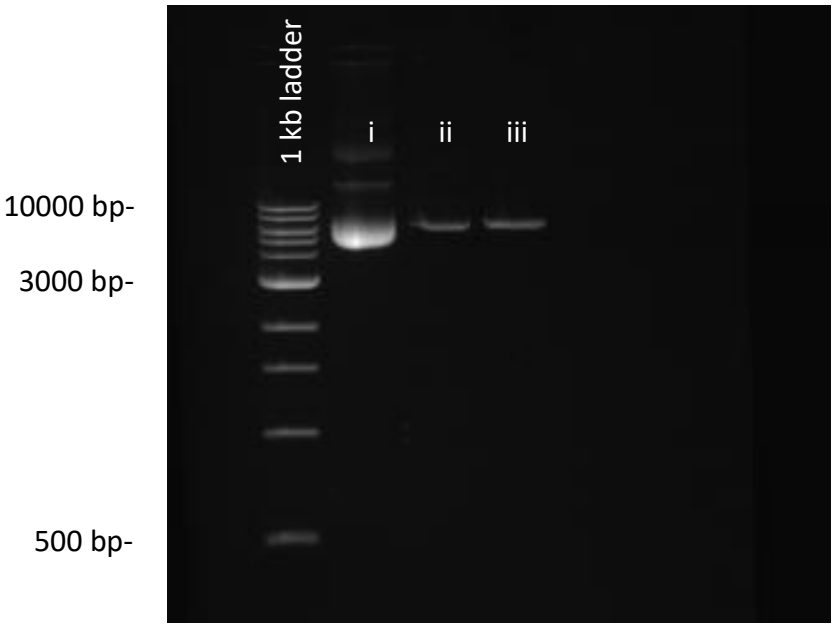
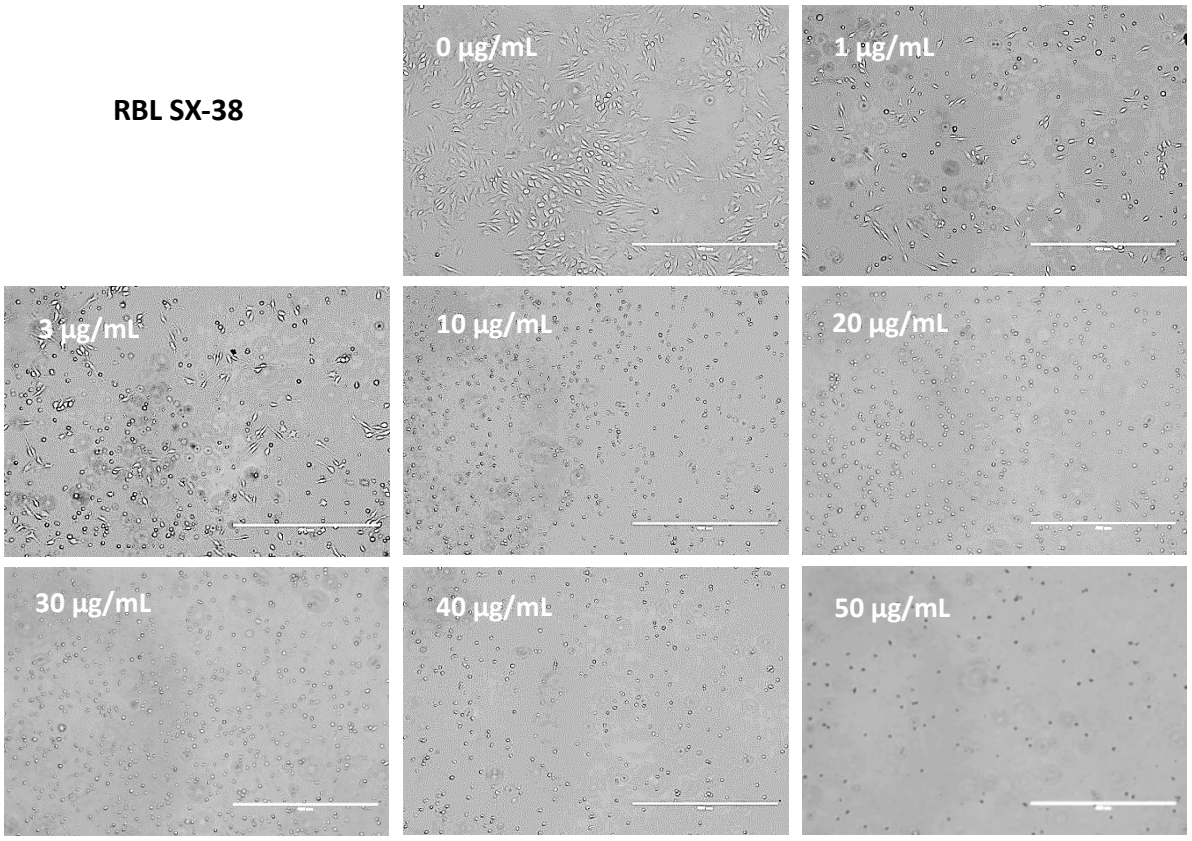


Figure 10. NFAT-DsRed-Express2 vector (5853bp) linearization using BglII restriction enzyme. Lane (i) uncut plasmid, lanes(ii & iii) linearized plasmid 34.7 ng/µL and 40.3 ng/µL, respectively. Samples run along a 1Kb Quick-load purple DNA ladder. Plasmid digestion was confirmed using a 1% w/v agarose gel and the DNA was visualized by ethidium bromide staining.

2.3.3. Blasticidin S optimization

Eukaryotic antibiotics were used to select for stably transfected cells. To determine the optimal concentration of blasticidin S for selection of RBL SX-38 cells stably transfected with NFAT-DsRed-Express2 reporter plasmid, “wild type” RBL SX-38 cells were cultured with RBL medium containing various concentrations of the selective antibiotic. After one week, the lowest concentration (20 µg/mL) that killed all cells was chosen to be used for further experiments (**Figure 11**).



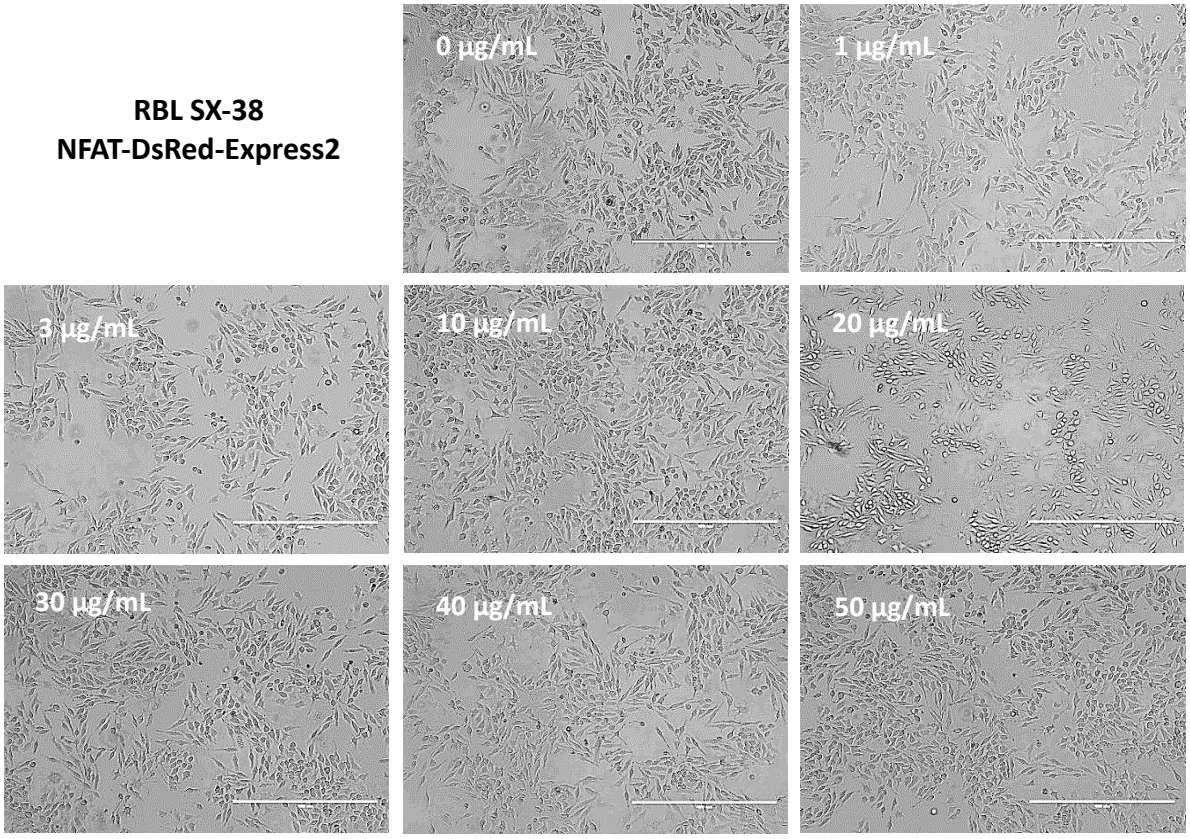


Figure 11. "Wild type" and transfected RBL SX-38 cells after one week of exposure to Blasticidin S. Cells were treated with various concentrations of the DsRed_Express2 selective antibiotic for selection of stably transfected cells. Bars represent 400 µm.

2.3.4. RBL SX-38 NFAT-DsRed cell activation

After one-month antibiotic selection of RBL SX-38 NFAT-DsRed-Express2, the cells were sensitized with human serum (1/50 dilution) and stimulated with anti-human IgE or ionomycin calcium salt (positive control) to select the highest responder cells by flow cytometry. To determine the optimal stimulatory concentration preliminary experiments were performed on RS-ATL8 and RBL 703/21 NFAT-

DsRed-Express2 cells (see Appendix III, **Figure 34** and Appendix IV,**Figure 35**) due to limited number of RBL SX-38 transfected cells. Stimulation of RS-ATL8 cells was achieved with 100 ng/mL stimulus generating the expected bell-shape curve whereas the RBL 703/21 NFAT-DsRed-Express2 cells were activated with higher concentrations of antihuman IgE (1 and 2 µg/mL), confirming the high sensitivity of RS-ATL8 cells. Therefore, for further experiments on RBL SX-38 transfected cells, we used the minimum concentration that induced activation on RBL 703/21 transfected cells. **Figure 12**. shows that stimulation of RBL SX-38 NFAT-DsRed-Express2 cells with 1 µg/mL anti-human IgE induced low levels of cell activation with low signal intensity and high background noise. Cells stimulated with 1 µg/mL anti-human IgE were chosen for further selection of clones.

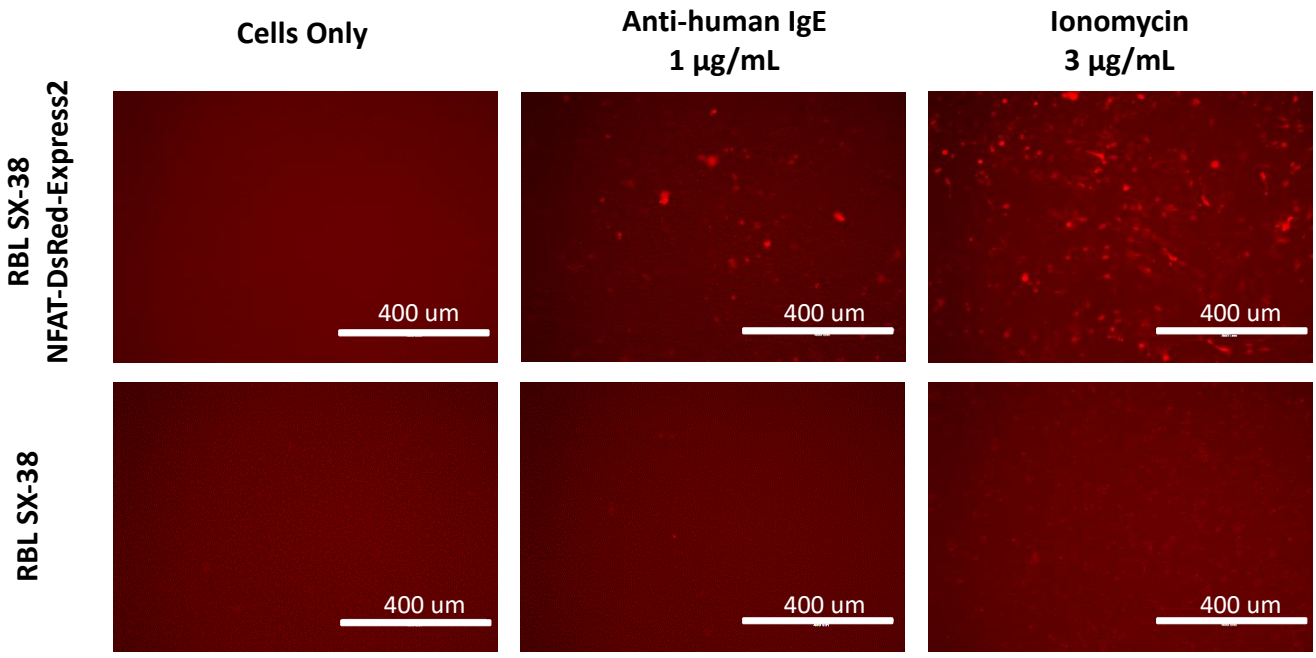


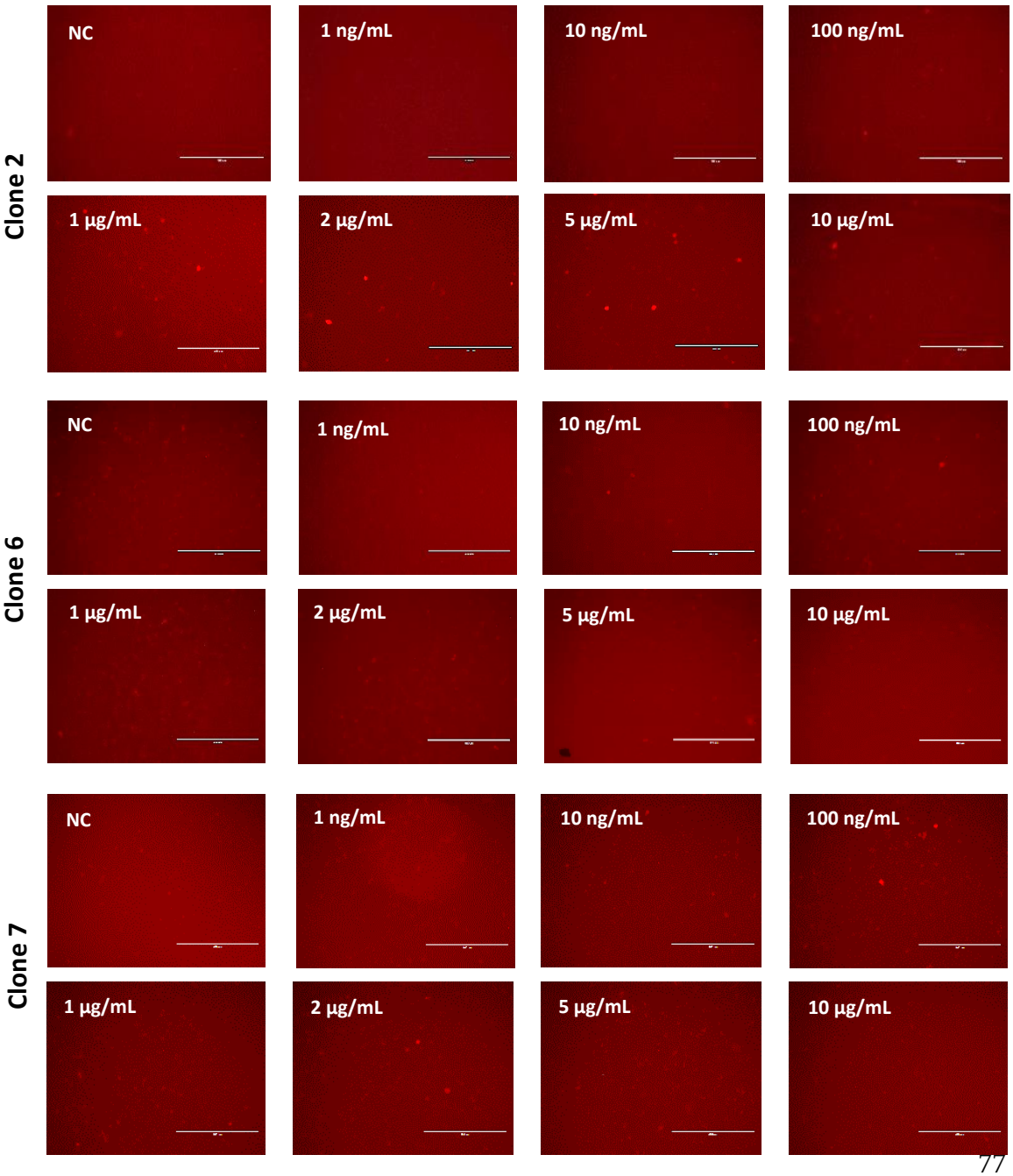
Figure 12. RBL SX-38 NFAT-DsRed-Express2 cell activation. After treating pNFAT-DsRed-Express2 transfected and non-transfected RBL SX-38 cells with selective antibiotic (20 µg/mL blasticidin S) for 30 days, cells were sensitized with human serum (1/50 dilution) and stimulated with 1 µg/mL anti-human-IgE or with 3 µg/mL ionomycin for 24 hours. Images represent single experiments that were captured with an Evos fl Digital Inverted Microscope using the RFP channel (Exc: 531 nm, Em: 593 nm).

2.3.5. Selection of clones

RBL SX-38 NFAT-DsRed-Express2 cells as described in 2.3.4 subchapter were stimulated with 1 µg/mL anti-human IgE and 3 µg/mL ionomycin (which was used as a positive control). Transfected cells stimulated with 1 µg/mL anti-human IgE were chosen for selection of highly fluorescent cells by flow cytometry. **Figure 13** A, shows that anti-human IgE stimulus did not strongly induce RBL SX-38 NFAT-DsRedExpress2 clone activation (clone 2, 6 and 7) after various concentrations of anti-human IgE tested (1 ng/mL to 10 µg/mL) compared to each clone negative

control (non-stimulated cells). **Figure 13** B, shows the net fluorescence intensity levels measured for each clone separately as depicted in **Figure 13** A against all tested concentrations. The net fluorescence intensity for each concentration was calculated by subtracting the values from non-stimulated cells that were used as a negative control.

A)



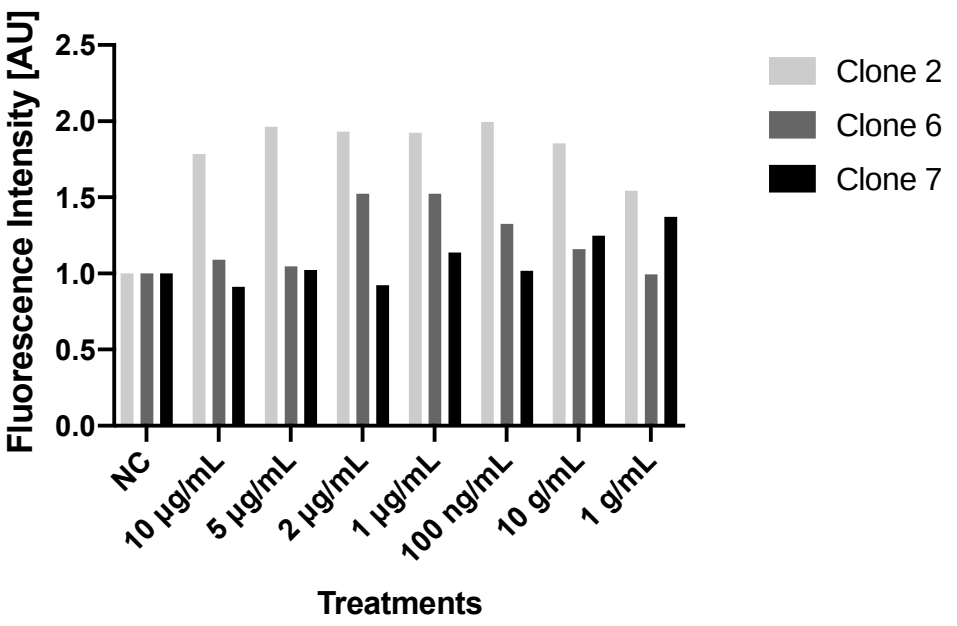


Figure 13. RBL SX-38 NFAT-DsRed-Express2 clone activation. Clones of RBL SX-38 transfected cells were sensitized for 16 hours with human serum and stimulated for 24 hours with various concentrations of anti-human IgE (1 ng/mL to 10 µg/mL). **A**) Images of activated clones with various concentrations of anti-human IgE. Images were captured with an Evos fl Digital Inverted Microscope using the RFP channel (Exc: 531 nm, Em: 593 nm). Bars represent 400 µm. **B**) Fluorescence intensity values of RBL SX-38 NFAT-DsRed-Express2 activated clones by different concentrations of anti-human IgE. Fluorescence intensity values corresponding to images in **A** were measured once and were compared with the negative control (NC) value of each clone (NC; non stimulated cells).

2.3.6. DsRed protein expression in RBL SX-38 NFAT-DsRed cells

Before performing Western Blotting analysis, expression of DsRed in pNFATDsRed-Express2 transfected RBL cells, (clone 6 described earlier in 2.3.5 Selection of clones), was observed as red fluorescence using fluorescence microscopy. Cells were sensitized with human serum overnight treated as described in 2.2.5.4 Selection of Clones and stimulated with anti-human IgE (1 µg/mL) for 24, 48 and 72 hours. Before cell harvesting, images were taken with an Evos fl Digital Inverted

Microscope using the RFP channel (Exc: 531 nm, Em: 593 nm). As shown in **Figure 14**, RBL SX-38 NFAT-DsRedExpress2 expressed very low levels of red fluorescence after 24 and 48 hours stimulation with anti-human IgE (1 µg/mL), and almost no detectable levels of fluorescence was observed after 72 hours. On the contrary, RBL 703/21 NFAT-DsRed-Express2 transfected cells which were used as a positive control expressed very high levels of red fluorescence after 24-hour stimulation with the same stimulus.

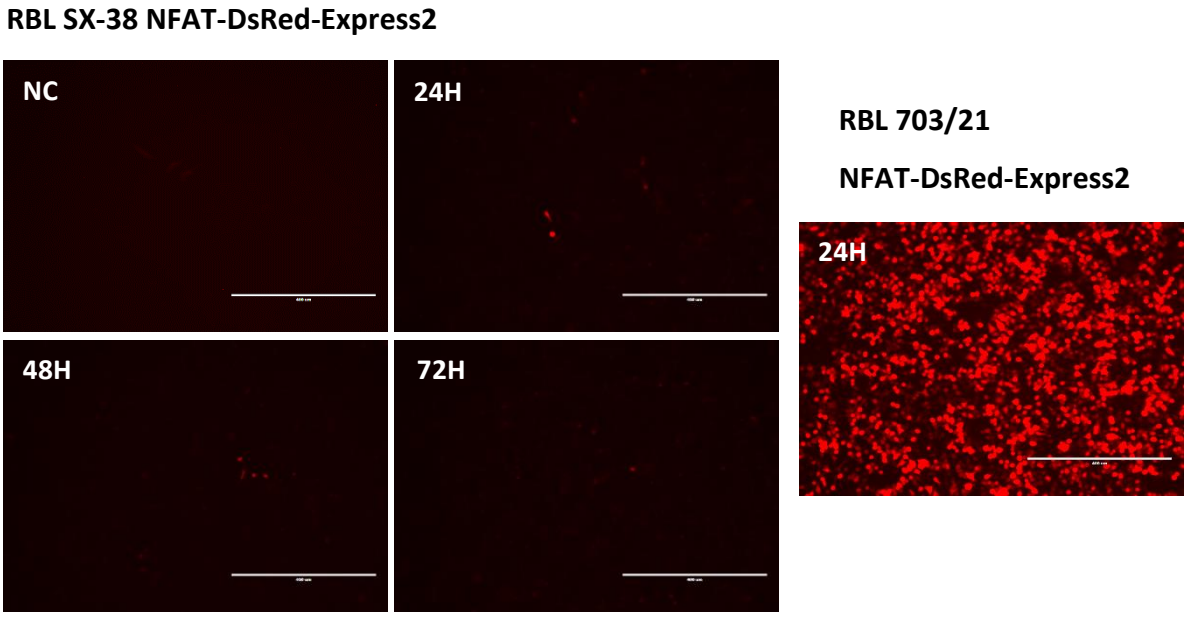


Figure 14. Fluorescence microscopy of pNFAT-DsRed-Express2 stably transfected RBL SX-38 and RBL 703/21 cells (the latter was used as a positive control), showing expression of red fluorescence after cell stimulation with human serum and anti-human IgE (1 µg/mL). Images represent single experiments captured 24, 48 and 72 hours after stimulation with anti-human IgE with fluorescence microscopy using the RFP light cube (Exc: 531 nm, Em: 593 nm) at 10x magnification. Bars represent 400 µm.

2.3.7. Red Fluorescence (RFP) expression in RBL SX-38 NFAT-DsRed cells

Western blot analysis was performed to detect DsRed protein (25.9 kDa) in RBL SX-38 and RBL 703/21 cells transfected with pNFAT-DsRed-Express2. Cells were first sensitized with human serum (see 2.2.5.4) and stimulated with anti-human IgE (1 µg/mL). Cell extracts were harvested at three different time points. As expected, no DsRed protein was detected in untreated RBL SX-38 NFAT-DsRed-Express2 cells. The DsRed protein was detected 24 and 48 hours after stimulation, reaching almost no detectable levels after 72 hours. In RBL 703/21 NFAT-DsRed-Express2 cells, expressed DsRed protein formed a strong band after sensitization with human serum and stimulation with anti-human IgE (**Figure 15**).

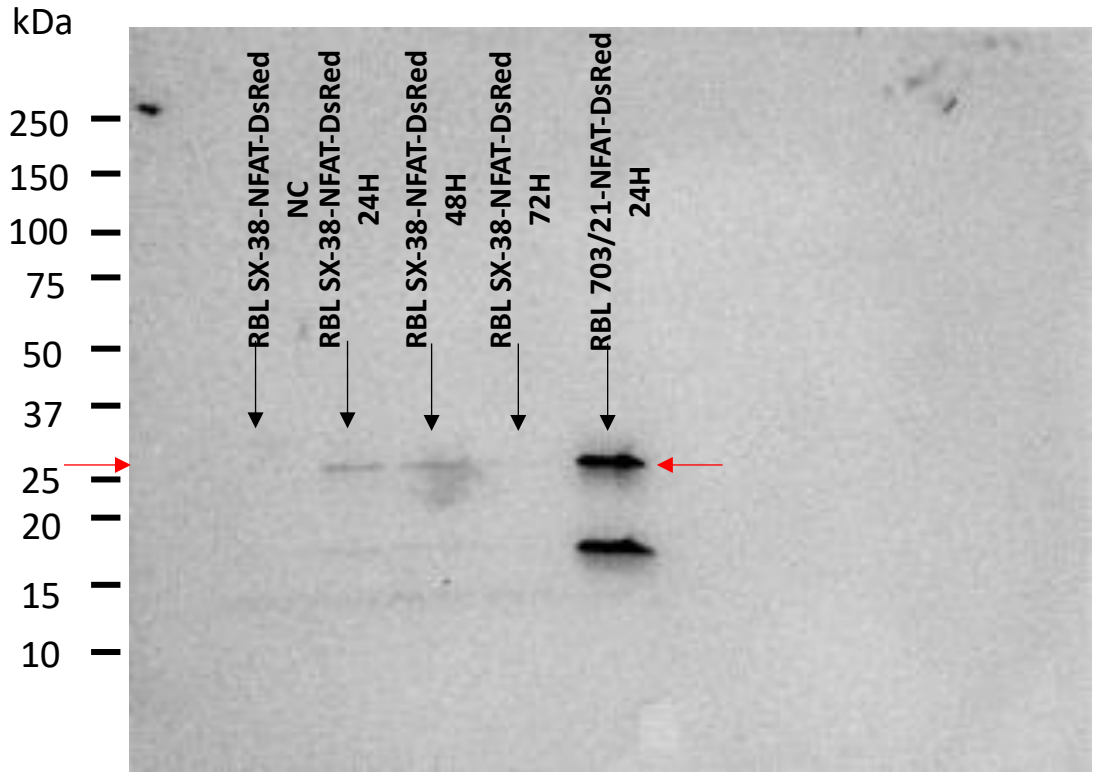


Figure 15. Immunoblot with anti-RFP antibody [6G6] for DsRed protein detection. Extracts of RBL SX-38 and RBL 703/21 pNFAT-DsRed-Express2 transfected cells, stably expressing DsRed red fluorescent protein. Samples transfected with pNFAT-DsRed-Express2 were harvested at 24, 48 and 72 hours after stimulation with anti-human IgE (except for the negative control (NC)). RBL 703/21 pNFAT-DsRed-Express2 was used as a positive control. The expected molecular weight of DsRed is 25.9 kDa and is indicated by the red arrows. The expression of DsRed was detected using a mouse monoclonal antibody [6G6] for Red Fluorescent Proteins as a primary antibody (1:1000 dilution) and goat anti-mouse IgG, HRP conjugated as a secondary antibody (1:2000 dilution). Membrane visualization was performed using a Pierce ECL chemiluminescent substrate.

2.4. Discussion

Already existing reporter cell lines such as the RS-ATL8 generated by Nakamura R., *et al*. (2010) (329) and RBL 703/21 NFAT-DsRed-Express2 generated by Wang, X *et al*. (2013) (344), contain the NFAT promoter to drive expression of their reporter genes, firefly luciferase and red fluorescent protein DsRed-Express2, respectively, after FcεRI receptor crosslinking. Both fluorescent and luminescent NFAT reporters are suitable to use in soluble allergen assays, however, only the DsRed-Express2 is suitable for an array format, as the firefly luciferase requires cell lysis before measurement of luciferase activity (344). This limitation excluded the RS-ATL8 cell line for use in diagnostic experiments using protein microarrays. Nevertheless, RS-ATL8 cells due to their highly sensitive reporter system (329) and the high surface expression of FcεRIα_H receptor compared to other RBL cell lines (323), were used as a positive control for all subsequent experiments. On the other hand, although the RBL 703/21 NFAT-DsRed-Express2 cells are efficiently activated on a microarray format, the low FcεRIα_H receptor expression compared to RS-ATL8 and RBL SX-38

(see **Table 2**), may result in a less sensitive reporter system. To generate an equally sensitive reporter cell line (i.e. lower threshold of detection) similar to RS-ATL8, we stably transfected the NFAT-DsRed-Express2 reporter gene in RBL SX-38 cells. Application of an optimized electroporation protocol resulted in the highest transfection efficiency achieved in this project (TR 62%). After activation of RBL SX38 transfected cells and the selected highest responding clones with predetermined concentrations of grass pollen reference serum (1/50 dilution) and anti-human IgE (1 µg/mL) previously tested on RBL 703/21 NFAT-DsRed-Express2 cells (Appendix III-**Figure 34**), we observed very low RBL SX-38 NFAT-DsRed cell activation compared to RBL 703/21 transfected cells. However, this observation was in agreement with previous findings that reported poor responsiveness of RBL SX-38 transfectants to IgE/a-IgE stimulation (see Xiaowei Wang and Daniel Wan PhD Theses, University of Nottingham, 2014). To confirm expression of the DsRed protein in RBL SX-38 transfected cells, we performed Western Blot analysis using an RFP protein-specific antibody. The expected size of the red fluorescence protein (RFP), 25.9 kDa, was confirmed for both NFAT-DsRed-Express2 transfected cells, RBL 703/21 and RBL SX-38, respectively. However, expression of RFP in induced RBL SX-38 transfectants was clearly lower compared to RBL 703/21 transfectants. This might have resulted from the low percentage of RBL SX-38 clones transfected with the DsRed reporter gene. Thus, the majority of the cells do not express DsRed or possess a defective DsRed, or a mutation in the NFAT promoter region. Expression of DsRed was assessed before in preliminary experiments (Appendix

VII, **Figure 36** and **Figure 37**) on RBL 703/21 NFAT-DsRed-Express2 cells that were used as a positive control. Western blot analysis revealed that in induced RBL 703/21 cells, the RFP was strongly expressed after 24 and 48 hours while gr adually declined after 72 hours. DsRed-Express2 is a variant of the red fluorescent protein DsRed which is homologous to green fluorescent protein (GFP). DsRed has higher extinction coefficient and quantum yield compared to GFP and is less sensitive to photo bleaching with a wider pH tolerance range (4.5-12) (346, 347). Strack et al. (2008) reported that the average fluorescence in most RFPs remains nearly constant from 24 h to 48 h, as in DsRed-Express2, and then progressively declines. Similar findings are presented in **Figure 15** and Appendix VII-**Figure 37**, showing gradual decrease on DsRed expression at 72 hours post stimulation with anti-human IgE. The authors also reported that cells containing DsRed-Express2 proliferated equally to those containing EGFP, it is minimally cytotoxic to a variety of cells, has low phototoxicity, high solubility and overall DsRed-Express2 shows strong and stable expression in bacterial and mammalian cells (348). On the other hand, Zhou *et al*. (2011) reported that despite the great potential in application DsRed has several limitations. Initially, its maturation is slow and might take up to several days at room temperature. They also reported that DsRed and its variants are more prone to oligomerization and aggregation, related to cytotoxicity, growth defects and/or instability (348- 350). Zhou *et al*. findings could provide a possible explanation on our observations, provided that both transfected cell lines (RBL 703/21 and RBL SX-38 showed similar results. However, this is not evident from

our results. Collectively, our findings are in agreement with Strack, R. L *et al*., as cell viability was not affected in both DsRed-Express2 transfected cell lines. It remains though to be understood why stimulation of NFAT-DsRed-Express2 transfected RBL SX-38 cells with anti-human IgE results in lower DsRed expression compared to RBL 703/21 transfected cells.

2.5. Conclusion

Transfection of the linearized NFAT-DsRed plasmid by electroporation in RBL SX-38 cells resulted in high transfection efficiency. However, the NFAT-driven fluorescent reporter system generated low signal fluorescence intensity after stimulation with anti-human IgE. Thus, we concluded that this reporter system was not suitable yet to be used for our future experiments with protein microarrays.

Chapter 3: NFAT-DsRed-FCER1G Hygromycin Reporter System

3.1. Introduction

In Chapter 2, stably transfected RBL SX-38 cells showed low DsRed-Express2 expression levels and unconvincing levels of fluorescence intensity after activation. However, in order to generate a cell line capable to bind human IgE in serum samples used for sensitization, thus defining a lower threshold of detection, i.e. sensitivity, as RBL SX-38-derived RS-ATL8 we also tested the RBL NFAT-DsRed cells. As described earlier, these cells are suitable to use in a microarray format since detection of cell activation relies on measurement of intracellular fluorescence that does not require cell lysis (322). However, as shown in **Table 2**, RBL NFATDsRed cells have very low expression levels of human FcεRIα chain (α_H 16,000) compared to RS-ATL8 reporter cells (α_H 500,000). To increase cell sensitivity, we transfected RBL NFAT-DsRed cells with the human ɣ-chain. As described in 1.2.5 FcεRI Signal Transduction subchapter, co-expression of γ-chain is important for masking ER retention signals resulting in assembly, maturation and export of the FcεRI complex from the ER, and transport to the cell surface (112, 139- 141). Importantly, higher FcεRI α-chain expression levels on the cell surface facilitate increased binding of human serum IgE used for sensitization, resulting in stabilization of the IgE-FcεRI complex. Additionally, this stabilization prevents degradation of the high affinity IgE receptor while sustaining basal synthesis (133).

Aim and Objectives

Here, we attempted to increase human FcεRIα chain expression levels in RBL NFAT-DsRed cells by stably transfecting FcεRIγ$_H$. We also compared human α-chain expression in various RBL cell lines (RBL 2H3, RBL SX-38, RS-ATL8, RBL 703/21 and NFAT-DsRed). Lastly, we assessed whether the increased FcεRIα$_H$ surface levels in NFAT-DsRed resulted in a higher response than the single transfectant parental cell line.

3.2. Materials and methods

3.2.1. FCERIG-Hygromycin Plasmid Construction

pBJ1 neo-hu FcεRI gamma (Plasmid #16540) encoding the full length human FcεRI gamma chain was obtained from Addgene (351). To perform stable transfection of pBJ1 neo-hu FcεRI gamma plasmid into RBL NFAT-DsRed cells, a new selective marker needed to be cloned into the plasmid. This would facilitate selection of cells only expressing FcεRIγ$_H$. The Hygromycin gene was chosen as a suitable marker to select stably transfected cells. Before cloning could be planned, it became necessary to fully sequence the pBJ1 neo-hu FcεRI gamma, as no sequence (except the insert sequence) was available on Addgene. Full map coverage was obtained after performing seven consecutive rounds of Sanger sequencing (Appendix VIII, **Figure 38**), using the primers listed in **Table 3** .

Table 3. Primers for complete coverage of pBJ1 neo-hu FcεRI gamma plasmid.

Oligo Name	Sequence (5'-3')
ROUND 1	
FCER1G-1 FOR	GAATTGTCCTCACCCTCCTCT
FCER1G-1 REV	ACTGTGGTGGTTTCTCATGC
ROUND 2	
FCER1G-2 FOR	CTGGCTTAACTATGCGGCATC
FCER1G-2 REV	CACCCTAACTGACACACATTCC
ROUND 3	
FCER1G-3 FOR	CGCCTTATCCGGTAACTATCG
FCER1G-3 REV	GCCTAATTTAAATGAGGACTTAACCTG
ROUND 4	
FCER1G-4 FOR	TGCTGAATGCCAGTTACCTTCG
FCER1G-4 INT FOR	TGCTAACACACCCTGCAGCTC
FCER1G-4 REV	GCTGTTTACCAACACTTCTGG
ROUND 5	
FCER1G-5 FOR	CTTCAGCATCTTTTACTTTCAC
FCER1G-5 REV	AGTCAGCAGTAGCCTCATCATC
ROUND 6	
FCER1G-6A FOR	CTACCTACAGAGATTTAAAGCTCTAAGG
FCER1G-6B FOR	GGTTTTCCAGCACCATTTTCATG
FCER1G-6C FOR	TGCCTCGCGCGTTTCGGTG
FCER1G-6D FOR	AGAGTTGGTAGCTCTTGATC
FCER1G-6E FOR	CACTCGTGCACCCAACTG
ROUND 7	
FCER1G-7A FOR	TGAACTGCAGGACGAGGCAG
FCER1G-7B FOR	ATCAAGAAGCACTGTGGTTGC
FCER1G-7C FOR	GTAGCTTTAGAATAGATGCGGTC
FCER1G-7D FOR	GGTTCCGCGCACATTTCC

All sequencing was outsourced to Source Bioscience in Nottingham. Results were assembled in SnapGene (see Map and complete sequence in Appendix VIII, **Figure 38**). Oligonucleotide primers were ordered from SigmaGenosys UK (now Merck). Lyophilised DNA oligos were dissolved in sterile water (RNase/DNase free) to a final stock concentration of 100 µM, and kept at -20 °C.

pTK-Hyg vector (GenBank Accession No.: U40398) (plasmid map in Appendix IX, **Figure 39**) was purchased from Clontech (now TakaraBio) and was propagated in E. coli using standard molecular biology techniques (Subchapter 2.2.2).

3.3.1.1. Hygromycin gradient PCR

For Hygromycin gene (1038bp) amplification, gradient polymerase chain reaction (PCR) (25 µL) was set up (Appendix IX, **Table 13**) in 0.2 ml thin walled PCR tubes (Starlab, UK) in a PTC 2000 Peltier Thermal Cycler (MJ Research, Canada). Various annealing temperatures were tested ranging from 62°C to 68°C. The reaction programme was set to 94 °C/1 min initial denaturation, 30 sec annealing and extension at 72 °C/1,5 min, for a total of 35 cycles, using pTK-Hyg plasmid as template (2.4 ng/ µL) and primers Hyg+BglII (Forward) ATATAT**AGATCT**ACCATGAAAAAGCCTGAACTCACCGCGACG and Hyg+BstBI (Reverse) TATATA**TTCGAA**TCAGTTAGCCTCCCCCATCTCCCGATC (Appendix IX,

Table 12). Optimized PCR samples/reactions were analysed by 1% agarose gel electrophoresis and the DNA was visualized by ethidium bromide staining as described in 2.2.5.3 subchapter.

3.3.1.2. FCER1G-Hygromycin cloning

In order to subclone the Hygromycin gene insert into the pBJ1 neo-hu FcεRI gamma vector we first amplified the selection marker coding sequence by PCR. PCR reactions were performed to a final volume of 50 µL, containing 5 µL (1X) High Fidelity PCR buffer, 1 µL (0.2 mM) dNTP mix, 0.2 µL (1U) Platinum™ Taq DNA Polymerase High Fidelity, 0.2 µL (2 mM) $MgCl_2$, 1.5 µL (5 µM) of the pre-designed Hyg+BglII primer pair in one reaction and Hyg+BstBI primer pair in the second reaction and 38.8 µL molecular biology grade water as described in Appendix IX,

Table 14 in 0.2 ml thin walled PCR tubes (Starlab, UK) in a PTC 2000 Peltier Thermal Cycler (MJ Research, Canada).

The annealing temperature was experimentally determined to 64.6 °C see 3.3.1.1. subchapter. A reaction program was set as follows, initial denaturation at 94 °C/30 sec, denaturation at 94 °C/15 sec, annealing 64.6 °C/30 sec and extension 68 °C/1minute, using pTK-Hyg plasmid as template (39.1 ng/µL) and primers Hyg+BglII (Forward) ATATAT**AGATCT**ACCATGAAAAAGCCTGA ACTCACCGCGACG and Hyg+BstBI (Reverse) TATATA**TTCGAA**TCAGTTAGCCTCCCCCATC TCCCGATC (Appendix IX,

Table 12). The obtained (expected 1038 bp) PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Germany) before setting up the restriction digest with BglII and BstBI (NEB, USA). Double digestion was initially performed for 2 hours at 65°C for BstBI following by another 2 hours at 37 °C for BglII by adding 100 mM NaCl (Appendix IX, **Table 15**). Both enzymes did not require heat inactivation according to the manufacturer. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Germany).

The NeoR (Neomycin) gene (795 bp) included in the original pBJ1 neo-hu FcεRI gamma vector (Appendix VIII, **Figure 38**) was double digested using BglII and BstBI under the same conditions described above. The restricted plasmid, from which the NeoR gene had been removed, was separated in 1 % agarose gel and extracted using Gel Extraction Kit (Qiagen, Germany).

The ligation reaction between the final insert product (Hygromycin + BglII/BstBI) and the resulting digested vector (pBJ1 neo-hu FcεRI gamma) were carried out in two different conditions regarding insert: vector molar ratios (3:1 and 5:1). The ligation mix was incubated overnight at 12 °C (Appendix IX, **Table 17**). Then, the ligated product was transferred to DH5α E. coli using a standard bacterial transformation protocol. Successfully transformed plasmids were purified using QIAprep® Spin Miniprep Kit (QIAGEN, Germany). Purified plasmids mixed with (0.8 µL) gel loading dye, purple (6x) (NEB, USA) were run alongside 1kb DNA ladder purple Quick-load (BioLabs, UK) in 1% agarose gel with 2.5 mg/ml ethidium bromide (Sigma, UK) in 0.5xTBE buffer (Alfa Aesar, UK) at 100 V for 60 minutes then imaged by GeneGENIUS (SYNGENE, UK). DNA sequencing analysis was performed using the primers listed in Appendix IX,

Table 12 by Source Bioscience, Nottingham. After comparing DNA sequence analysis between samples iii and iv (Appendix IX,**Figure 40**), plasmid FCERIG-Hygromycin iv was used for all subsequent experiments (see complete FCERIG-Hygromycin plasmid map at Appendix IX, **Figure 40**).

3.2.2. Transfections with human FcεRI gamma chain

The plasmid FCERIG-Hygromycin iv was used for all subsequent transfection of NFAT-DsRed cells. Before nucleofection, cell culture confluency was checked under the light microscope, since the optimal confluency for nucleofection is 75–80%. For

each nucleofection, 2 µg FcεRI gamma plasmid was transfected in $4x10^6$ cells in 100 µL cell culture medium in Nucleocuvette vessels using the SF Cell Line Optimization 4D-Nucleofector XL kit (Lonza, UK). To determine transfection efficiency, an equal number of cells were transfected using 2 µg pmaxGFP vector, constitutively encoding a green fluorescent protein. Post nucleofection, 700 µL transfected cells resuspended in fresh warm low calcium medium (RPMI 1640, Merck, USA) were transferred into a clear, flat-bottomed, tissue-cultured treated 6-well polystyrene plate (Corning, UK) containing 1 mL fresh warm medium without antibiotics and placed in a 37°C cell incubator for 24h. Transfected cell images were then taken using an Evos fl Digital Inverted Microscope. For green fluorescence, the GFP light cube was used (470 nm excitation, 525 nm emission). Images were taken at 10x magnification, and transfection efficiency was calculated as the percentage of GFP cells (green) among the total DAPI-stained cells (total population, blue) using a PureBlu™ DAPI Nuclear Staining Dye (Bio-Rad, UK). 24h post transfection, fresh warm medium was added to the transfected cells; this was supplemented with 600 µg/mL Hygromycin B (Invivogen, UK), based on kill curves determined in preliminary experiments. Cells were kept under the selective antibiotic pressure for three weeks. Next, surviving transfected cells were transferred to a 24-well Upcell cell culture dish (ThermoFisher, USA) containing $2x10^6$ cells in each well in a total volume of 500 mL.

3.2.3. RT-PCR

Isolation of mRNA and cDNA synthesis was carried out using µMACS One-step cDNA Synthesis Kit (Miltenyi Biotec, Germany) following the manufacturer's protocol with oligo(dt) MicroBeads for priming. The relevant genes were amplified by PCR using species-specific (subscript H = human, R = rat) oligonucleotide primers (Sigma-Aldrich, UK), with the expected amplicon sizes given in brackets for cDNA and genomic DNA (**Table 4**).

Table 4. Sequences and predicted amplicon sizes for oligonucleotides primers.

Transcript target	Primer direction	Oligonucleotide sequence (5'⟶3')	cDNA/gDNA product size (bp)
βACT_R	Forward	TGAGAGGGAAATCGTGCGTG	278/368
	Reverse	TGTTGGCATAGAGGTCTTTACGG	
GAP-DH_H	Forward	TGATGACATCAAGAAGGTGGTGAAG	240/240
	Reverse	TCCTTGGAGGCCATGTGGGCCAT	
FcεRIα_H	Forward	AATGGCAGCCTTTCAGAAGA	360/2165
	Reverse	CTCATAGTCCAGCTGCCACA	
FcεRIβ_H	Forward	TCCTGGACAGCTCGGTTAAT	338/1722
	Reverse	TCCCCAGAATGGATAACCTG	
FcεRIγ_H	Forward	GGAGAGCCTCAGCTCTGCTA	218/946
	Reverse	CATCTATTCTAAAGCTACTGTGGTGGT	

PCR reactions (20 µL) were set up in 0.2 ml thin walled PCR tubes (Starlab, UK) in a PTC 200 Peltier Thermal Cycler (MJ Research, USA) using the following cycling conditions: 2 min initial denaturation, followed by 35 cycles of denaturing (30 sec, 94°C), annealing 45 sec, 60°C) and extension (90 sec, 72°C) followed by a final

extension (5 min, 72°C). Each polymerase chain reaction (PCR) for analysis was made up by mixing 10 µL 2x GoTaq Hot Start Green Master Mix (Promega, UK), 7 µL molecular biology-grade water, 1 µL 10 µM (500 nM final concentration) of each appropriate forward and reverse oligonucleotide primer and 1µL DNA template. PCR product sizes were verified on agarose gel electrophoresis (1% w/v) in 0.5x Tris/borate/EDTA buffer (Thermo Fisher Scientific, UK) alongside 100 bp TriDye DNA ladders (New England Biolabs, USA) before imaging under UV light using a GeneGenius Gel Imaging System (Syngene, UK) using a 400ms exposure for image capture.

3.2.4. Assessment of Human FcεRI alpha chain expression by flow cytometry

Cell suspensions from RBL 2H3 and the five humanized RBL cell lines (RS-ATL8, RBL SX-38, RBL 703/21, NFAT-DsRed and NFAT-DsRed FCERIG) were collected as described in Subchapter 2.2.1 in a 24-well Nunc™ Multidish with UpCell™ Surface (Thermo Fisher Scientific, USA) to a final concentration 5 x 10^5 cells/well in 500 µL/well. To block endogenous immunoglobulin receptors, cells were incubated with 2% rat serum (Sigma-Aldrich, UK) for 4h at 37°C. Cell viability was previously assessed with various concentrations of rat serum after 24 h incubation (Appendix X, **Figure 41**). Cells were then washed once with phosphate buffered saline (PBS) (Merck, UK), and sensitized with 1 µg/mL human IgE (BioPorto) for 16 h at 37°C. After this incubation, cells were kept at room temperature for 30 min in order to detach from the plate surface and then transferred to labelled FACS tubes. Subsequently, sensitized cells were stained with 5 µl APC-labelled anti-human FcεRI α-chain monoclonal antibody (BioLegend, AER-37 (CRA-1)) and washed three

times with 4 mL DPBS until performing FACS analysis using a Beckman Coulter FC500 Cytometer.

3.3. Results

3.3.1. FCERIG-Hygromycin Plasmid Construction

3.3.1.1. Hygromycin gradient PCR

To determine the optimum annealing temperature for Hygromycin gene amplification by PCR, we tested three different temperatures ranging from 62°C to 68°C. As shown in **Figure 16**, lane ii corresponding to 64.6 °C was chosen as the optimum annealing temperature having a clearer band than lane i, which also had a smear and thicker band than lane iii.

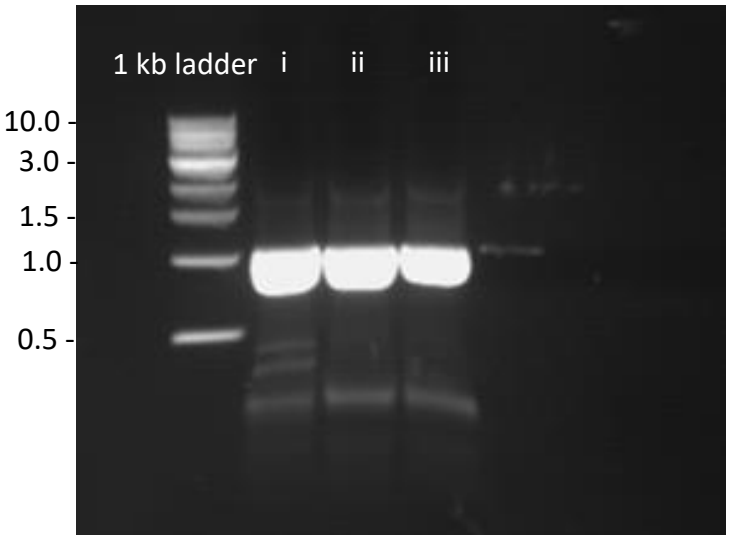


Figure 16. Hygromycin gene (1038bp) gradient PCR. Different lanes represent i) 62 °C ii) 64.6 °C & iii) 68 °C annealing temperatures. Samples run along a 1Kb DNA ladder (New England Biolabs, USA). Plasmid annealing temperatures were confirmed using a 1% w/v agarose gel and the DNA was visualized by ethidium bromide staining**.**

3.3.1.2. FCERIG-Hygromycin Ligation

After digestion of both the insert (Hyg PCR product) and the vector (pBJ1 neo-hu FceRI gamma) with BglII and BstBI restriction enzymes, the purified DNA were ligated in either 3:1 or 5:1 molar ratio (insert: vector), followed by bacterial transformation. Recombinant clones were selected for plasmid DNA extraction with subsequent confirmation of the cloning by double digestion with the aforementioned restriction endonucleases. The resulting DNA samples were analysed in 1% w/v agarose gel. As shown in **Figure 17**, purified DNA samples iii and iv generated distinct bands at desired size on the agarose gel, indicating the presence of the target sequence (Hygromycin – 1038 bp) in the constructed plasmid.

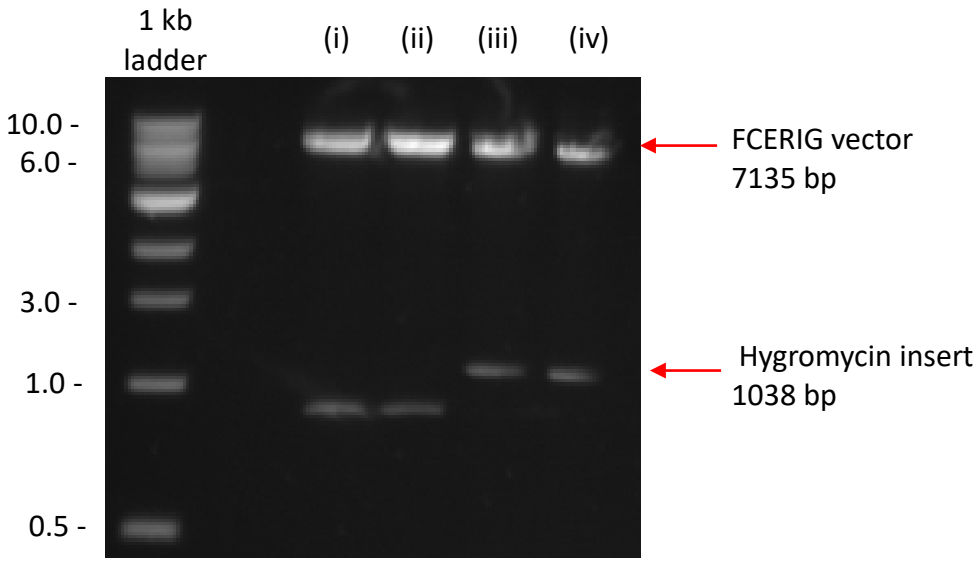


Figure 17. Confirmation of Hygromycin gene subcloning in pBJ1 vector. Four colonies (i-iv) (Hygromycin+ BglII/BstBI and pBJ1 neo-hu FcεRI gamma) were selected for confirmation of cloning by restriction enzyme double digestion. Samples were analysed in 1% agarose gel and DNA was visualized with ethidium bromide staining.

3.3.2. Transfections

RBL NFAT-DsRed cells were transiently transfected with 2 µg pBJ1 neo-hu FceRI gamma plasmid and transfection efficiency was assessed 24 hours post transfection using the pmaxGFP™ Vector, which encodes the green fluorescent protein (GFP). Transfection efficiency was determined by the transfection rate: Transfection Rate (TR %) = No. of green cells/No. of total cells in the same area in each well. In each well, four different areas were measured and the average of those four measurements resulted in the final transfection rate. As shown in **Figure 18** (overlay image), FcεRI gamma plasmid transfection rate in RBL NFAT-DsRed cells was assessed to 42%.

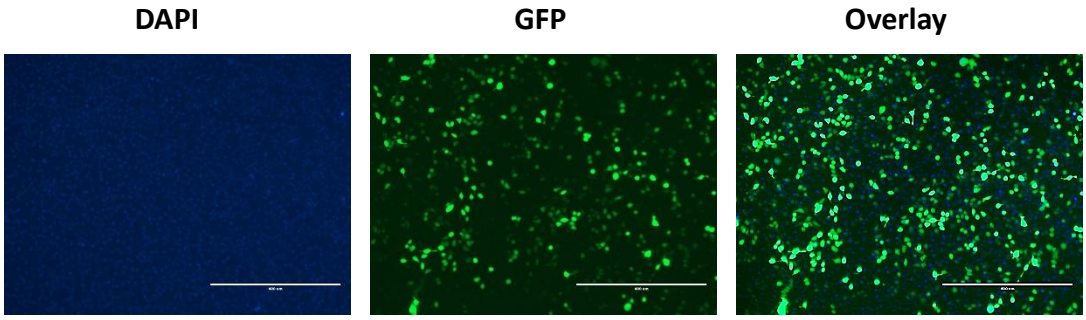


Figure 18. Transient transfection of FcεRI ɣ-chain in RBL NFAT-DsRed cells. Representative images, 24 hours post transfection, of RBL NFAT-DsRed cells transfected with pmaxGFP plasmid. The transfection rate was determined after quantitative analysis of the percentage of GFP cells (green, middle image) among the total DAPI stained cells (total population, blue, left image) as shown in the overlay image (right) .

Stable transfection of RBL NFAT-DsRed cells with FCERIG-Hyg plasmid. Transfection efficiency was assessed 24 hours post transfection using the pmaxGFP™ Vector. In **Figure 19**, GFP image shows the transfection efficiency determined to 48% after

counting the number of green fluorescent protein cells over the total number of cells.

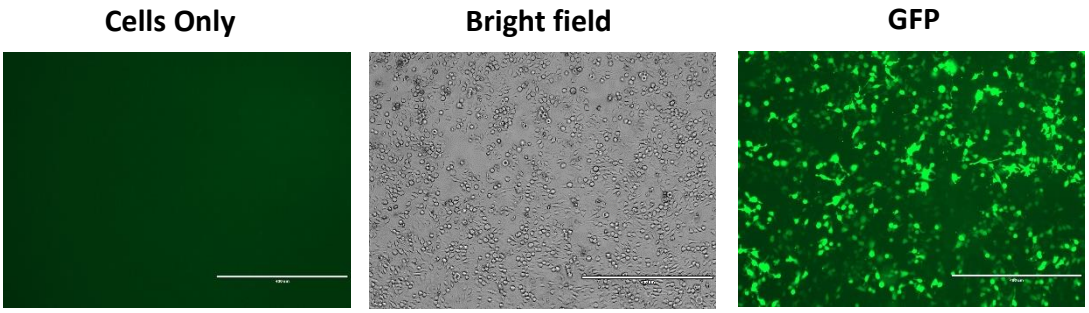
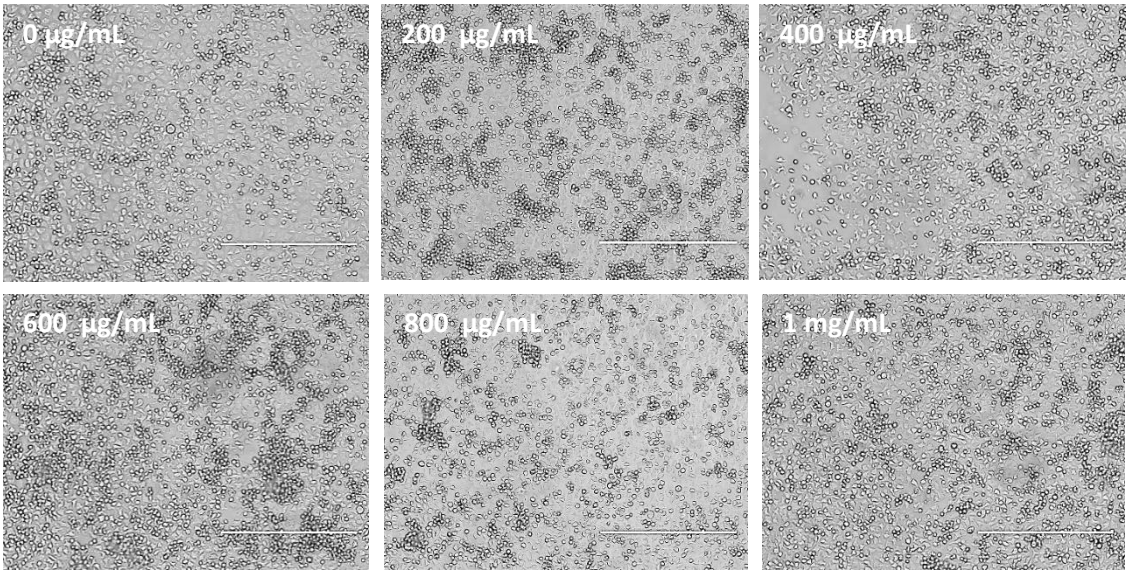


Figure 19. Stably transfected NFAT-DsRed cells with FCERIG-Hyg plasmid. Representative images, 24 hours post transfection, of RBL NFAT-DsRed cells transfected with pmaxGFP plasmid. The transfection efficiency was determined after quantitative analysis of the percentage of GFP cells (green) among the total number of cells. Left: cells only; non-transfected cells, middle: bright field; transfected cells, right: GFP channel; GFP transfected cells .

3.3.3. Hygromycin B optimization

To determine the optimal concentration of Hygromycin B selection antibiotic for selection of RBL NFAT-DsRed cells stably transfected with FCERIG-Hygromycin plasmid "wild type" RBL NFAT-DsRed cells were cultured with RBL medium containing various concentrations of the selective antibiotic. After 13 days, the lowest concentration that killed most cells was determined (1 mg/ mL) which was used for maintenance of the Hygromycin gene (**Figure 20**).

RBL NFAT-DsRed FCERIG-Hygromycin



RBL NFAT-DsRed

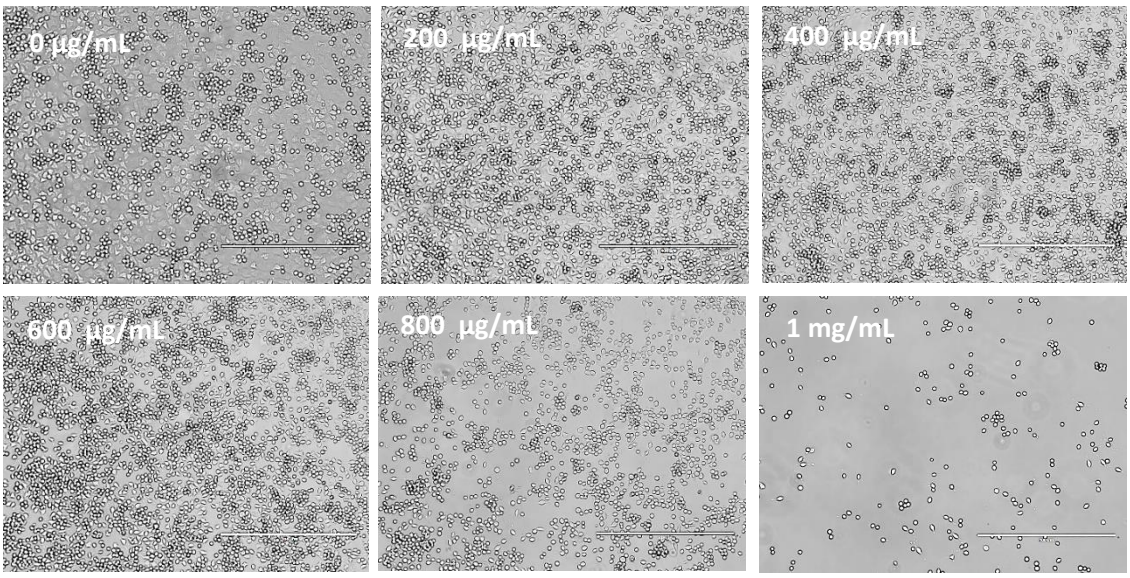


Figure 20. "Wild type and transfected RBL NFAT-DsRed cells after two weeks of exposure to Hygromycin B. Cells were treated with various concentrations of the selective antibiotic for selection of stably transfected cells. Bars represent 400 µm.

3.3.4. RT-PCR

To confirm mRNA expression of the human FcεRIα and FcεRIγ chains we compared transfected and non-transfected RBL NFAT-DsRed cells by RT-PCR. Messenger RNA was isolated and reverse transcribed into single stranded cDNA, which was subjected to PCR using primers specific for the human subchains of the FcεRI receptor. The housekeeping gene rat β-actin for RBL was included as positive control. As shown in **Figure 21**, the primers specific for human FcεRIɣ generated a strong single band for the γ_H chain for the transfected NFAT-DsRed FCERIG cells but not for the non-transfected, confirming the successful integration of the inserted human FcεRIɣ.

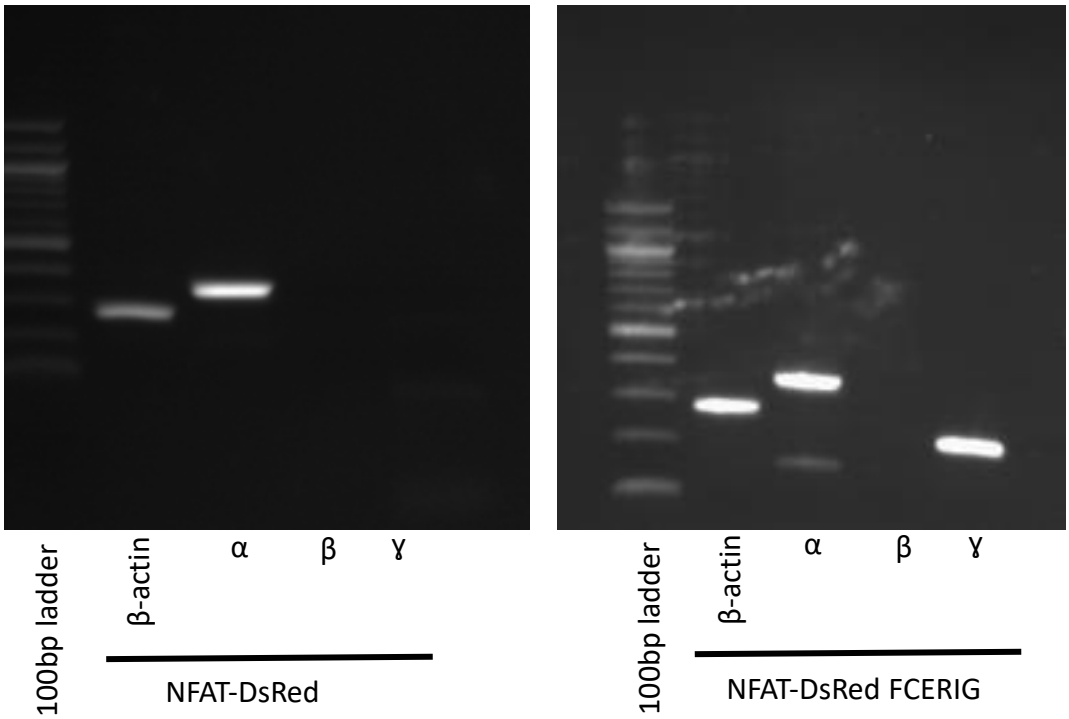


Figure 21. RT-PCR demonstrating expression of FcεRIγ chain in humanized RBL NFAT-DsRed cells. Left; non-transfected NFAT-DsRed cells, right; stably transfected NFAT-DsRed FCERIG showing FcεRIγ_H expression.

3.3.5. Human FcεRIα expression

Next, we examined whether transfection of NFAT-DsRed reporter, which showed the lowest FcεRIα_H surface expression, could be increased by transfection with FcεRIγ_H. This was based on the observation that the humanized cell lines which co-expressed FcεRIα_H and FcεRIγ_H (RS-ATL8 and its parental RBL SX-38) had the highest FcεRIα_H surface expression, whereas single α-chain transfectants had much lower expression.

This is a pivotal parameter, as it determines the ability of these cell lines to efficiently bind human IgE in serum samples used for sensitization, particularly those with low IgE concentrations, and therefore defines the lower threshold for detection, i.e. sensitivity.

A monoclonal fluorescently-labeled, CRA-1, antibody specific for human FcεRIα_H-chain was used to analyze directly levels of surface FcεRI α-chain expression. As shown in **Figure 22**, stably transfected NFAT-DsRed cells with a plasmid encoding the FCER1G cDNA with a constitutive promoter resulted in increased median fluorescence compared with the non-transfected cells as determined by flow cytometry. The parental RBL-2H3 cell line which expresses only the rat FcεRI receptor was used as a negative control while the RS-ATL8 and its parental line SX-38 as positive controls.

This results demonstrates how co-expression of FcεRIα$_H$ and FcεRIγ$_H$ chain in RBL can restore higher levels of surface FcεRIα$_H$ expression, albeit still not to a level comparable with SX-38 and RS-ATL8. This may suggest the existence of additional factors governing FcεRI cell surface expression.

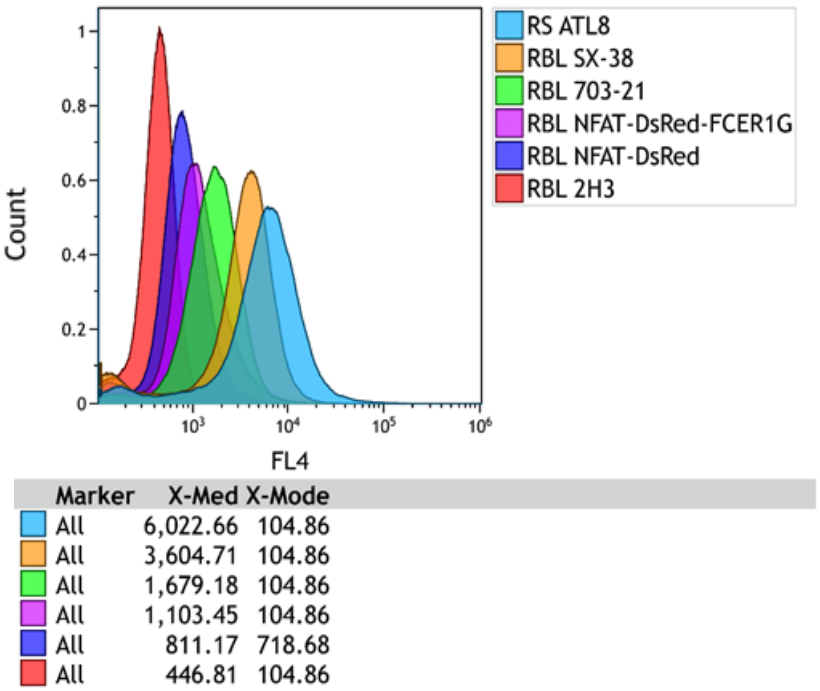
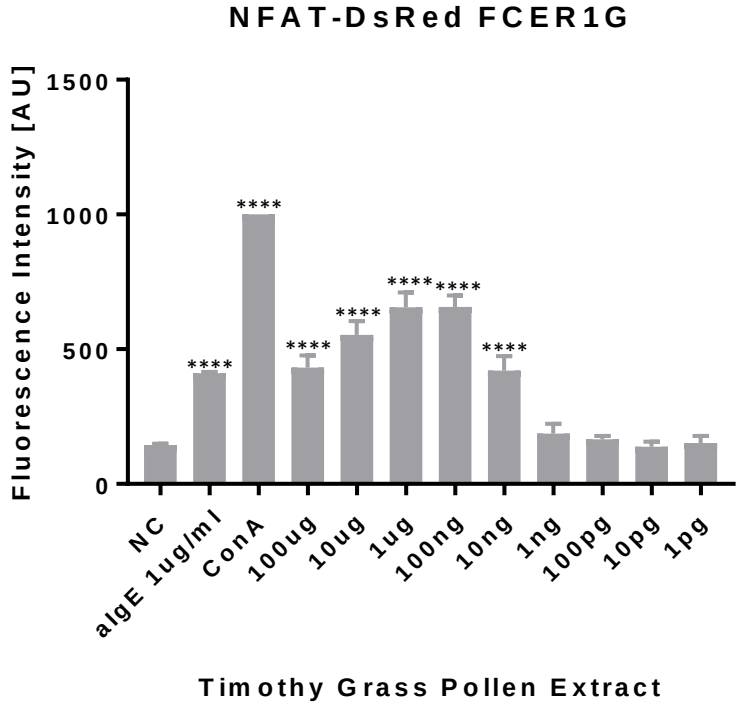


Figure 22. Histograms of FcεRIα$_H$ surface expression in RBL cell lines. The blue histogram shows non-transfected NFAT-DsRed cells compared to stably transfected cells with pBJ1 neo-hu FcεRI gamma (NFAT DsRed FCERIG) in purple and RS-ATL8 cells in light blue. All cells were sensitized overnight with 1 µg/mL recombinant human IgE and the next day they were stained with APC-labelled anti-human FcεRI α-chain monoclonal antibody (CRA-1). Representative of four separate experiments (**Figure 42**, Appendix XI).

After comparing FcεRIα$_H$ surface expression levels in NFAT-DsRed cells transfected with FcεRIγ$_H$ and non-transfected cells, we assessed whether this would lead to higher response than the single human FCER1A transfectant cell line. Cells were

sensitized overnight with grass pollen reference serum and stimulated the next day with timothy grass pollen extract for 18 hours. In **Figure 23**, we see that stable transfection of FCER1G cDNA resulted in 10x higher sensitivity as cell activation was detected up to 10 ng/mL in the transfected cells compared to 100 ng/mL in non-transfected cells but 10.000x less than RS-ATL8 (**Figure 24**). As expected RS-ATL8 cells expressing the highest human FcεRI α-chain levels showed the highest response after stimulation with timothy grass pollen extract. Cell activation was detected up to 1 pg/mL of allergen stimulation compared to 10 ng/mL and 100 ng/mL in NFAT-DsRed-FCER1G and NFAT-DsRed cells, respectively.



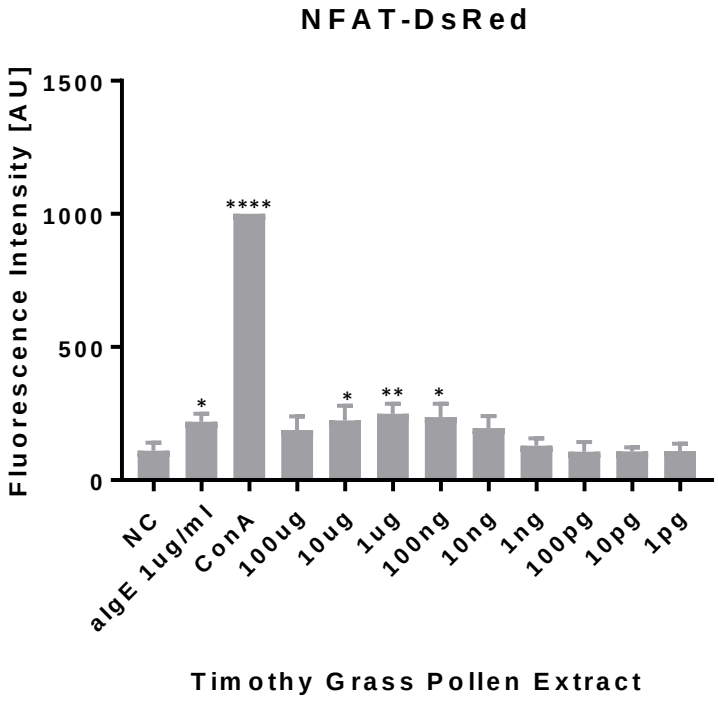


Figure 23. Dose response curves showing the increased response of the FCER1G-transfected NFAT-DsRed cells versus non-transfected and RS-ATL8. All cells were sensitized overnight with serum from a healthy donor with a known grass pollen allergy (diluted 1:50) and stimulated with serial dilution of Timothy grass pollen extract the next day. Fluorescence was measured after 16-18 hours of incubation. Data were normalized for ConA (1 µg/mL), used as a positive control. Negative control (NC); cells treated with human serum only. *p< 0.05; **p<0.01; ***p<0.001, or ****p<0.0001 (Dunnett's multiple comparisons test). Representative of four separate experiments performed in triplicates with comparable results.

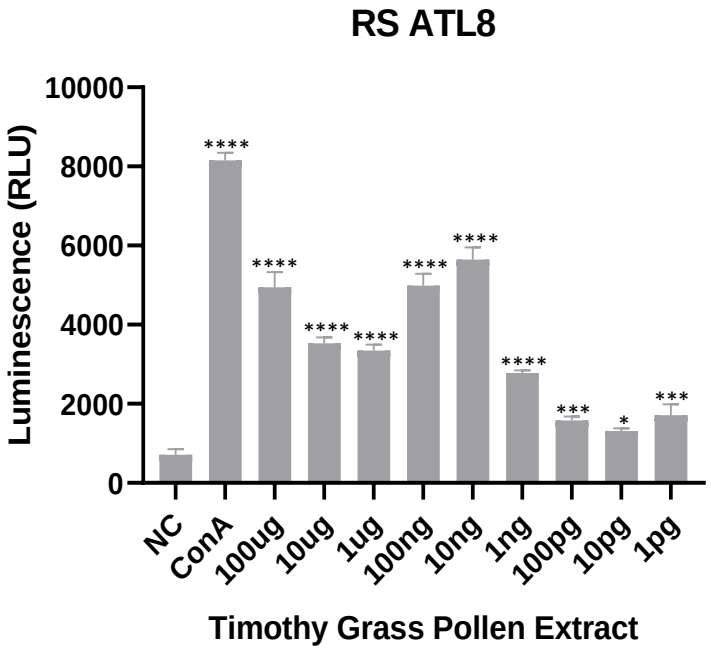


Figure 24. RS-ATL8 cells were sensitized overnight with 1:50 dilution serum from a donor allergic to timothy grass pollen. After 16 h sensitization, cells were stimulated with various concentration of timothy grass pollen extract, and ConA (1 µg/mL) . Negative control (NC); cells treated with human serum only. Activation was measured as luminescence 4 h after stimulation. Data are shown as mean ± s.d. for triplicate determination.

3.4. Discussion

A number of studies have shown that FcεRI densities vary 100-fold among subjects' basophils with higher densities being responsible for unusual behaviours of the cells, such as sensitivity to certain monomeric IgE antibodies or spontaneous release (352). Alternatively, humanized reporter cell lines do not have the same variations, as they are derived from the same parental cell lines and are of clonal origin (323). The expression of FcεRI on the cell surface depends on several

parameters such as synthesis of FcεRIα, FcεRIβ, FcεRIγ, and free IgE levels (353). Similarly, FcεRI levels have a direct impact on cell line's sensitivity to detect allergen-specific IgE. The RS-ATL8 and its parental SX-38 humanized RBL cell line, were previously considered as triple human FcεRI αβγ transfectants. However, RT-PCR data generated previously in our group Ali *et al*. (2019) (323) showed the loss of the transfected β-chain in the parental SX-38 cell line, and consistently also in the RS-ATL8 cells deriving from it. Despite the loss of human β-chain expression, both cell lines showed the highest surface FcεRIα_H receptor expression (approximately 450,000 – 500,000 FcεRIα_H molecules per cell), similar to human peripheral blood basophils (up to 600,000/ cell) (100), while RBL 703/21 and NFAT-DsRed cells appeared to have only 52,000 and 16,000 human α-chains per cell, respectively. Considering that the latter two cell lines are single α-chain transfectants, this might explain why the surface levels of the human FcεRI α-chain are lower in these two humanized RBL cell lines than the SX-38 and RS-ATL8. Ra et al. (354) showed that although in mice the β-chain is an absolute requirement for the α and γ chain to reach the cell surface, in humans they can reach the cell surface in the absence of β. The human α-chain when expressed alone is kept in the endoplasmic reticulum (ER) (140). Letourneur F. and Cauvi D. *et al*. (139, 355) identified that the intracellular (IC) tail of α-chain contains two dilysine retention motifs, and an Asp in the transmembrane (TM) domain. The presence of γ-chain, with or without the β-chain, masks these retention motifs and facilitates transport of FcεRIα to the cell surface. This suggests that absence of human γ-chain in the

ER, will lead to reduced surface expression of the human α-chain in the single transfectant NFAT-DsRed compared to RS-ATL8 or RBL SX-38, which is also confirmed from our data (**Figure 21** and **Figure 22**). This would also explain the increased human FcεRIα surface expression after introducing human FcεRI ɣ-chain (**Figure 22**), albeit, unexpectedly, to a limited extent compared to the NFAT-DsRed cells that do not express human FcεRIγ (**Figure 21**). The lower-than-expected human alpha chain ($FcεRIα_H$) surface expression, is possible to result from different levels of expression of the mRNA encoding the human FcεRIγ between the humanised cell lines. This, leads to different levels of competition between the endogenous rat and the introduced human γ-chains at the protein level for assembly into the tetrameric receptor. FcεRI receptor expression on the surface of basophils is crucial for antigen-specific activation because of the receptor's ability to bind allergen-specific IgE (356). IgE antibodies upregulate FcεRI cell surface expression on basophils by stabilizing the receptor on the cell surface preventing its internalization (357). Consequently, FcεRIα surface expression levels affect the amount of allergen-specific IgE present on the cell surface, thus the overall cell line sensitivity to detect cell activation. This is in agreement with our findings, as higher FcεRIα expression levels is expected to result in a more sensitive cell line. The RS-ATL8 cells (expressing the highest human α-chain expression levels) used in this study responded positively to 1 pg/mL of timothy grass pollen extract after sensitization with serum of individual with a matching allergy. In contrast, after using serum from the same individual and the same stimulus, the NFAT-DsRed cells

responded to only 100 ng/mL as a result of the ~30-fold lower surface expression of FcεRIα$_H$. However, as shown in **Figure 23** after introducing the human γ-chain NFAT-DsRed cells detected as little as 10 ng/mL IgE, resulting in 10-fold higher sensitivity compared to single transfectant NFAT-DsRed cells..

3.5. Conclusion

In conclusion, despite the lower FcεRIαH expression levels and the low IgE concentration detection levels, the NFAT-DsRed and its derivative cell line (NFATDsRed FCER1G) have a distinct advantage over RS-ATL8 luciferase cell line. NFATDsRed cells can be used on microarray format allowing simultaneous screening of hundreds of allergens without the need for expensive luciferase agents, thus, reducing the overall cost and the volume of patient serum

Chapter 4: High-Throughout Allergy Diagnosis Combining Protein Microarrays with Fluorescent RBL Reporter Cells NFAT-DsRed

4.1. Introduction

As described in Subchapter 1.3, allergy diagnosis starts with a detailed medical history, followed by a physical examination. This is augmented by specific skin prick tests (SPT) and serum immunoassays which are performed to detect and quantitatively measure allergen-specific IgE (sIgE) antibodies in human serum (358).

Throughout the 20th century, allergic diseases were studied using allergen extracts for diagnostic and therapeutic purposes (265). Allergen extracts are complex heterogeneous biological mixtures containing different proteins, glycoproteins and polysaccharides (359). However, their standardization has been challenging due to inconsistencies in source materials and production processes, which led to considerable variations in test results (360). Since the turn of the century, advances in molecular biology and biochemistry have facilitated the characterization and production of recombinant proteins (361). This, together with the development of microarray technology, allowed the simultaneous screening of multiple proteins or DNA sequences on a miniaturized scale against thousands of samples and conditions for the first time (362). This resulted in the generation of array formats of proteins, which could then be used to detect binding of sIgE to individual allergen components (340) a process referred to as molecular or component resolved diagnosis (CRD) (see 1.3.2.1 Allergen-specific IgE) (266). One important

advantage of CRD to the previous allergen extract technology has been the ability to distinguish between genuine sensitization and cross-reactivity (266). Many allergic individuals show allergic symptoms after contact with various unrelated allergen sources. For example, patients allergic to birch pollen often experience oral allergic reactions after consumption of certain fruits or vegetables. This may be due to the existence of structurally similar and immunologically related crossreactive allergens in the different allergen sources (363, 364). Also, some allergic individuals may show inconsistent allergic reactions after contact with an allergen source at different times. This may result when the reaction is due to an allergen that occurs as a sporadic contamination in an unrelated allergen source such as mite allergens present in animal hair dander extracts (365) or not consistently present in the allergen source due to developmental or other reasons such as some pollen allergens expressed only in mature pollen (366) or the expression of mould allergens that varies depending on culture conditions (367). Moreover, a major limitation of the allergen-specific IgE reporting assays is that they do not necessarily document the crosslinking of the FcεRI/IgE complex on the surface of basophils and mast cells by allergens, which is closely associated with allergy clinical symptoms especially in food allergies (253, 368). In some cases, clinically irrelevant results in serum IgE tests such as the CAP test, can partly be caused by cross-reactive carbohydrate determinants (CCDs) (369). The CCD-specific IgE antibodies present in patients' sera can bind to the carbohydrate moieties in the allergen. However, carbohydrate determinants having only one site

per allergen fail to induce cell activation as they are not capable to crosslink the high-affinity IgE receptor (FcεRI) on basophils or mast cells (370).

In 2007, Lin et al. proposed an approach for clinically relevant detection of sensitization, which relied on coupling of protein arrays with purified peripheral blood basophilic cells (340). Thus, with this approach it appeared achievable to combine high-throughput screening of allergens using only minute amounts of patient serum with a readout reporting cellular activation, rather than mere sIgE binding (106). However, purifying basophils from peripheral blood of human donors is not a widely acceptable proposition for non-specialised labs, despite the fact that strongly improved and semi-automated protocols for their purification exist (371). As an alternative to human peripheral blood basophils, the use of humanized rat basophilic leukaemia (RBL) cell lines for detection of allergen-specific IgE has been made possible by stable transfection of the human FcεRI α chain. RBL cells have been used for many decades as a mast cell model to investigate the molecular basis of IgE-dependent signal transduction (322). Earlier methods relied on the relatively insensitive measurement of beta-hexosaminidase release, and were thus dependent on the use of a cell sensitivity enhancing agent such as deuterated water (D2O) (372). Almost twenty years ago, Nakamura R. *et al*. (2010) (329), were the first to develop a reporter system named EXiLE, for IgE crosslinking (x)-induced luciferase expression, after stably transfecting humanised RBL SX-38 cells (343) with a nuclear factor of activated T cells (NFAT)-responsive reporter gene. Activation of the RS-ATL8 reporter cells relied on measuring the

firefly luciferase reporter gene expression using a chemiluminescent substrate (329). This system could be used in 96- or 384-well format (373). It would be ideal to combine such a cellular, clinically relevant readout with the array format of CRD. However, RS-ATL8 cells and the ExiLE system are not compatible with a solid-phase format, as lysis of the cells is required prior to enzymatic activity measurement due to the inability of the substrate to cross the membrane. Therefore, an alternative reporter system expressing an intracellular fluorescent reporter gene such as DsRed was developed by our group (344).

Aim and Objectives

In this research, we aimed to create a diagnostic technology which combines allergens (and controls) printed in array format with the fluorescent IgE reporter NFAT-DsRed cell line. To achieve that we tried to identify suitable conditions for incubation and washes, by including extracellular matrix proteins for enhanced cell adhesion. We also worked on identification of suitable surface coatings which are not toxic to the cells and provide good protein binding. Lastly, we assessed different polymer-coated surfaces' ability to support reporter RBL cell activation.

4.2. Materials and methods

4.2.1. Cell culture

All experiments were performed with rat basophilic leukaemia cells (RBL-703/21-derived NFATp-DsRed-Express2) generated at The University of Nottingham (344). These are available from the authors for non-commercial purposes on the basis of a materials transfer agreement (MTA). Cells were grown as described in Subchapter 2.2.1 in Minimum Essential Medium Eagle (MEM) (Merck, UK) cell culture medium supplemented with 10% v/v heat-inactivated fetal bovine serum (GIBCO, UK), 100 U/mL penicillin, 100 µg/mL streptomycin and 2mM L-glutamine (Merck, UK) in a 37 °C/ 5% CO_2 humidified cell incubator. To maintain stable expression of the FcεRIαH gene, cells were cultured with 1 mg/mL G418 sulphate (Thermo Fisher Scientific, UK) and 20 µg/mL blasticidin S HCL (InvivoGen, USA) for selection of NFATp-DsRed-Express2 reporter gene expression.

4.2.2. Protein printing

The following proteins diluted in DPBS to a final concentration of 1 mg/mL, goat anti-human IgE (Merck, UK) and timothy grass pollen extract (Merck, UK) containing 10% FN and K-casein (Merck, UK) and timothy grass pollen extract w/o FN, were printed in triplicate onto the functionalised slides using a XYZ3200 dispensing workstation (Biodot, US) and 0.5 mm XtendTM microarray ceramic pins (LabNEXT Inc, US). The printing conditions were O2 < 2000 ppm, 25°C, and 34% humidity. The arrays were dried at room temperature (RT) for 24 hours.

4.2.3. Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS)

ToF-SIMS is a surface analysis technique that provides high chemical sensitivity and specificity to identify and analyse elements and molecules on sample surfaces. In a ToF-SIMS analysis, the sample is bombarded with a high-energy, primary ion beam that causes atoms, molecular fragments, and intact molecules to be sputtered from the sample surface. A fraction of these particles is ejected as ions (secondary ions). The secondary ions are extracted and mass analysed in a time-of-flight (ToF) mass analyzer, where they are separated with respect to mass to charge ratio (374). A wide range of biological samples have been successfully analyzed with ToF-SIMS, including proteins on nanoparticle surfaces (375).

Here, detection of the printed proteins on the basis of their amino acids (376) before and after washing the surfaces with deionized (DI) water was conducted using a ToF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany) using a Bi3+ cluster primary ion source operated at 25 kV. The primary ion dose density was maintained at $<1 \times 10^{12}$ ions/cm^2 to ensure static conditions. Positive polarity spectra were acquired in high current bunched mode over 1 X 1 mm areas at a resolution of 228 pixels per mm, using the macroraster stage function. Retrospective data analysis allowed ions indicative of amino acids (e.g., CH_4N^+, $C_2H_6N^+$, $C_4H_8N^+$) to be identified. Positive spectra were mass calibrated using CH_3^+ (*m/z* 15), $C_2H_5^+$ (*m/z* 29), $C_3H_7^+$ (*m/z* 43), and $C_4H_9^+$ (*m/z* 57) peaks. Data analysis was carried out using SurfaceLab 7 software (ION-TOF GmbH, Münster, Germany).

4.2.4. Cell adhesion assay

Glass slides functionalised with the following three chemistries: Aldehyde- (Nanocs, UK) Epoxy (Molecular Devices/Genetix, UK) and CodeLink activated Amine-binding/NHS ester-coated (Surmodics, US) were treated with five different ECM proteins diluted in Dulbecco's phosphate-buffered saline (DPBS; Ca2+/Mg2+ free) (Merck) to a final concentration of 10 µg/mL. Non-functionalized glass slides were used as the negative control. Adhesion assays were carried out in 16-well ProPlate® Multiwell Chambers (Grace Bio Labs, US) in sterile Petri dishes (100 x 15 mm diameter) containing 200 µL of each ECM. The functionalized glass slides were coated with vitronectin (VN) (Advanced BioMatrix, US), laminin (LN) from Engelbreth-Holm-Swarm murine (Merck, UK) and fibronectin (FN) from human plasma (Merck, UK) in a 37°C/ 5% CO_2 humidified cell incubator and collagen type I solution from rat tail (CO) (Roche Diagnostics, UK) at 4°C for 48h. Prior to the assay, the incubated glass slides were washed three times with sterile DPBS. Cultured cells were collected using trypsin/EDTA (Merck, UK) for 15 minutes in a 37°C/ 5% CO_2 humidified cell incubator. The cells were resuspended in fresh medium to a final concentration of 6×10^4 cells/200 µL. They were then added in each chamber-well and kept at 37°C for maximum three days. Every 24h, 100 µL (1 µg/mL) Hoechst 33342 solution (ThermoFisher Scientific) dissolved in DPBS was added for 20 minutes at 37°C. After incubation, cells were washed once with sterile Glass slides functionalised with the following three chemistries: Aldehyde- (Nanocs, UK) Epoxy (Molecular Devices/Genetix, UK) and CodeLink activated

Amine-binding/NHS ester-coated (Surmodics, US) were treated with five different ECM proteins diluted in Dulbecco's phosphate-buffered saline (DPBS; Ca2+/Mg2+ free) (Merck) to a final concentration of 10 µg/mL. Non-functionalized glass slides were used as the negative control. Adhesion assays were carried out in 16-well ProPlate® Multiwell Chambers (Grace Bio Labs, US) in sterile Petri dishes (100 x 15 mm diameter) containing 200 µL of each ECM. The functionalized glass slides were coated with vitronectin (VN) (Advanced BioMatrix, US), laminin (LN) from Engelbreth-Holm-Swarm murine (Merck, UK) and fibronectin (FN) from human plasma (Merck, UK) in a 37°C/ 5% CO_2 humidified cell incubator and collagen type I solution from rat tail (CO) (Roche Diagnostics, UK) at 4°C for 48h. Prior to the assay, the incubated glass slides were washed three times with sterile DPBS. Cultured cells were collected using trypsin/EDTA (Merck, UK) for 15 minutes in a 37°C/ 5% CO_2 humidified cell incubator. The cells were resuspended in fresh medium to a final concentration of 6×10^4 cells/200 µL. They were then added in each chamber-well and kept at 37°C for maximum three days. Every 24h, 100 µL (1 µg/mL) Hoechst 33342 solution (ThermoFisher Scientific) dissolved in DPBS was added for 20 minutes at 37°C. After incubation, cells were washed once with sterile.

4.2.5. Cell activation assay

RBL cells were seeded at a concentration of 6 X 10^4 cells/200 µL in each well of 6-well tissue-culture treated plates (Corning, UK). After seeding, cells were sensitized with 20 µL/mL serum from a healthy donor allergic to grass pollen (see Appendix

XIII, **Table 18**) (1:50 diluted in cell culture medium) for 16h. The next day, printed surfaces were sterilized using ultraviolet light in a Class II biosafety cabinet at 30,000 µJ/ cm2 for 30 minutes. Following this, surfaces were washed three times with 0.05% v/v Tween 20 (Merck, UK) in DPBS. Next, they were blocked with 100 µL bovine serum albumin (Merck, UK) diluted in DPBS to a final concentration 1 mg/mL. After 1h incubation at 37°C, they were washed 3x with 0.05% v/v Tween 20 and 1x with sterile DPBS before coating with 100 µL FN for 3 hours at 37°C. After incubation, the slides were washed 3x with DPBS. Sensitized cells were collected using sterile cell scrapers (Corning, UK) and 200 µL cell suspension was added in each well of the 16-well Multiwell Chambers for 24h in a 37°C/ 5% CO_2 humidified cell incubator. The next day, cell-protein activation was assessed using an Evos fl Digital Inverted fluorescence microscope at 10x magnification using the RFP channel (531 nm excitation, 593 nm emission). The developed slides were then scanned with a GenePix 4000B scanner (Axon Instruments Inc., Union City, CA, USA) using a 635 nm laser, followed by image 110 analysis with GenePix Pro 6.1 software (Axon Instruments Inc.). The fluorescence intensity of each cell-protein spot was calculated by subtraction of the local background from the mean intensity of the spot.

4.2.6. Cell activation in soluble phase format

RBL cells were seeded at a concentration of 5 X 10^4 cells/50 µL in each well of 96-well tissue-culture (TC) treated plates (Corning, UK). After seeding, cells were sensitized with 1:50 diluted serum from timothy grass pollen allergic patient in cell

culture medium for 16h. The next day, after removing the medium, wells were washed once with DPBS (Merck, UK) and cells were sensitized with 5 µg/mL Concanavalin A (Merck, UK) (positive control), 1 µg/mL anti-human IgE (polyclonal goat anti-human IgE; Merck) as second positive control and various concentrations ranging from 1 µg/mL to 1 pg/mL of timothy grass pollen extract kindly donated by G. Schramm (Research Centre Borstel, Germany) for 24 hours. The following day, the stimuli were carefully aspirated from each well, and 100µL lysis buffer 1% v/v Triton X-100 (Merck, UK) in DPBS was added to each well. After one minute of lysis, lysates were transferred to black, flat bottomed, untreated 96-well polystyrene plates (Corning, USA). Fluorescence in each well was measured with an Infinite M200 plate reader (Tecan, Switzerland) using 530 nm excitation and 590 nm emission filters.

4.2.7. Statistical analysis

Cell activation statistical analysis was performed using one-way ANOVA, followed by Dunnett's test. Cell binding was analysed using two-way ANOVA followed by Tukey's multiple comparisons test compared with negative controls cells only without treatment. P values < 0.05 were considered statistically significant (*p< 0.05; **p<0.01; ***p<0.001; ****p<0.0001).

4.3. Results

4.3.1. Comparative profiling of cell adhesion on ECM treated polymer-coated surfaces

In this study, three different surface chemistries (aldehyde-, epoxy- and NHS estercoated) were tested for their ability to support and maintain adhesion of RBL cells to four different ECM proteins (vitronectin, fibronectin, laminin and collagen). All proteins were tested in triplicate to reduce variations in the number of cells remaining attached after incubation. The number of cells attached during the different incubation periods (24, 48 and 72 hours) are shown in **Figure 25**. Treatment of aldehyde- and NHS ester-coated surfaces with vitronectin and fibronectin significantly increased cell attachment after 24 hours. Cell attachment was also increased in all surfaces after 48h incubation with fibronectin, and 72h after vitronectin and fibronectin treatment only on epoxy-treated surfaces. In contrast to NHS ester-treated slides, aldehyde- and epoxy-coated slides promoted cell attachment also in the absence of ECM proteins (cells only). **Figure 26** A-B, show images of RBL cell attachment onto the different polymer coated surfaces after 24h treatment with ECM proteins (vitronectin, collagen, fibronectin, laminin or cells only (no treatment) captured with a fluorescent microscope. During the first 24h, vitronectin and fibronectin supported higher number of cells on all surfaces as well laminin equally to VN and FN only on epoxy-coated slides. Cell binding after 48h and 72h treatment with ECMs or without treatment is also depicted in **Figure 43** Appendix XII. Overall, NHS ester slides compared to aldehyde and epoxy supported the lowest number of cell attachment even after vitronectin and

fibronectin treatment. In addition, fibronectin and vitronectin induced significant cell attachment in all surfaces compared to laminin and collagen.

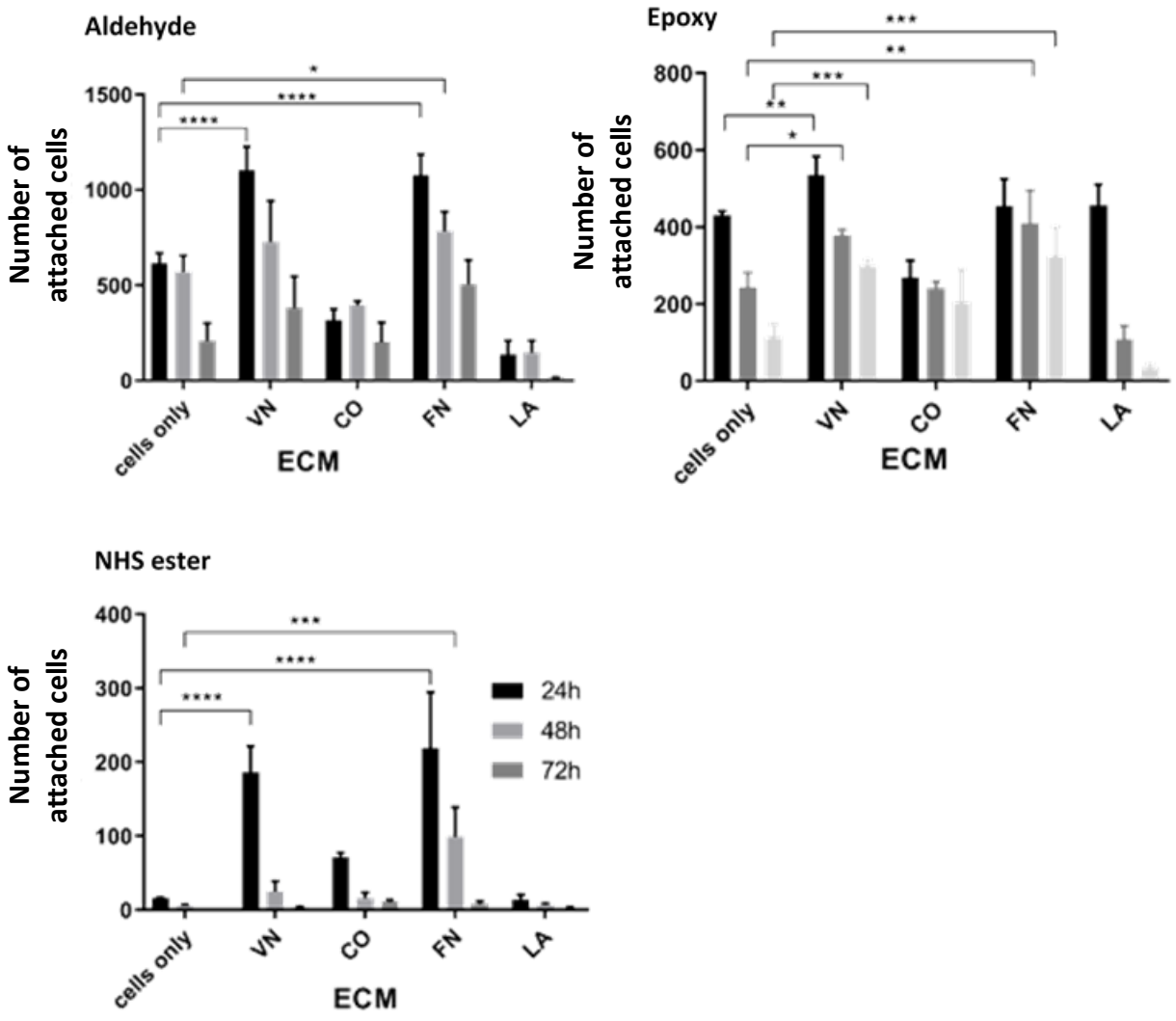
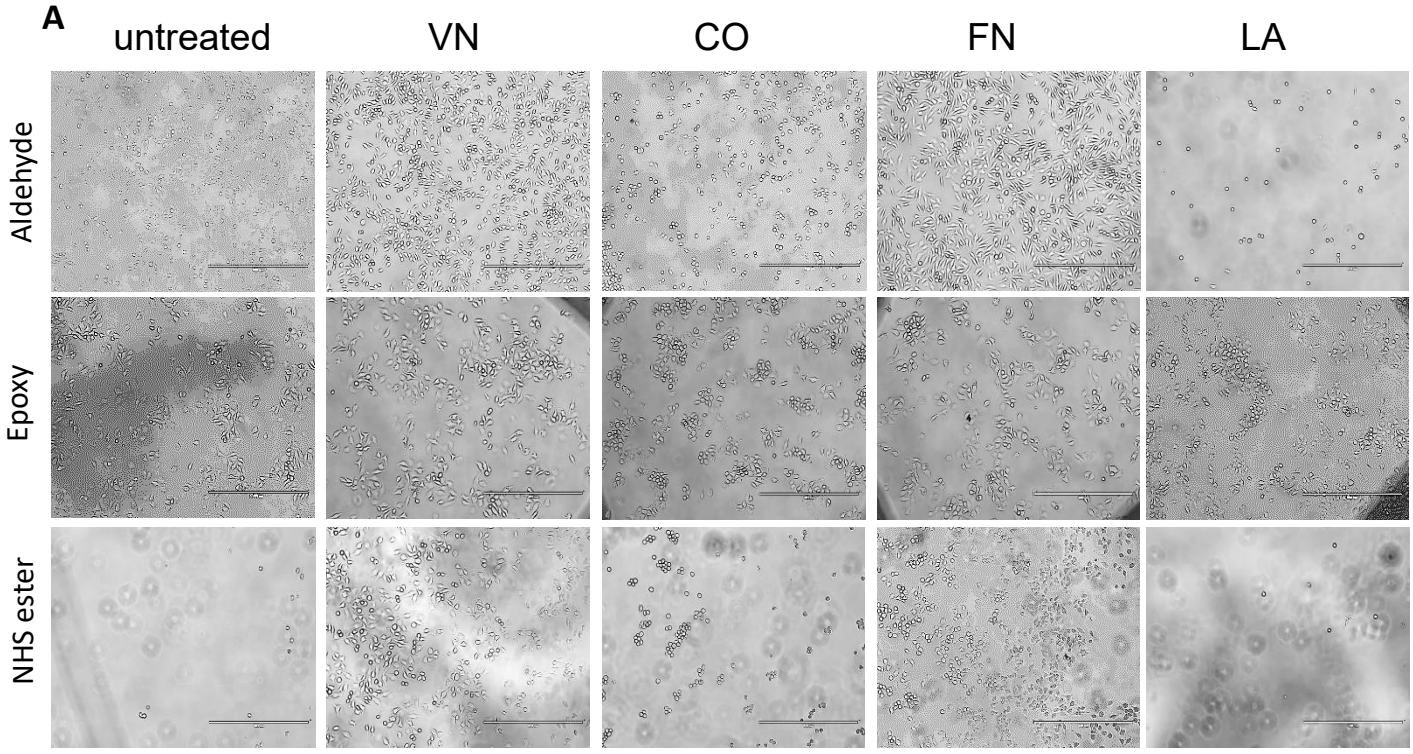


Figure 25. RBL 703-21/NFAT-DsRed cell attachment on aldehyde, epoxy and NHS ester polymer-coated surfaces treated with vitronectin (VN), collagen (CO), fibronectin (FN), laminin (LA) or no treatment (cells only), after 24, 48 and 72 hours (Data are means ± SEM (n=3). *p< 0.05; **p<0.01; ***p<0.001, or ****p<0.0001 (Tukey's multiple comparisons test).


A
untreated
VN
CO
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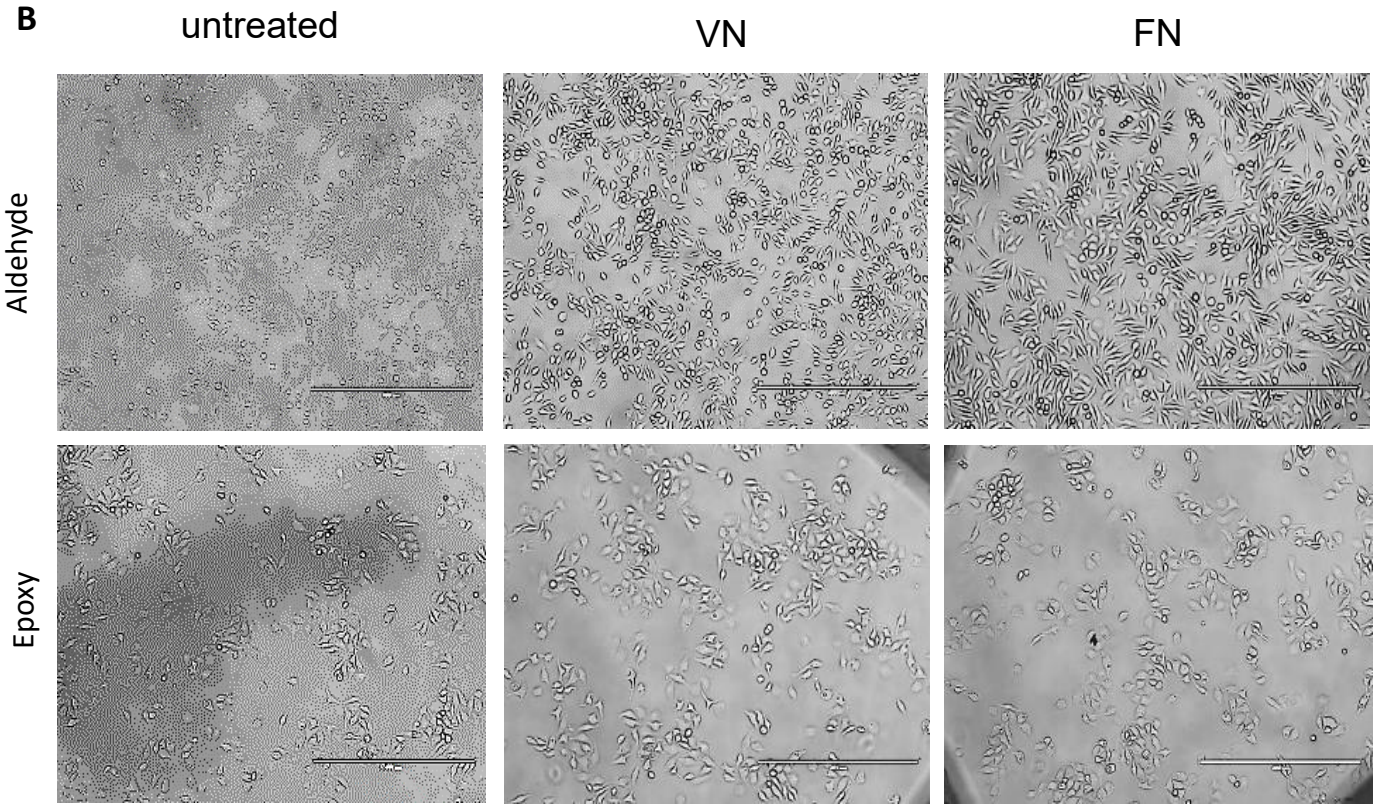
Epoxy
NHS ester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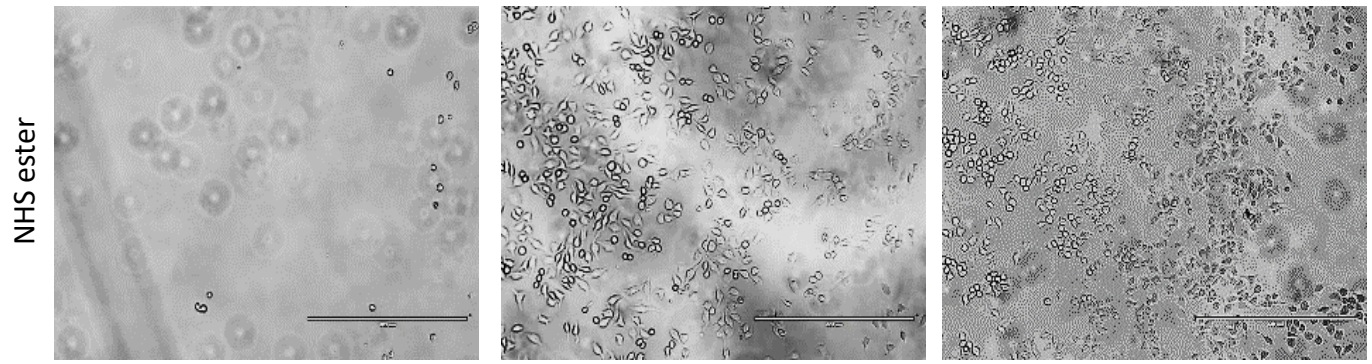


Figure 26. A. RBL 703-21/NFAT-DsRed cell attachment on aldehyde, epoxy and NHS ester-coated surfaces treated with vitronectin (VN), collagen (CO), fibronectin (FN), laminin (LA) or no treatment (cells only), after 24-hour incubation. **B**. Enlarged images of A. after RBL 703-21/NFAT-DsRed cell attachment on vitronectin (VN), fibronectin (FN) or no treated surfaces for 24 hours. Images were captured using fluorescence light microscopy with a 10x objective lens. Images scale bar 400 µm.

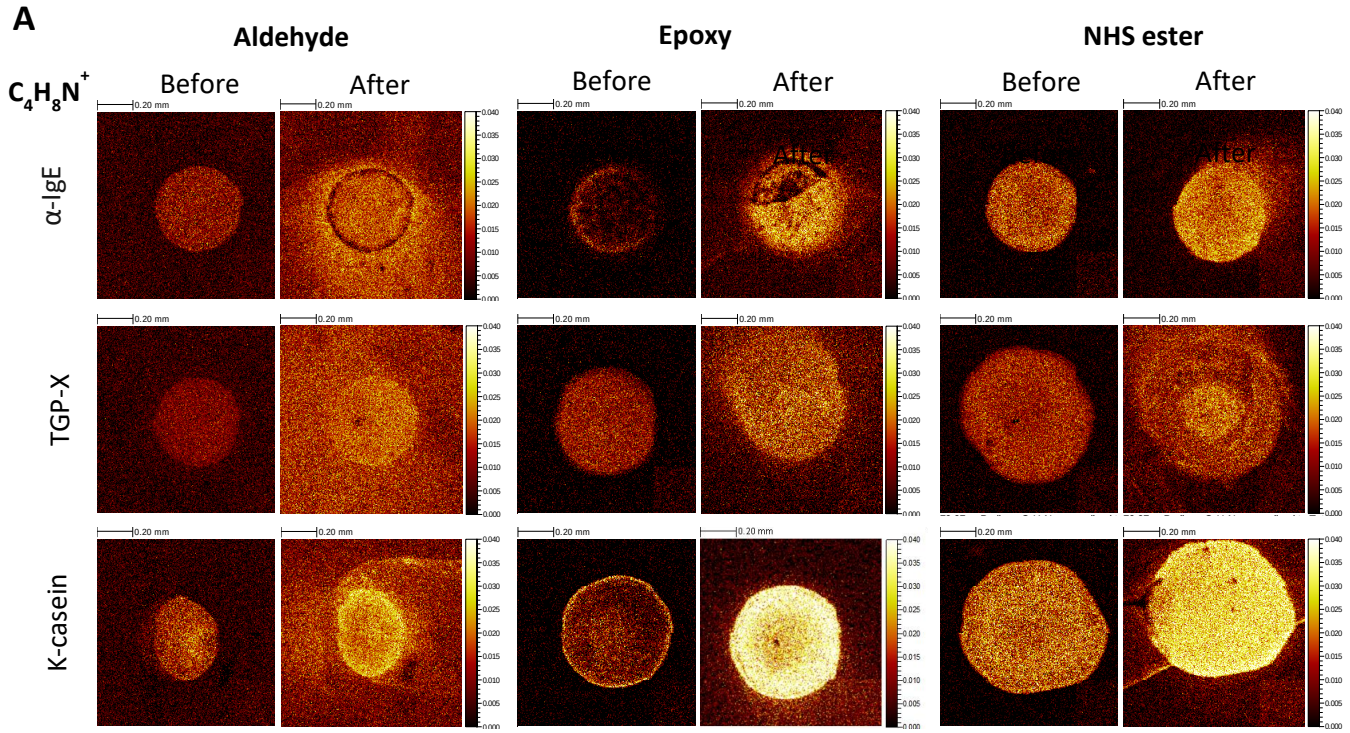
4.3.2. Printed protein stability

To confirm the presence of the printed proteins before and after washing the surfaces with deionized water, surface analysis of the protein microarrays using ToF-SIMS was performed. The ability of the three surface chemistries to bind and retain spotted protein before and after the washing procedure to remove nonadherent protein was assessed for the three key proteins; anti-human IgE (α-IgE; positive control), timothy grass pollen extract (TGP-X; allergen test sample) and kcasein (k-casein; negative allergen control). Characteristic secondary ion fragments cleaved off from amino acids for mass spectrometric analysis of proteins as described by Saleem *et al*. (2010), were used as a reference when examining the generated ToF-SIMS spectra to detect the presence of these proteins (377).

ToF-SIMS ion distribution maps of printed proteins

ToF-SIMS ion distribution maps of characteristic amino acid fragments help to reveal the presence of printed proteins. Each protein was printed using a 500 µm-sized ceramic pin on aldehyde-, epoxy- and NHS ester-coated surfaces. ToF-SIMS detects printed proteins on the three different surfaces before and after the washing steps. Normalized ion images of the characteristic proline fragment (*m/z* 70, $C_4H_8N^+$) are displayed in **Figure 27** A, indicating the distribution of proteins. Additional amino acid ions analyzed of normalized ion images of characteristic glycine (*m/z* 30, CH_4N^+) and alanine (*m/z* 44 $C_2H_6N^+$) fragments are presented in Appendix XII, **Figure 44**. Overall, clear and distinct protein dots were found for all printed proteins before washing procedure, except that anti-human lgE on epoxy shows a discontinuous pattern, possibly due to an artefact. After washing, a more intense protein distribution was noticed for all samples, which is also clearly confirmed in the corresponding statistic plots in **Figure 27** B. Considering that buffer salts can potentially negatively affect ToF-SIMS analysis e.g. by masking protein signals (378), we determined salt ion distribution before and after washing as well. Na^+ was chosen to illustrate salt distribution before and after the washing step. It is evident from **Figure 45**, Appendix XII that Na^+ intensity decreases after washing in all surfaces, hence, revealing the previously masked protein signal. **Figure 27** A also shows diffusion of proteins on substrates after washing procedure. **Figure 27** B shows that after wash, all three substrates show a similar proline intensity for anti-

human IgE and timothy-grass pollen, while epoxy shows a much higher proline intensity for k-casein.



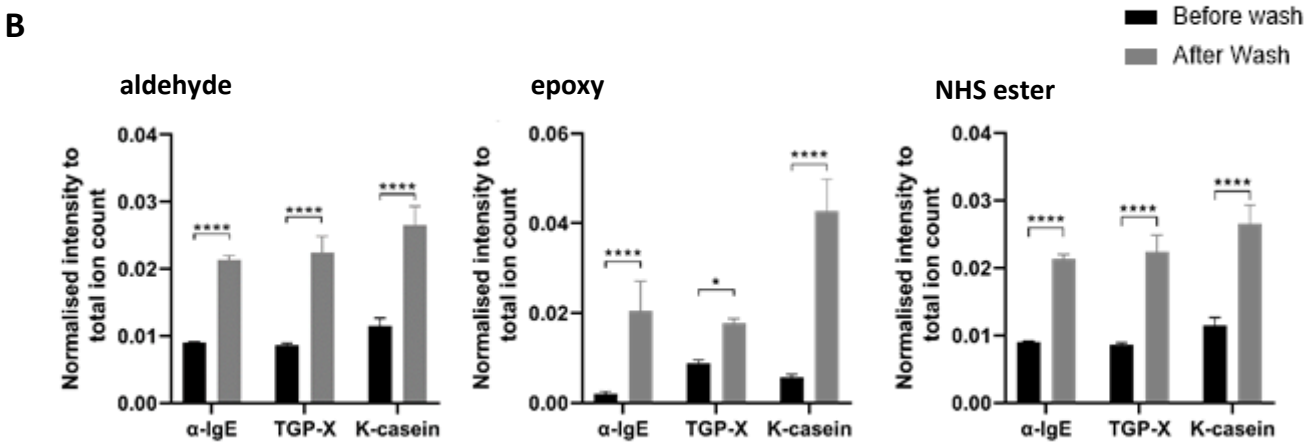


Figure 27. A. ToF-SIMS ion distribution maps for the characteristic proline fragment (*m/z* 70 $C_4H_8N^+$) on aldehyde, epoxy and NHS ester coated surfaces. Each image was normalised to the corresponding total ion map. Brighter colour corresponds to higher normalised ion intensity. **B**. Statistic Plots displaying normalised proline ($C_4H_8N^+$) ion intensities of the three proteins (anti-human IgE, timothy grass pollen extract and k-casein) printed on aldehyde, epoxy and NHS ester polymer coated surfaces. Data expressed are mean ± SD from quadruple determinations. Asterisks show results of Sidak's multiple comparisons test (Two-way ANOVA); n.s. not significant, *p<0.01, ****p<0.0001.

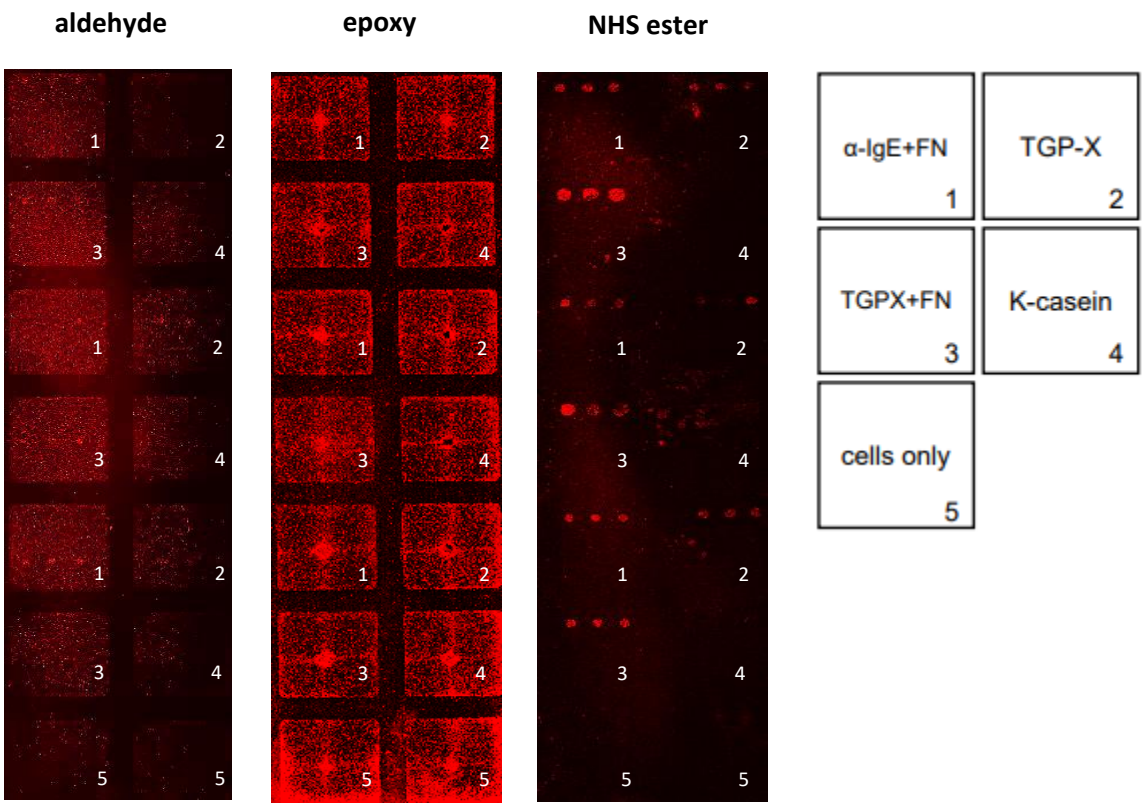
4.3.3. Detection of activated RBL cells on polymer-coated surfaces

NFAT-DsRed IgE reporter cells were sensitized overnight with serum from a donor allergic to timothy grass pollen, but not to milk (verified by clinical analysis). The next day, sensitised cells were added to the slides onto which timothy grass pollen extract (TGP-X; with or without fibronectin), as well as a positive (anti-IgE plus fibronectin) and a negative control (k-casein) had been printed and incubated for a further 24 hr. After this final incubation, cell activation on each surface was determined using a microarray laser scanner at 635 nm and an EVOS fl. fluorescence microscope using RFP channel (Exc: 531 nm/ Em: 593 nm). The raw fluorescent scanner images in **Figure 28** A, show that the surfaces supported cell activation to varying degrees. Images revealed distinct round-shaped dots especially on NHS ester and to a lesser extent on aldehyde-coated surfaces, showing successful fluorescent reporter gene activation on the printed spots. Epoxy surfaces showed the least activation, as well as high background noise was evident by recurrent high fluorescence intensity spots in the middle and the edges of every well, which was not related to cell activation. GenePix software was used

to quantify the fluorescence intensity generated from each individual protein-spot. As shown in **Figure 28** B, NHS ester treated slides show a consistent activation pattern with timothy grass pollen+10% fibronectin (TGP-X+FN) having the highest activation, following by anti-human IgE+10% fibronectin, timothy grass pollen extract (TGP-X) and k-casein showing very low to limited activation similar to negative control (cells only).

Timothy grass pollen+10% fibronectin (TGP-X+FN) fluorescence intensity in NHS ester-coated surfaces is 3x higher compared to aldehyde- and epoxy- treated surfaces. On the aldehyde and epoxy slides the fluorescence intensity observed was highly variable between spots. Epoxy-coated slides showed high fluorescence intensity levels in wells that no protein was printed (cells only) and overall the lowest intensity values due to more prone to artefacts surface (see activation on edges of incubation chamber in **Figure 28** A). Allergen-specific activation of the NFATDsRed IgE reporter cells was confirmed with fluorescence microscopy using the RFP channel (531 nm excitation, 593 nm emission). As shown in **Figure 29**, timothy grass pollen extract plus 10% fibronectin (TGP-X+FN) resulted in higher RBL cell activation compared to cells only (no protein) on all surfaces. Image comparison showed higher DsRed protein expression on NHS ester-coated surfaces compared to aldehyde- and epoxy-treated ones. However, weak cell activation was also observed in the cell only slides (no protein), which most likely resulted from cell autofluorescence and/or surface-induced activation.

A


aldehyde
epoxy
NHS ester
α-IgE+FN
1
TGP-X
2
TGPX+FN
3
K-casein
4
cells only
5

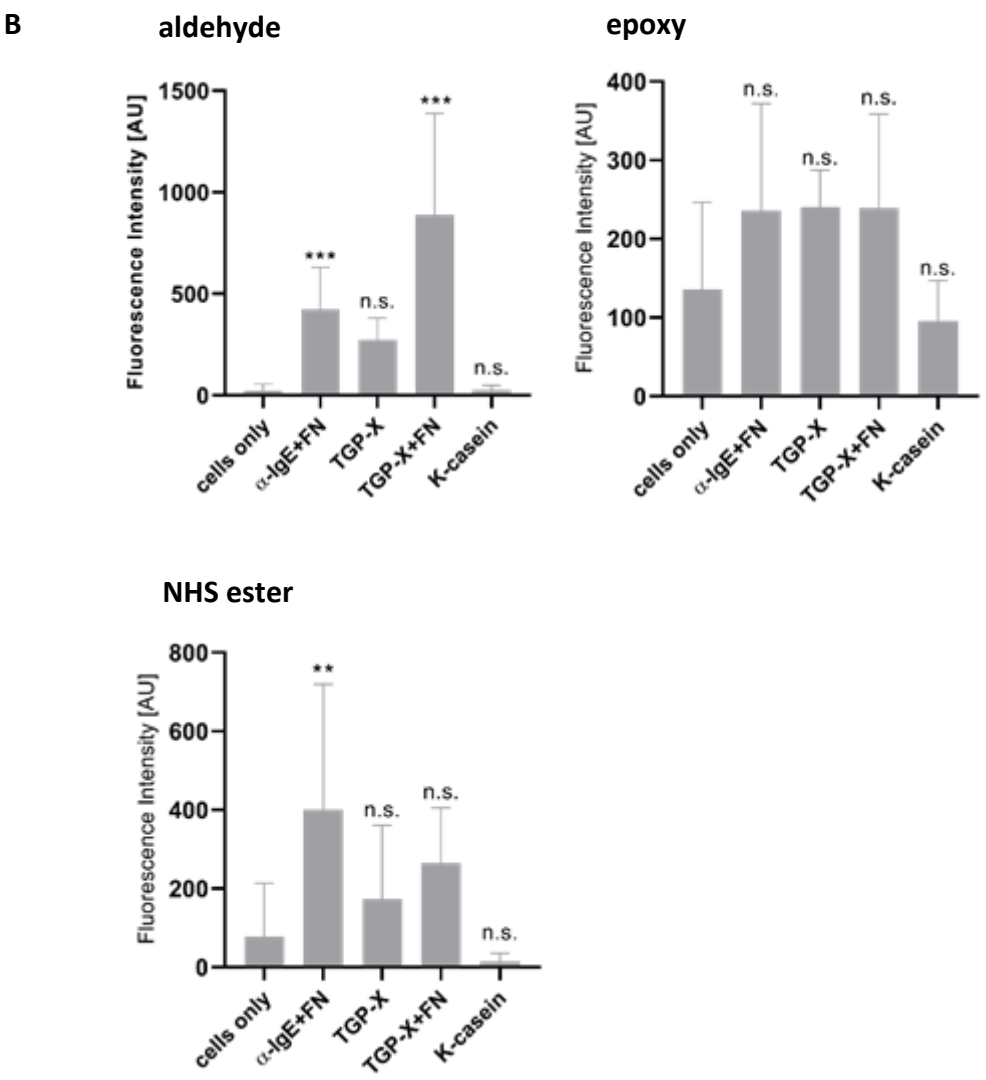


Figure 28. A) Cell binding of NFAT-DsRed IgE reporter cells to immobilized proteins printed in triplicate (1. anti-human IgE+10% fibronectin (FN), 2. timothy grass pollen extract (TGP-X), 3. TGP-X+10% fibronectin (FN), 4. K-casein and 5. cells only) on aldehyde-, epoxy- and NHs ester-coated slides. RBL cells were sensitised with the serum of a timothy grass pollen allergic patient overnight and incubated with the slides ($8.4x10^5$ cells/slide) for 24h. Images of each protein spot were scanned using a GenePix 4000B laser scanner at 635 nm. **B**) Fluorescence intensity values derived from each activated protein-spot on aldehyde-, epoxy- and NHS ester-coated slides after allergen-specific cell activation. Spot fluorescence intensity values were compared to negative control (cells only) and collected in triplicate. Data analysis was performed using one-way ANOVA, followed by Dunnett's test. P values < 0.05 were considered statistically significant (*p< 0.05; **p<0.01; ***p=0.001, or ****p<0.0001).

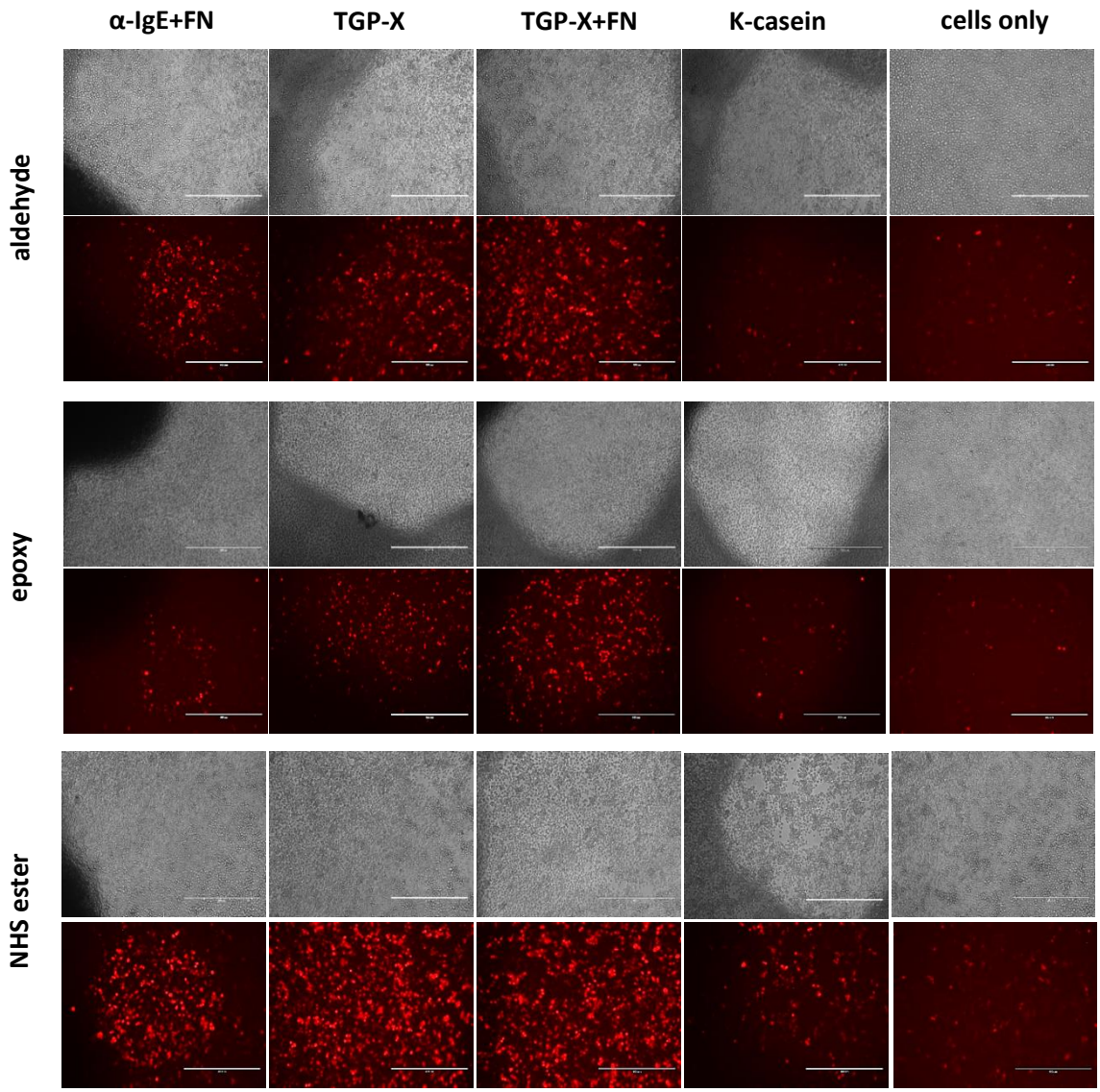
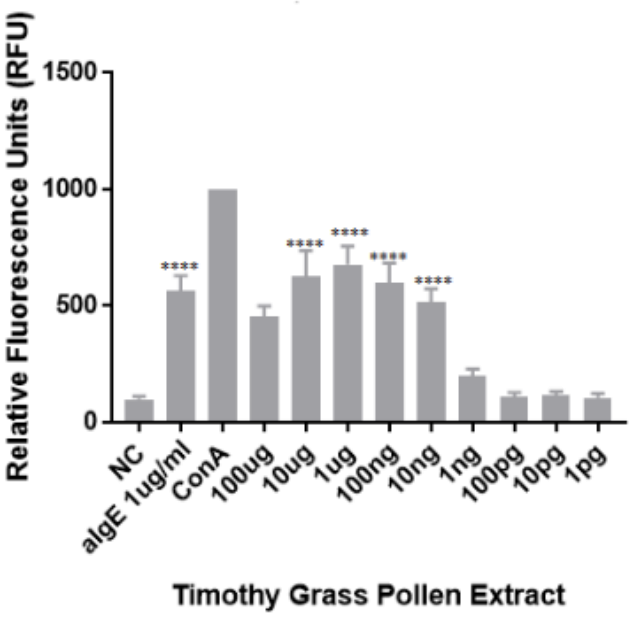


Figure 29. Cell activation images of NFAT-DsRed IgE reporter cells immobilized onto anti-human IgE, timothy grass pollen extract (TGP-X) with and without Fibronectin (FN) or k-casein printed on aldehyde-, epoxy- and NHS ester-treated surfaces. After 24-hour incubation at 37˚C, images of each protein spot were captured using fluorescence light microscopy (RFP channel) with a 10x objective lens. Image scale bar 400 µm.

Lastly, we assessed NFAT DsRed IgE reporter activation in the conventional 96-well soluble phase format, using the same materials used for the array format, in order to eliminate any artefacts caused by the different coated array surfaces. NFAT-

DsRed cells were sensitised overnight with serum of healthy donor with a known grass pollen allergy, and the allergens (TGP-X and k-casein) added at concentration range from 1 pg/mL to 100 µg/mL the next day. After a further 18 hours of incubation, fluorescence was measured using a fluorescent plate reader. As shown in **Figure 30**, results obtained from the 96-well format were very similar to those obtained with the NHS ester-coated arrays. The NFAT-DsRed cells were activated in the concentration range between 10 ng/mL and 100 µg/mL after stimulation with TGP-X, indicating the presence of timothy grass pollen specific IgE in human serum. However, k-casein gave negative results in all tested concentrations, except for a small but statistically significant ($p<0.5$) positive signal at the highest concentration 100 µg/mL. The positive controls anti-IgE and ConA (the latter was used for normalization of data), gave strong positive signals.



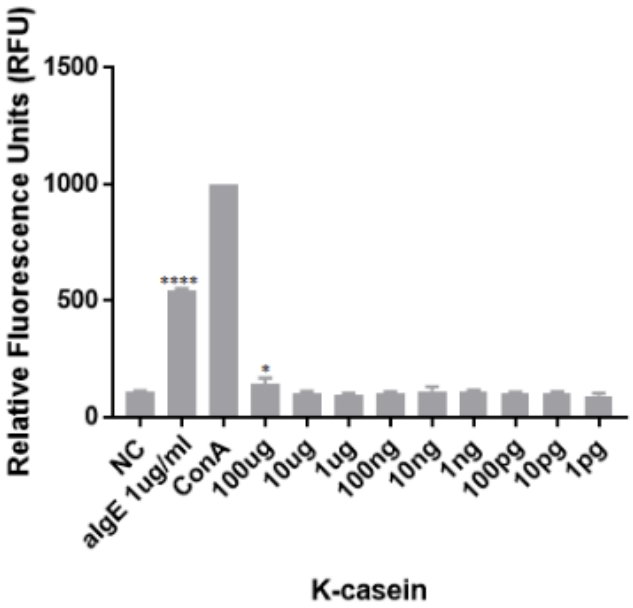


Figure 30. Fluorescence of NFAT-DsRed in conventional 96-well format. Anti-human IgE, ConA (both 1 µg/mL) and timothy grass pollen extract (TGP-X) or k-casein (both 1 pg/mL to 100 µg/mL) were added to reporter cells sensitized overnight with serum of healthy donor with a known grass pollen allergy. After a further 18 hours incubation, to allow for production and maturation of fluorescent reporter protein DsRed in activated cells, the plates were measured in a Tecan Infinite M200 plate reader using 530 nm excitation, and 590 nm emission. Data are mean ± SD (n=3). *p< 0.05; **p<0.01; ***p<0.001, or ****p<0.0001 (Dunnett's multiple comparisons test).

4.4. Discussion

Advances in biochip technology led in the development of a valuable tool for quantitative measurement of allergen-specific IgE levels in allergic individuals (268). More specifically, protein microarrays provide a miniaturized allergy test where hundreds of purified proteins are applied onto glass slides to diagnose allergic sensitization using only minute amounts of patient sera (379). However, allergy diagnosis is not fully mirrored by measuring allergen-specific IgE in vitro, as merely IgE binding to the high affinity IgE receptor FcεRI does not always correlate

with clinical symptoms (88). Fleischer *et al*. (2011) reported that almost all 125 patients who participated in a retrospective study were misdiagnosed of food allergy based on serum IgE testing while all of whom had atopic dermatitis (380). Sampson H. A. (1999) reported that misdiagnosis of food allergy may be attributed to the low positive predictive accuracies of PSTs (Prick/puncture skin tests), less than 50 %, compared with DBPCFCs (286, 381- 383). To overcome this limitation, Lin et al. (2007) (340) proposed coupling of protein arrays with effector basophilic cells as a feasible method to diagnose allergy.

Toward this end, basophilic cell activation results in up-regulation of surface markers that could be linked with an allergic response. However, a number of limitations such as the low abundance of human peripheral blood basophils (<1%) of total white blood cells), the need for basophil purification and the large volumes of patient sera to isolate the required amount of cells, rendered the practical application of this system difficult.

This paved the way for basophilic cell lines to be used as surrogate of patient basophils. The introduction of humanised rat basophilic leukaemia cell lines (RBL) stably transfected with the human FcεRIα chain consisted a promising alternative as human IgE could bind with high efficiency to induce cell activation upon crosslinking with a matching allergen (323). First attempts using CD63 as cell surface activation marker were unsatisfactory, due to high pre-activation levels of CD63 on RBL cells (340). As mentioned earlier, the use of the RS-ATL8 reporter cell line that relies on NFAT-dependent expression of a firefly luciferase reporter gene

was examined. Although, RS-ATL8 cells express high sensitivity, capable to detect as little as 1 pg/mL IgE when stimulated with a matching allergen, are not suitable to a microarray format. In such system, measurement of luciferase activity would require cell lysis, which in turn would disconnect signals from the allergen spots (322). Experiments with cell penetrating luciferase formulations were attempted but were not further pursued. As a viable alternative, our lab developed a reporter system expressing intracellular fluorescence (RBL 703/21 NFAT-DsRed) showing high stability in solid-phase formats (344).

In order to establish proof-of-principle our fluorescent reporter cell line suitability on printed-array format, we investigated several parameters that could determine the system's effectiveness, such as printed protein stability before and after washing the surfaces, cell binding to solid surfaces, and the ability of different polymer coatings to support cell activation. The ideal material would support high protein binding and retention during washes, promote cell attachment and, importantly, not induce toxic effects. The two first properties were fulfilled by the widely used FAST slides (384) which were used in the original work by Lin et al. (2007) (340). FAST slides have a very high binding capacity resulting in near quantitative protein binding and retention. However, subsequent experiments showed that the proprietary nitrocellulose matrix of FAST slides was toxic to RBL cells during the necessary overnight incubation and were thus characterised as not suitable from this purpose. Therefore, we explored alternative surface chemistries

that would provide satisfying protein binding but also promote long term binding of RBL cells, without toxicity.

One mechanism of cell attachment onto the polymer surfaces is via absorption of extracellular matrix proteins (ECMs) onto the polymer slides. In addition to attachment, ECM proteins facilitate diverse cell functions such as cell growth, adhesion and migration, mediate the indirect interaction of cells with the polymer (385) and can directly facilitate activation of RBL cells (386-388).

In our experiments though, the aldehyde- and epoxy-activated glass slides supported RBL cell binding during the first 48h, regardless of the presence of ECM proteins (**Figure 25**, **Figure 26** & **Figure 43**, Appendix XII). Nonetheless, RBL cell binding was significantly increased after treating all surfaces (aldehyde, epoxy and NHs ester) with fibronectin (FN) and vitronectin (VN), whereas no significant binding was observed after collagen (CO) and laminin (LA) treatment. These results were expected, as nearly forty years ago, a number of studies demonstrated that fibronectin and vitronectin, are two key serum glycoproteins that facilitate attachment and spreading of culture cells (389- 393) via a tripeptide sequence, arginine–glycine –aspartic acid (RGD) which allows specific interactions with a5β1 and avβ3 integrin cell surface receptors in fibronectin and vitronectin, respectively (394). Similarly, Ra C. *et al*. (1994) (387), showed that mast cells attached more prominently to FN and VN rather than CO and LA. Wang et al. (2014) (344), also reported that RBL 703/21 cells attached strongly to FN coated surfaces but also, although less prominently, to CO and LA. Other authors (395, 396) have also

reported similar findings on RBL-2H3 cell binding to collagen type I and laminin. Following, in order to confirm the presence of the printed proteins before and after washing the surfaces with deionised water, we performed ToF-SIMS analysis that enables label-free detection of individual molecules (377). Recently, ToF-SIMS has drawn interest, especially as a means of examining bio/organic surfaces, owing to its surface sensitivity and chemical specificity (397).

For proteins, small mass ions are detected by ToF-SIMS which usually correspond to fragments from the 20 common amino acids present in various combinations in the proteins. To interpret low mass fragmentation patterns, researchers have identified characteristic ions that correspond to individual amino acids (398). However, protein identification can be challenging, since proteins derive from the same set of 20 amino acids unique peaks from adsorbed proteins cannot be easily differentiated (399). Alternatively, multivariate methods such as principal component analysis (PCA), provide detailed interpretation of spectral data by reporting differences between each spectrum (scores) which can then be related to the different fragmentation pattern of the spectra (400- 402).

Nonetheless, for the purpose of this study, we used only the characteristic peaks of individual amino acid fragments generated by ToF-SIMS to identify printed proteins. The characteristic peak of the essential amino acid proline ($C_4H_8N^+$) was chosen as a marker to illustrate the presence of the protein. As shown in **Figure 27**, A & B, all surfaces were suitable to use with protein microarrays. Washing procedure is usually expected to move loosely bound protein and buffer salts. The

increased intensity of proline ion ($C_4H_8N^+$) distribution (**Figure 27** A) after washing the surfaces, is perhaps attributed to the removal of buffer salts that were masking amino acid ion related intensity signals, which is confirmed by the distribution of Na^+ , a chosen marker of butter salts. It is clear from **Figure 45** B, Appendix XII, that Na+ ion intensity levels were higher before the washing step and greatly decreased after washing all three surfaces tested, mediating signal detection of amino acid related ion fragments. However, diffusion patterns of proteins on different substrates (**Figure 27** A), indicate that aldehyde is less able to retain bound protein during washes. Next, after testing RBL cell activation on three different polymer coated surfaces, we observed that NHS ester-treated surfaces had larger printed surface diameter which we speculated would have a direct effect on protein binding (more active sites for protein to bind to) and ultimately on cell activation. This was expected as the (NHS ester) CodeLink activated slides are prepared using a hydrophilic polymer containing N-hydroxysuccinimide ester groups. According to the slide manufacturers, due to the hydrophilic nature of the coating, the spot size would be slightly larger compared to less hydrophilic coatings (403). In 2011 Kimzey and co-authors, after printing GFP fluorescence protein on various chemistry surfaces from different manufacturers, reported that N-hydroxysuccinimide (NHS) -ester coated slides were able to bind significantly higher amount of protein compared to epoxide- and aldehyde-coated surfaces (395). Yang J. *et al*, (2011) reported similar findings, after examining the relationship between the cell number and the surface chemistries analysed by ToF-

SIMS. They concluded that ToF-SIMS ions corresponding to the tertiary butyl moiety ($C_4H_9^+$) and phenyl group ($C_6H_5^+$) showed lower cell adhesion whereas NHS ester treated surfaces with the characteristic ion $C_3H_8N^+$ resulted in high cell adhesion (396). In this study, we report for the first time RBL reporter cell activation on allergenprinted arrays. Our data provide proof-of-principle data that the proposed system can be used to detect the presence of allergen-specific IgE in the tested sera. The serum used for this study was obtained from a healthy donor with a known grass pollen allergy, but without any allergy to milk proteins. Our results are in agreement with serological tests and the soluble phase assay performed in a 96-well assay with the NFAT DsRed reporter, showing significantly increased fluorescence intensity only to anti-human IgE (positive control) and timothy grass pollen extract (test allergen) but not to k-casein (negative control allergen).

4.5. Conclusion

Collectively, our results show the feasibility to develop a novel tool able to diagnose clinically relevant allergic sensitization to multiple allergens. Our future efforts will be geared towards the assessment of large number of allergens using well-characterized patient sera to confirm the robustness of our newly developed system in allergy diagnosis, and ultimately determine key parameters such as specificity, sensitivity as well as negative and positive predictive value.

Chapter 5. General Discussion, Conclusion & Future Work

Being an *in vitro* surrogate of an allergic reaction that occurs *in vivo* in allergic individuals, combining protein microarrays with humanized reporter cell lines can potentially be used to support the diagnosis of type I hypersensitivity reactions (291). This system has the potential to supersede classical methods of measuring basophil degranulation. In the past, the basophil response to allergens was assessed by measuring mediators released by cells into the supernatant *in vitro*, including histamine and leukotrienes. Here, protein arrays provide a miniaturized allergy test where purifed proteins are applied onto glass slides coated with polymers to diagnose allergic sensitization using only small amounts of patient sera. The fluorescent reporter cell response is then assessed by measuring the fluorescence intensity resulting from the interaction between the cell-bound sIgE and the surface-immobilized allergens.

Nonetheless, since the diagnostic performance of this system on different allergic conditions has not been defined yet, it is implausible to determine its diagnostic supremacy compared to other methods. In the healthcare setting, clinical decisions, patient care and treatment methods rely on available diagnostic tools. Consequently, utilization of these diagnostic tests should go in hand with evidence to support them. Sensitivity and specificity are key indicators of a test's validity which help to determine the suitability of the diagnostic methods (404). These parameters can be assessed, for every new laboratory assay, by comparing the

results of the novel test to that of a gold standard (405). However, for sIgE the optimal diagnostic method does not currently exist. *In vitro* assays are mainly based on allergen which are bound on solid phases, often in surplus amount. Thus, a low-affinity immune response cannot be easily distinguished from a high-affinity one. In addition, in serology, sIgE tests have certain characteristics in regards to the structure of the solid surface (if used), the allergen concentration, the time of incubation and the dynamic range of the reading. For example, certain solid phases such as cellulose, express CCDs that can lead to false positive results in patients with sIgE to CCD (406).

The sensitivity of serum specific-IgE measurement assays is estimated to be comparable to that of SPT for food allergy, albeit only complementary for venom and drug allergy diagnosis performed by intradermal allergy testing (405). As the field of molecular-based allergy diagnostics advances, the use of purified natural or recombinant allergen components in singleplex assays resulted in increased sensitivity, specificity and accuracy of the diagnostic tests (407). Several advantages and disadvantages lie behind the use of whole allergens and molecular components similar to skin prick testing. First, the diagnostic sensitivity of molecular components is lower to that of complex allergenic preparations (e.g. pollen extracts) as rarely a single component is responsible for the sensitization. One example is Phl p 1, a major component of *Phleum pratense,* which is positive in 70-80 % of the patients positive to the whole extract. Phl p 1, in order to reach 90% sensitivity has to cojoin with Phl p 5. (407). Second, not every allergen is

available as recombinant or purified native protein. However, in certain cases such as multiple IgE sensitivities, recombinant allergens can provide more information compared to whole allergen extracts as they can distinguish the presence of cross-reactivities (408), (409).

Cellular assays, such as the basophil activation test, play an increasingly important role *in vitro* diagnostic allergy tests (410). In case of food allergies such as cow's milk allergy as reported by Rubio et al., the BAT showed the highest sensitivity (91%) and specificity (90%) compared to SPT and cow's milk-specific IgE levels. Another important application of BAT is in immediate drug hypersensitivity. In drug challenges measured by skin testing, the results were viewed with great uncertainty (411). Reviews by Mangodt et al. and Ebo *et al*. (412), (413) highlighted that neuromuscular blocking agents (NMBAs), antibiotics (β-lactams and fluoroquinolones), and iodinated contrast media (ICM)-induced immediate hypersensitivity when tested by BAT showed 50% and 60% sensitivity. To date, BAT represents the only valid assay to detect IgE-mediated opiate hypersensitivity (414).

Today, in suspected IgE-mediated allergy, several singleplexed or multiplexed *in vitro* diagnostic systems have been developed to diagnose allergen sensitization. However, the most widely used methods (e.g. measurement of serum specific IgE antibodies (ImmunoCAP or ImmunoCAP ISAC)) by clinicians or allergists possess a number of advantages and disadvantages. A major limitation is that they often only

detect allergic sensitization which does not always correlate with clinically relevant symptoms triggered by the causative allergen(s) (253). A comparison between the most widely used *in vitro* diagnostic systems is described in the following pages.

Most tests that detect specific IgE in patients' blood are immunoassays that involve chemiluminescent assays, fluorescent enzyme immunoassays (FEIAs), enzyme-linked immunosorbent assays (ELISAs) or radioallergosorbent assays (RASTs). However, in 2010 the National Institute of Allergy and Infectious Diseases (NIAID) urged the replacement of RAST with alternative fluorescence enzyme-labeled assays where the amount of IgE present is determined by the amount of fluorescence. At present, ImmunoCAP is the next generation of RAST and is considered the reference method for determination of *in vitro* specific IgE. In 2002, a multiplex assay was developed for the diagnosis of allergic sensitization based on proteomic microarray approach, widely known as ISAC (Immuno Solid-phase Allergen Chip) system (268) .

A key difference between the two systems is that the singleplex assay allows clinicians to determine a single analyte in serum or other body fluids while in the multiplex system 112 recombinant or purified native allergen components deriving from more than 50 allergen sources are fixed on an array. The assay principle is similar between the two methods, however in the singleplex assay the allergens are immobilized on a large cellulose matrix, whereas in the multiplex format they are spotted in triplicate on a polymer-coated glass slide. The main differences

between the two assays are presented in **Table 5** (Adapted by Marianne van hage *et al*., (2017)(415).

Table 5. ImmunoCAP singleplex vs multiplex.

ImmunoCAP	ImmunoCAP ISAC
Quantitative results, IgE values traceable to World Health Organization reference preparation.	Semiquantitative results
High sensitivity per allergen.	Broad sensitization profile
No interference with IgG.	Interference with IgG
A selection of crude extracts and/or allergen molecules from many sources.	A fixed selection of 112 highly defined proteins (allergen molecules)
One result.	One hundred twelve results
50 µL of serum per 1 allergen/assay.	40 µL of serum per 112 allergens/assay
Cost advantage in small screening.	Cost advantage in large screening
Worldwide experience and use.	Less frequently used, comparably demanding in result interpretation and assay quality control.
Extract-specific caveats:	
Detection of low-affinity antibodies with little or low clinical relevance.	
Allergens in low abundance in extracts might lead to low sensitivity.	

The singleplex ImmunoCAP and the multiplex ISAC 112 assay were compared in a study including 101 patients sensitized to grass pollen. The two methods tested correlated well with a positive percent agreement (PPA) varying between 60 and 100% and negative percent agreement (NPA) varying between 78-97% of corresponding allergens (416).

Cellular assays such as the Basophil Activation Test (BAT), are used to monitor specific functions of effector cells in allergic responses. They have superseded traditional histamine release assays, therefore they have resulted in important tool within in vitro allergy diagnosis tests (410)

In principle, basophils, a lineage of effector cells implicated in immediate hypersensitivity responses, as described in 1.2.3.2 Basophils, degranulate upon crosslinking of the sIgE/FcεRI complex by a matching allergen. This effect can be assessed by flow cytometry techniques (417). Despite the inceasing interest and wide acceptance of the assay within the scientific community, similar to the *in vitro* specific-IgE measuring assays, the two diagnostic approaches have a number of advantages and disadvantages. Kim Y. H. and Kim Y. S. *et al*., compared the diagnostic performance of BAT with serum-specific immunoglobulin E (sIgE) levels measured on an ImmunoCAP system (UniCAP®, Thermo Fisher Scientific, Waltham, MA, USA), using sera from children with concomitant cow's milk and egg allergy and with or w/o atopic dermatitis (AD). In their study, the diagnostic performance of BAT was significantly correlated with sIgE. In addition, the diagnostic value of BAT was also evident when testing for milk allergy in children with AD, since sIgE results were challenging to interpret in patients with AD due to high total IgE levels or polysensitization to many different allergens (418) . The main advantages and disadvantages of sIgE reporting assays (ImmunoCAP) and the BAT are described in **Table 6**.

Table 6. ImmunoCAP vs Basophil Activation Test (BAT).

ImmunoCAP	BAT
Assesses only sensitization	Reflects a functional response induced by crosslinking of FcεRI by the allergen
Samples can be store at 2 °C to 8 °C up to one week, or else at –20 °C.	Should preferably be performed within 4h of sampling for optimal results.
Using allergen extracts cannot single out the molecular target of the IgE reactivity.	Assesses IgE-dependent as well as IgE-independent mechanisms.
Some allergen molecules may be in low abundance or even missing in allergen extracts giving false negative results.	Requires specialized personnel trained in flow cytometry technique.
Validated and reproducible (fully automated).	Standardization and harmonization of the BAT techniques and interpretation of outcome values and results are still lacking
Not every allergen extract or molecular allergen is available in diagnostics	Approx. 10% of donors present “anergic” basophils (‘non-responders’). This needs to be controlled by introducing non-IgE dependent stimuli, usually fMLP

As described in 1.3.2.4 Protein microarrays, the development of chip technology has allowed the evolution of miniaturized IgE antibody assays that use microarrays of allergenic proteins applied onto activated silica chips. This technology, permitted the use of very small quantities (~ 10 nL) of purified or crude mictures of proteins that are spotted in triplicate usually at concentrations of 1 mg/mL. Jahn-Schmid B. and co-authors, compared the analytical performance of a proteomics chip IgE assay with the ImmunoCAP system against recombinant grass and tree pollen allergens. The authors reported that analytical sensitivities for the two methods tested were comparable with the exception of Phl p 6 where the chip assay was analytically more sensitive than the CAP method (419). A list of advantages and limitation of allergen microarrays are described in **Table 7**.

Table 7. Microarrays: advantages and disadvantages.

Allergen Microarrays

Advantages	Disadvantages
Minimal volumes of serum needed.	All allergen sources are not yet completely represented on the arrays.
Parallel broad-spectrum allergy evaluation.	Lower analytical sensitivity compared to singleplex assays.
Potential for complete automation, reduced assay variation.	Antigenic competition among cross-reactive allergens.
Precise quantitation by using multiple replicates.	Competition of IgG and IgE antibodies for limited number of spotted allergens (patients with current or previous immunotherapy)
Rapid turnaround time.	Provide semiquantitative measurements.

In 2010, Renault *et al*. (336) reported that the variable nature of protein microarrays allows testing of multiple parameters simultaneously such as different immunoglobulin classes present in human sera from small volumes of samples with good accuracy. Their preliminary data correlated well with existing commercial CAP results to determine allergen specific IgE. The food extract protein microarray system they tested in their study also showed good discrimination between atopic and non-atopic individuals for certain food-scpecific IgE.

A few years earlier, an advanced protein microarray concept was introduced in 2007 by Lin *et al*, demonstrating the application of purified peripheral blood basophils or humanized basophilic cell lines in combination with PM platform. Their results, although preliminary, demonstrated that both human peripheral basophils and the RBL cells tested showed higher surface expression of CD63 after

incubation on allergen printed on FAST slides. However, RBL cells also showed high CD63 levels before stimulation, resulting in an unsuitable signal/noise ratio (340).

Here, our efforts were geared towards the advancement of the system proposed by Lin *et al*. We proposed the application of a fluorescent RBL reported system together with protein microarrays to detect the presence of allergen specifc IgE and the feasibility of developing a clinically relevant allergy diagnosis system.

A key difference between e.g. the ImmunoCAP and similar technologies is that the first measures the amount of allergen-specific IgE bound onto the solid phase, whereas the array technology proposed here measures sIgE indirectly, by measuring cell activation response induced by sIgE-FcεRI complex crosslinking by a matching allergen. Therefore, it is less at risk of producing false positive results by cross-reactive carbohydrate determinants (CCDs). In parallel, the cellular signal transduction pathway provides a powerful signal amplification cascade, which goes beyond signal amplification in traditional sIgE measurement without cells. Lastly, all humanized IgE reporter cell systems involve washing steps after overnight sensitization with patient serum. These washing steps result in removal of unbound IgG antibodies, as the cells do not express human IgG receptors, and RBL cells only express low affinity IgG receptors (420). Consequently, competition between IgE and IgG for the same epitope is prevented. An overview of the main characteristics of a system measuring levels of allergen-specifc IgE (ImmunoCAP

ISAC) and our newly developed diagnostic system used to detect allergen-specific IgE is presented in **Table 8** below.

Table 8. ImmunoCAP multiplex vs reporter system allergen microarrays

Multiplex ImmunoCAP ISAC	Protein Microarrays & RBL Cells
Quantifies allergen-specific IgE antibody levels in patient serum (40 µl).	Minute amounts of patient serum needed per slide (20 µl).
Low analytical sensitivity (cut off 0.3 ISU) compared to singleplex ImmunoCAP.	Contain a powerful signal amplification cascade.
Measure the amount of allergen-specific IgE bound to allergens immobilized on a solid-phase.	Measure cell activation response induced by crosslinking of IgE-FcεRI by a matching allergen.
Highly cross-reactive allergens are not spotted on the ISAC from each source available, can report false-positive results.	Less prone to report false positive results caused by CCDs due to the highly sensitive reporter system.
Allergen-bound IgE antibodies are detected by labeled anti-human IgE	No labeled antibodies used.

The miniaturized allergy test proposed here, can potentially have several applications in research and clinical setting. It offers the ability to determine patients' individual sensitization profiles by detecting sIgE antibodies against numerous allergen molecules with minimal effort. Tripodi *et al.* determined in his study, 39 different sensitization profiles in 176 Italian children allergic to grass pollen after testing eight *Phleum pratense* (Timothy grass, Phl p) allergens with serum IgE assays (ImmunoCAP, Phadia) and SPT (421). In addition, similar to ISAC microarray analysis, the sensitization pattern of children could be monitored. In children for example, the sIgE response to *Phleum pretense* allergen molecules begins with monosensitization to a single allergenic molecule and gradually develops to a complex polymolecular pattern over time (422), (423). More

specifically, in timothy grass pollen allergy, usually Phl p 1 is the starting molecule most commonly recognized. Peadiatric patients during the early stages of sensitization usually only respond to this protein. After several months or years, IgE sensitization results from more timothy grass pollen proteins most frequently in the following order: Phl p 1> Phl p 4 and Phl p 5> Phl p 2, Phl p 6, and Phl p 11> Phl p 12 and Phl p 7 (423). Another potential application is the development of allergen-specific immunotherapy (AIT) customized for each patient. An important parameter in order to apply AIT is that clinical symptoms should derive from IgE sensitization to markedly defined allergen sources. Including the major allergens, while excluding cross-reactivity toward panallergens that demonstrate questionable clinical relevance (424). The use of reporter RBL cells in combination with allergen microarrays has the potential to distinguish “primary” genuine sensitization from antibody reactions resulting from cross-reacting molecules. Thus, it could influence physicians’ decisions regarding the composition of allergen preparations to be tested and prescription of AIT (286).

However, in order to demonstrate the robustness of the novel system used to detect clinically relevant allergic sensitization over the existing diagnostic assays, a number of parameters are to be determined such as the analytical specificity and analytical sensitivity, the negative and positive predictive value as well as validation of the assay procedure.

In the future, to improve sIgE reporting assays, it is important to ensure that allergen extracts and individual allergen molecules are standardized, the number of allergen molecules tested and applied in multiplex arrays is increased, as well as the sensitivity of multiplex chip-based systems. This is particularly important for allergy diagnosis in pediatric patients with low IgE levels but clear clinical symptoms of allergic reactions.

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Appendices

Appendix I

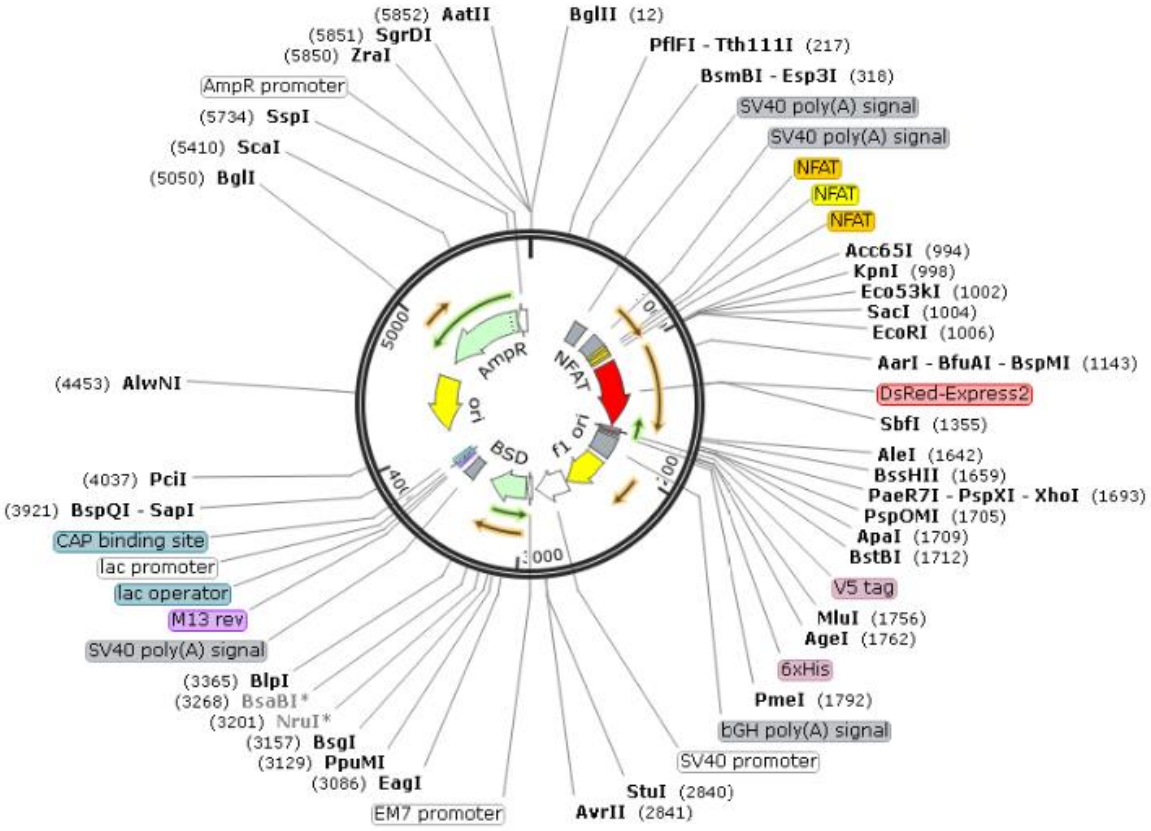


Figure 31. Plasmid map of pNFAT-DsRed-Express2 (pUB6/V5-His A) (5853bp).

Table 9. Primers for DsRed_Express2 detection in NFAT-DsRed-Express2 plasmid

Oligo Name	Sequence (5'-3')
DsRed-Express2 (FOR)	AGAGGTTTTCACCGTCATCAC
DsRed-Express2 (REV)	CGAGACCGAGGAGAGGGTTAG

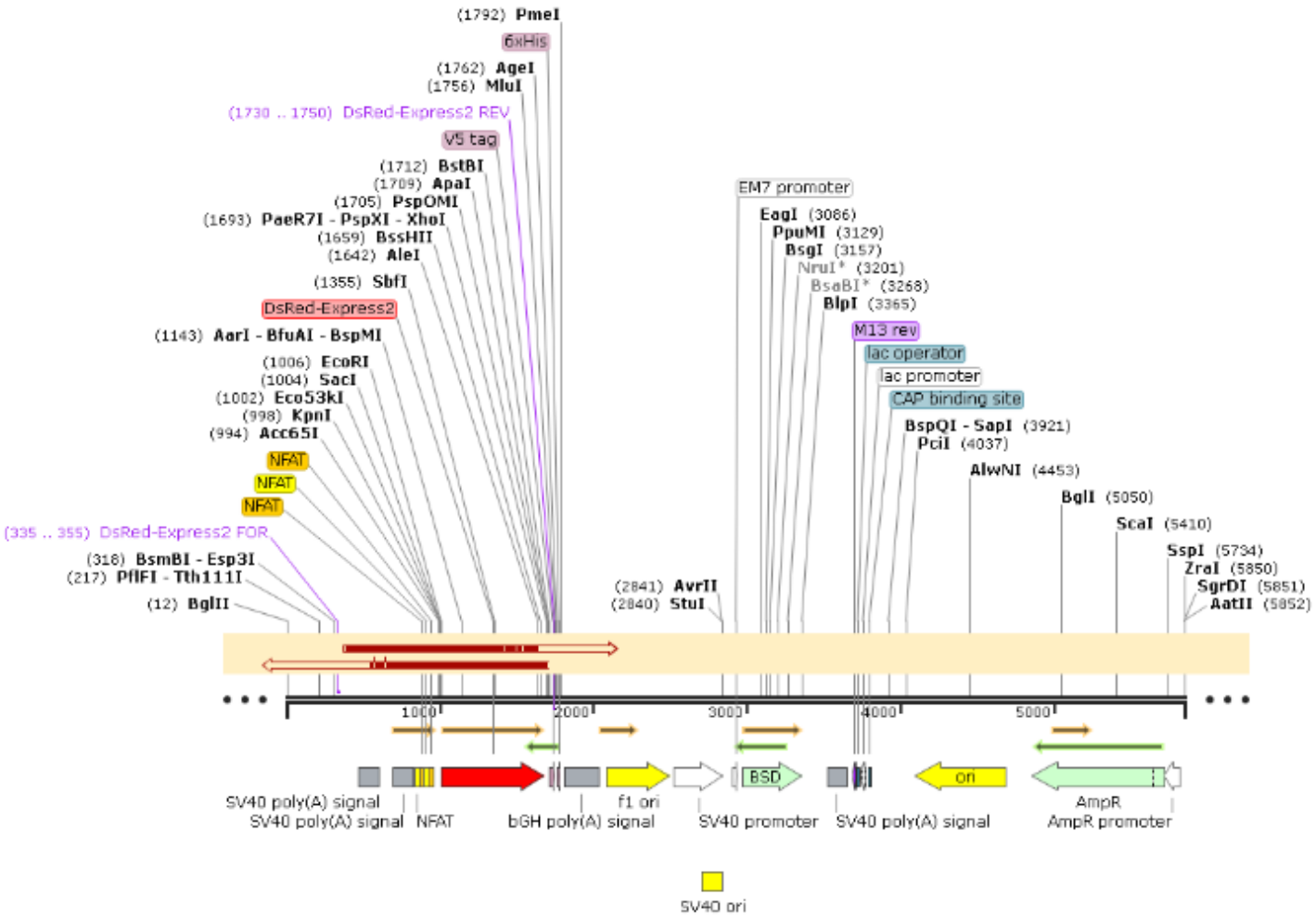


Figure 32. DsRed-Express2 gene sequencing analysis using specific primers for protein detection (**Table 9**).

Appendix II

RBL SX-38 NFAT-DsRed-Express2 Activation

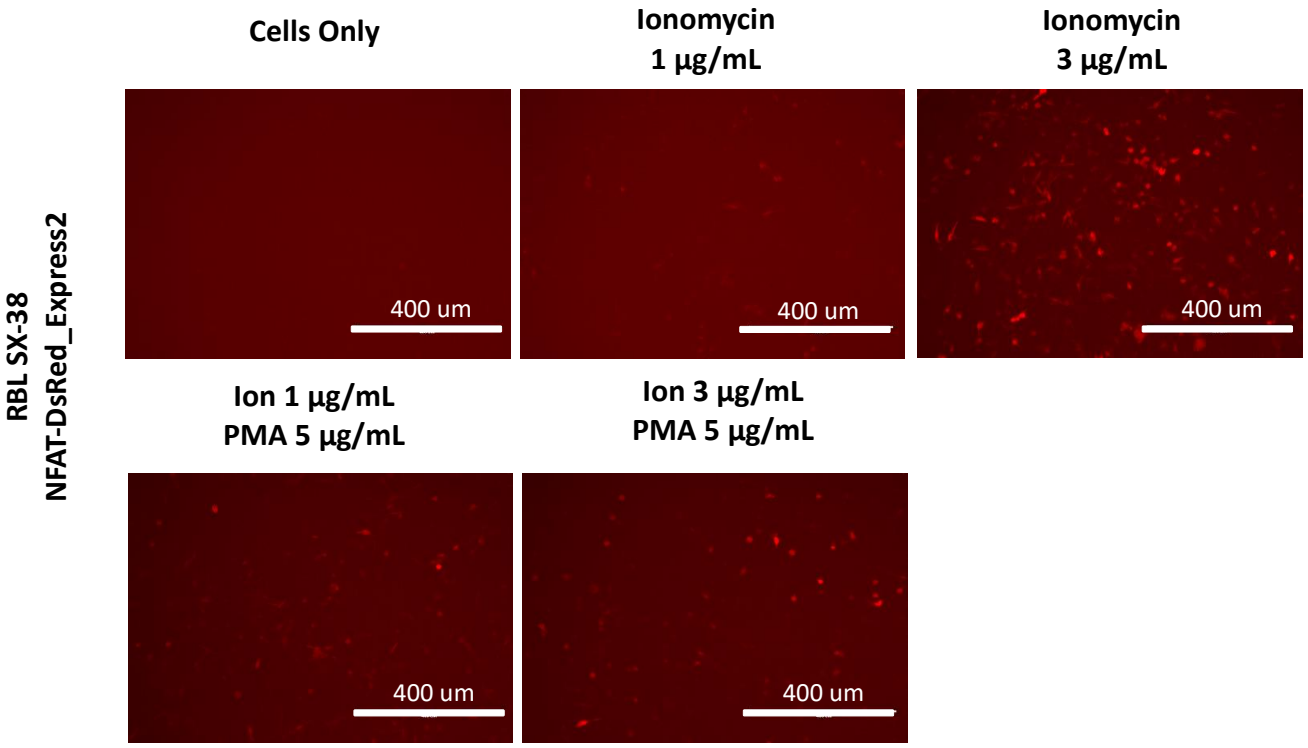
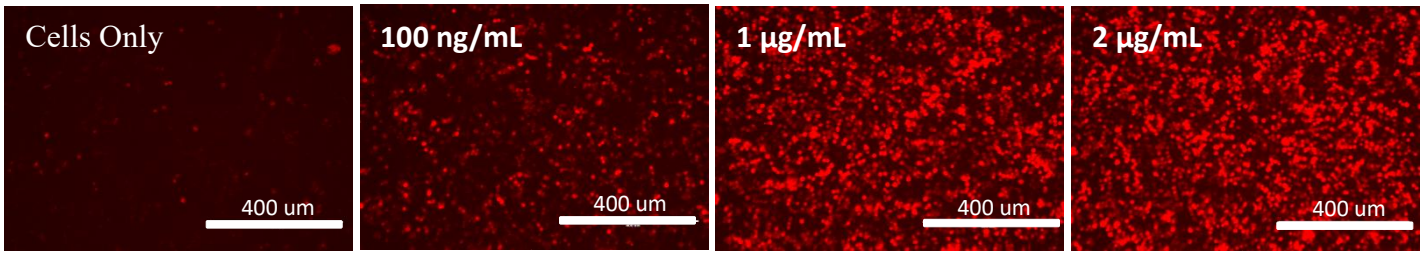


Figure 33. RBL SX-38 NFAT-DsRed-Express2 cell activation. $5x10^4$ transfected cells per well were sensitized overnight with human serum (1/50 dilution in RBL medium) and activated with 1 and 3 µg/mL ionomycin and 1 and 3 µg/mL ionomycin together with 5 µg/mL phorbol 12-myristate 13-acetate (PMA). Cell activation was assessed after 24 hours with a Evos fl Digital Inverted Microscope using the RFP channel (Exc: 531 nm, Em: 593 nm).

Appendix III

RBL 703/21 NFAT-DsRed_Express2 Activation with Human Serum

A)



B)

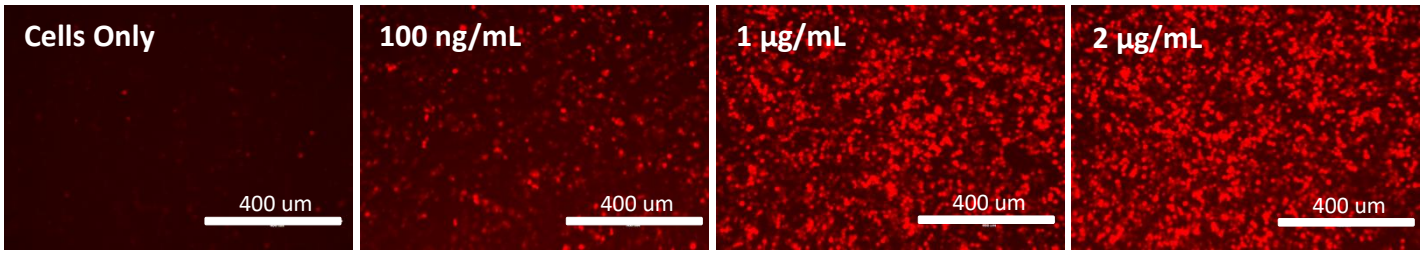


Figure 34. RBL 703/21 NFAT-DsRed-Express2 cell activation. $5x10^4$ transfected cells per well were sensitized overnight with two different concentrations of human serum and stimulated with 100 ng/mL, 1 µg/mL and 2 µg/mL anti-human IgE. **A**) Cells sensitized with 1/50 serum dilution after preheating the serum in 56 °C for 5 minutes. **B**) Cells sensitized with 1/100 serum dilution without pretreatment. Cell activation was assessed after 24 hours with a Evos fl Digital Inverted Microscope using the RFP channel (Exc: 531 nm, Em: 593 nm).

Appendix IV

RS-ATL8 Activation

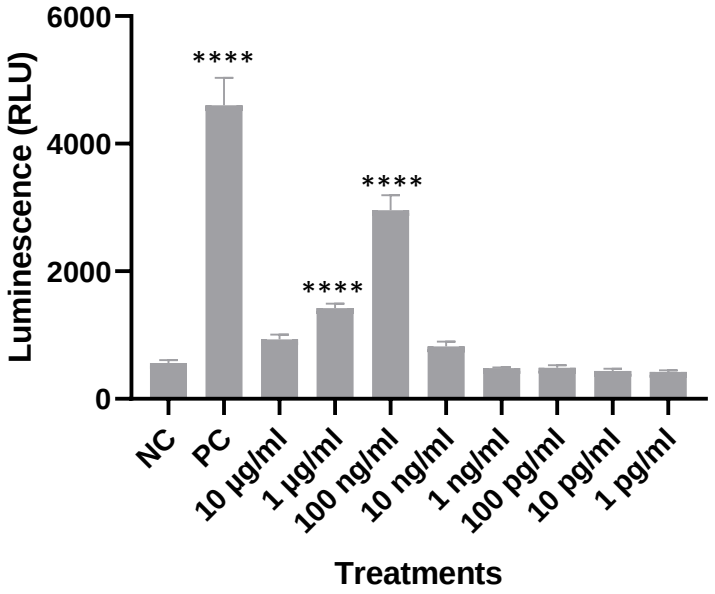


Figure 35. RS-ATL8 cell activation. Cells were sensitized overnight with 1 µg/mL human IgE. After 16 hours, cells were stimulated with a wide range of concentrations of anti-human IgE and activation measured as luminescence 4 h after stimulation. Data are shown as mean ± s.d. for triplicate determination.

Appendix V

NFAT-DsRed-Express2 Plasmid Digestion

Table 10. pNFAT-DsRed-Express2 linearization. Each PCR tube contained a total reaction volume of 100 µl. Sample master mixes were kept at 37°C for 6 hours. 1U (Unit) BgLII is used to digest 1 µg of DNA in 1h at 37°C in a total reaction volume of 50 µl.

Reagent	Concentration	Volume µl	Final Concentration
NEBuffer 3.1	10x	10	1x
Res. Enzyme **BgLII**	10x	1	1x
NFAT-DsRed-Express2 DNA	1310 ng/ µl	2.3	3 µg
H2O (RNase/Dnase free)	-	86.7	-

Appendix VI

Transfer Buffer-Western Blot

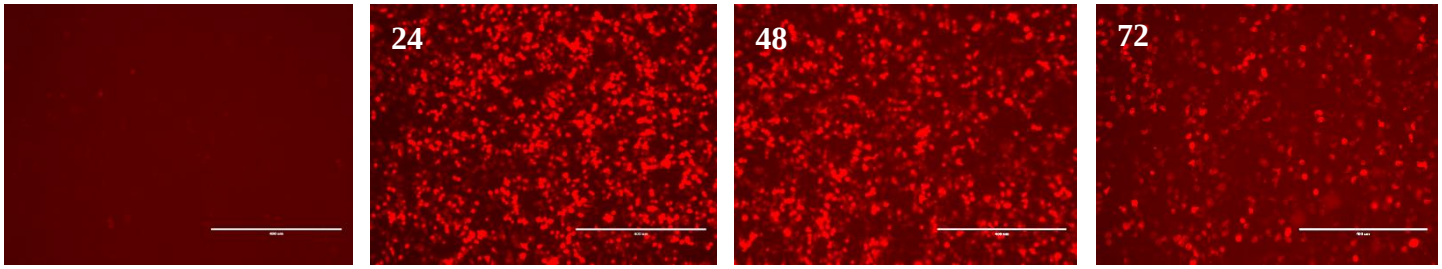
Table 11. Western Blot transfer buffer

Reagent	Volume mL	%
Methanol	40	20
Tranfer Buffer (Trans-Blot Turbo Transfer	40	20
H2O (Deionized)	120	60
Total	**200**	-

Appendix VII

DsRed protein expression in RBL 2H3 and RBL 703/21 pNFAT-DsRed-Express2 transfected cells.

RBL 703/21 NFAT-DsRed-Express2



RBL 2H3

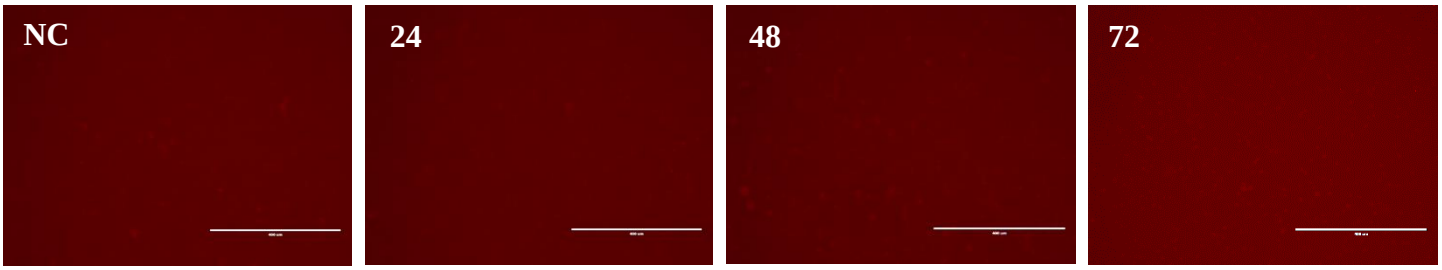


Figure 36. DsRed protein expression in pNFAT-DsRed_Express2 stably transfected RBL 703/21 cells. Images were taken 24, 48 and 72 hours after stimulation with anti-human IgE. RBL 2H3 cells do not express DsRed protein and were used as a negative control. Images were captured with fluorescent microscope set to RFP channel (Exc: 531 nm, Em: 593 nm) at 10x magnification. Bars represent 400 µm.

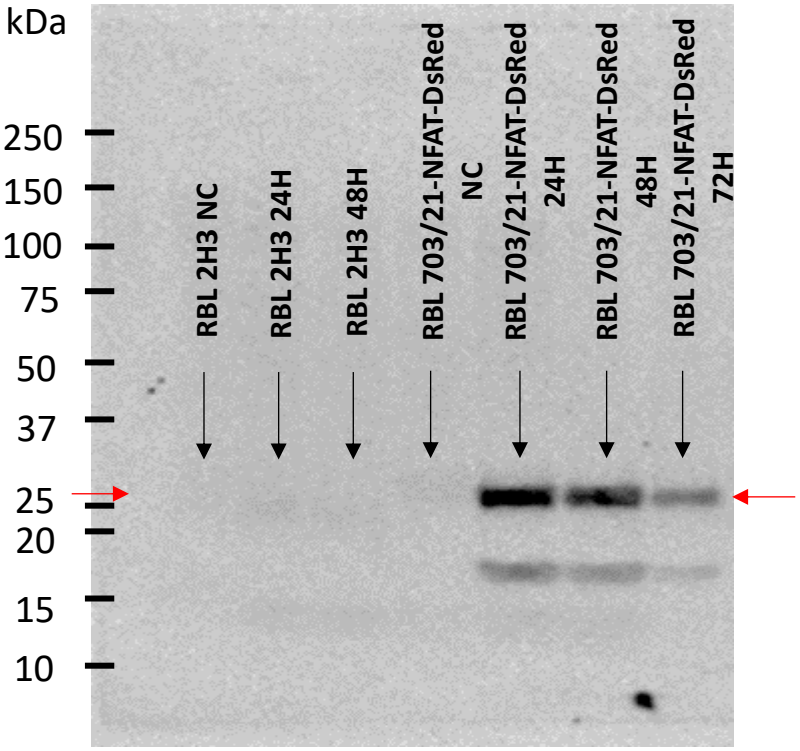
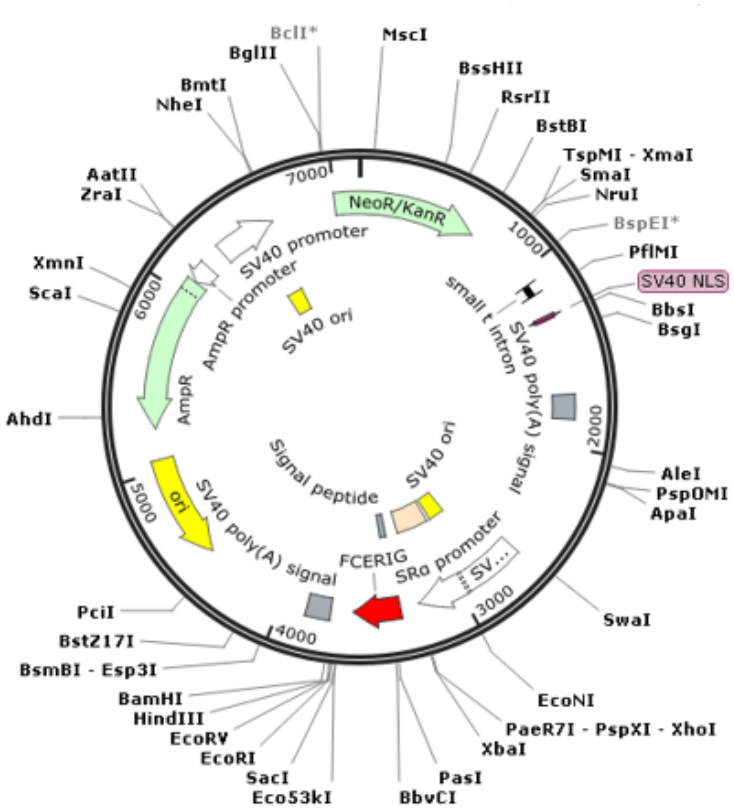


Figure 37. Immunoblot with anti-RFP antibody [6G6] for DsRed protein detection in RBL 703/21 NFAT-DsRed-Express2 cells. Extracts of RBL 2H3 non transfected cells and RBL 703/21 pNFAT-DsRed-Express2 transfected cells, stably expressing DsRed red fluorescent protein. Cell samples were harvested at 24, 48 for both cell lines and 72 hours only for RBL 703/21 transfected cells after initiation of stimulation with anti-human IgE (except for the negative control (NC)). RBL 2H3 was used as a negative control. The expected molecular weight of DsRed is 25.9 kDa and is indicated by the red arrows. The expression of DsRed was detected using a mouse monoclonal antibody [6G6] for Red Fluorescent Proteins as a primary antibody (1:1000 dilution) and anti-mouse IgG as a secondary antibody (1:2000 dilution). Membrane visualization was performed using a Pierce ECL chemiluminescent substrate.

Appendix VIII



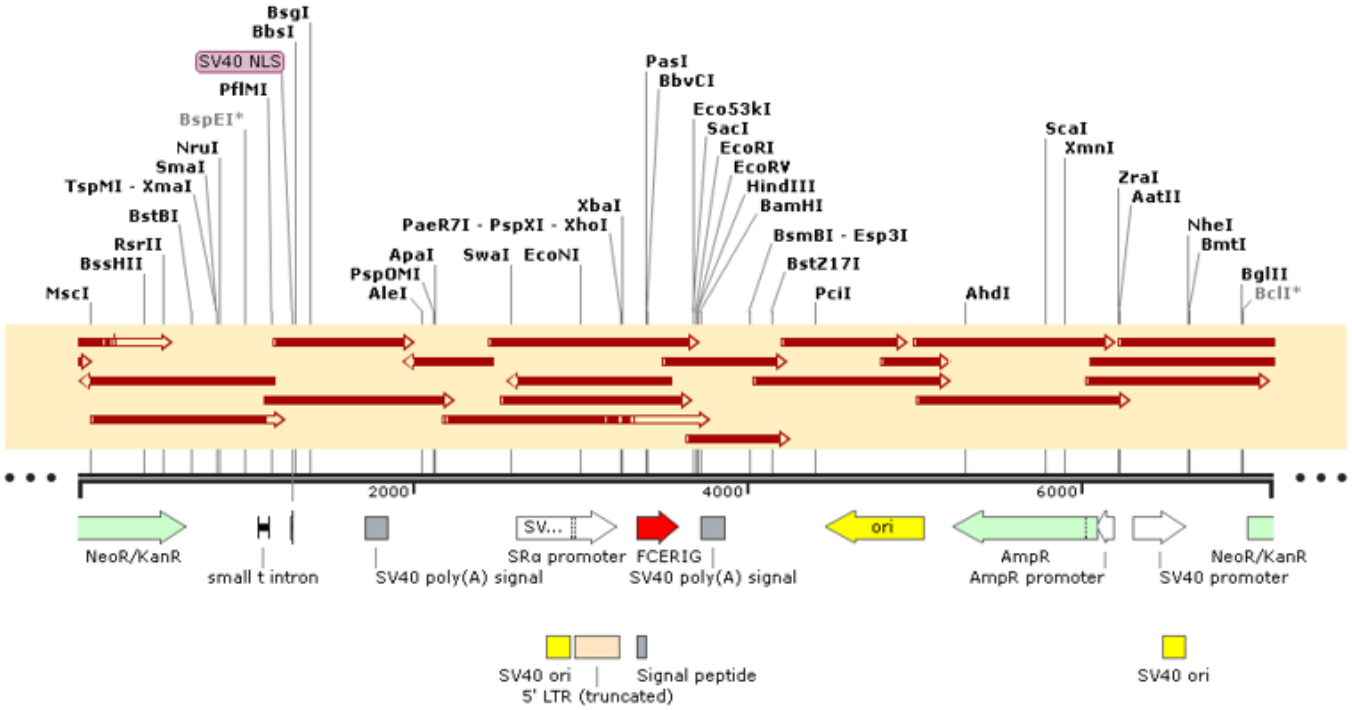


Figure 38. pBJ1 neo-hu FceR1 gamma plasmid map encoding neomycin/kanamycin resistance.

Appendix IX

pTK-Hyg Plasmid Map

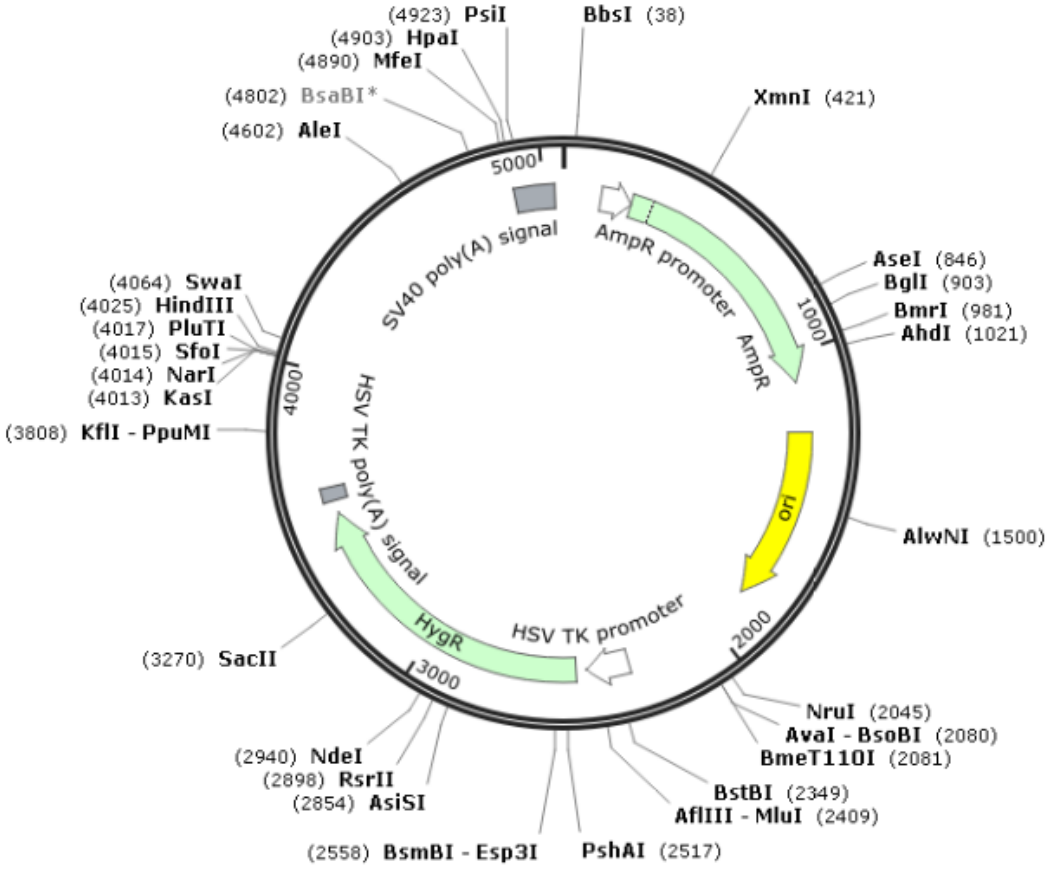


Figure 39. Plasmid map of pTK-Hyg (5066 bp).

Table 12. Primers for Hygromycin gradient PCR.

Oligo Name	Sequence (5'-3')
Hyg+BgLII (FOR)	ATATATAGATCTACCATGAAAAAGCCTGAACTCACCGCGACG
Hyg+BstBI (REV)	TATATATTCGAATCAGTTAGCCTCCCCCATCTCCCGATC

Table 13. Hygromycin gene gradient PCR reaction components. Master mix contained MgCl2 at a final concentration of 1.5 mM in the reaction.

Reagent	**Concentration**	**Volume µL**	**Final Concentration**
2x Platinum™ SuperFi™ Green PCR Master Mix[1]	2x	12.5	1x
Hygromycin DNA template	39.1 ng/µL	4	2.4 ng/ µL
Hyg+BgLII (F) Hyg+BstBI (R)	100 µM	6	5 µM
H2O (RNase/DNase free)	-	2.5	-
Total		25	

Table 14. PCR reaction components. A master mix of the constituents minus the DNA template was made up for both reactions including control, and all reagents were from Invitrogen (Thermo Fisher Scientific, US).

Reagent	**Concentration**	**Volume µL**	**Final Concentration**
10x High Fidelity PCR Buffer	10x	5	1x
dNTP Mix	10 mM	1	0.2 mM
Platinum® *Taq* DNA Polymerase High Fidelity	5 U/µL	0.2	1 U
$MgSO_4$	50mM	2	2 mM
Hyg+BgLII (F)	100µM	1.5	5µM
Hyg+BstBI (R)	100µM	1.5	5 µM
H2O (RNase/DNase free)	-	38.8	-
Total		50	

Table 15. Diagnostic restriction digestion. Restriction reactions were carried out in 20 of each in PCR tubes. 1U (Unit) of BstBI restriction enzyme is capable of digesting 1µg of DNA at 65°C for 1 hour. 1U (Unit) of BglII restriction enzyme is capable of digesting 1µg of DNA at 37°C for 1 hour.

Reagent	Concentration	Volume µL	Final Concentration
Hygromycin DNA template	39 ng/µL	10.25	400 ng/µL
CutSmart® Buffer	10x	1	1x
BstBI	20 U/µL	1	1 U/µL
BglII	10 U/µL	1	0.5 U/µL
NaCl	5M	0.4	100 mM
H_2O (RNase/DNase free)	-	6.35	-
Total		20	

Table 16. Setting up restriction sites for insert. Restriction reactions were carried out in 100 of each in PCR tubes. 1U (Unit) of BstBI restriction enzyme is capable of digesting 1µg DNA at 65°C for 1 hour. 1U (Unit) of BglII restriction enzyme is capable of digesting 1µg DNA at 37°C for 1 hour.

Reagent	Concentration	Volume µL	Final Concentration
Hygromycin DNA template	39 ng/µL	85	3.3 µg
CutSmart® Buffer	10x	10	1x
BstBI	20 U/µL	2	0.4 U/µL
BglII	10 U/µL	2	0.2 U/µL
NaCl	5M	1	100 mM
H_2O (RNase/DNase free)	-	-	-
Total		100	

Table 17. Reaction in 10 µL in PCR tubes

Reagent	Concentration	Volume µL	Final Concentration
Ligase buffer	10x	1	1x
T4 DNA ligase	10x	1	1x
V: FceR1G+ BglII/BstBI	2 µg/µL	1	0.2 µg/µL
F: Hygromycin+BgIII/BstBI	5 µg/µL	3	1.5 µg/µL
H_2O (RNase/DNase free)	-	4	-
Total		10	

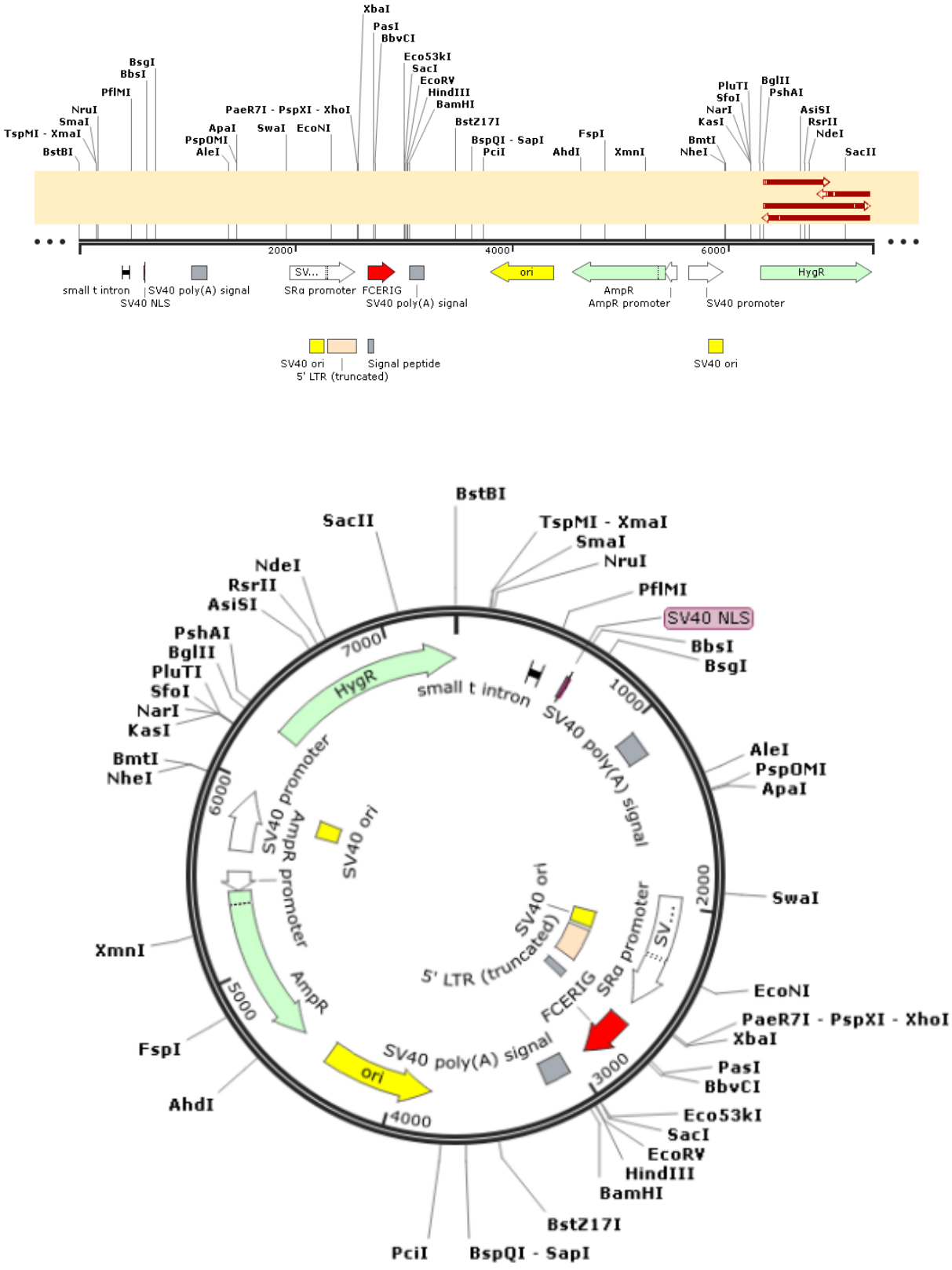


Figure 40. DNA sequence comparison of FCERIG-Hygromycin plasmids (iii & iv).

Appendix X

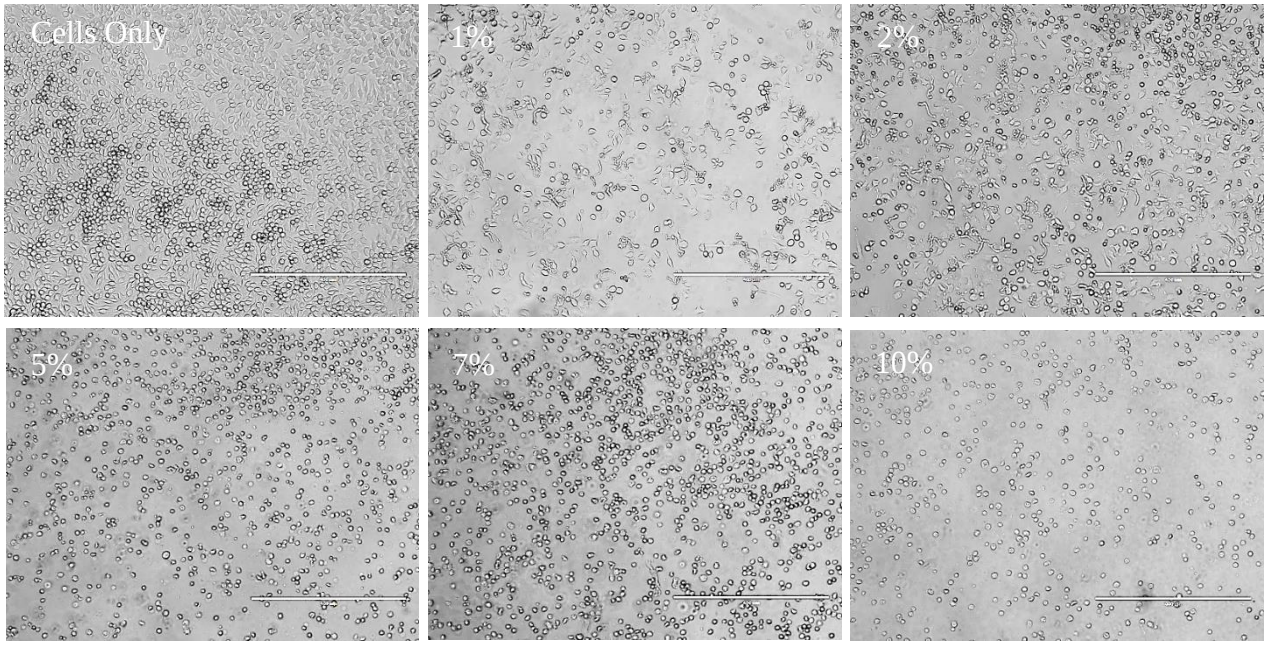
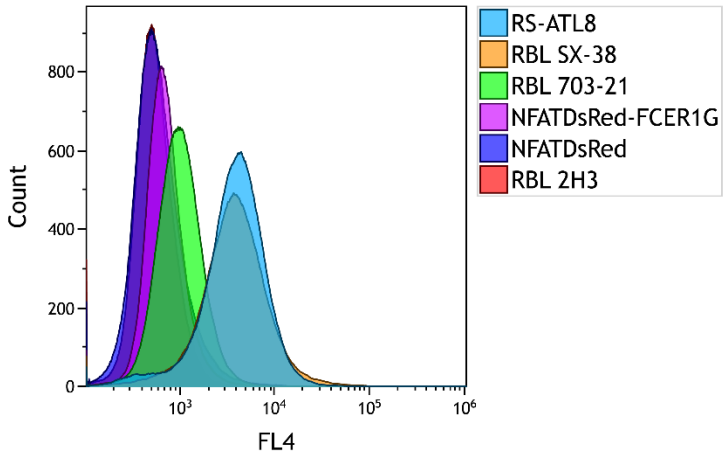
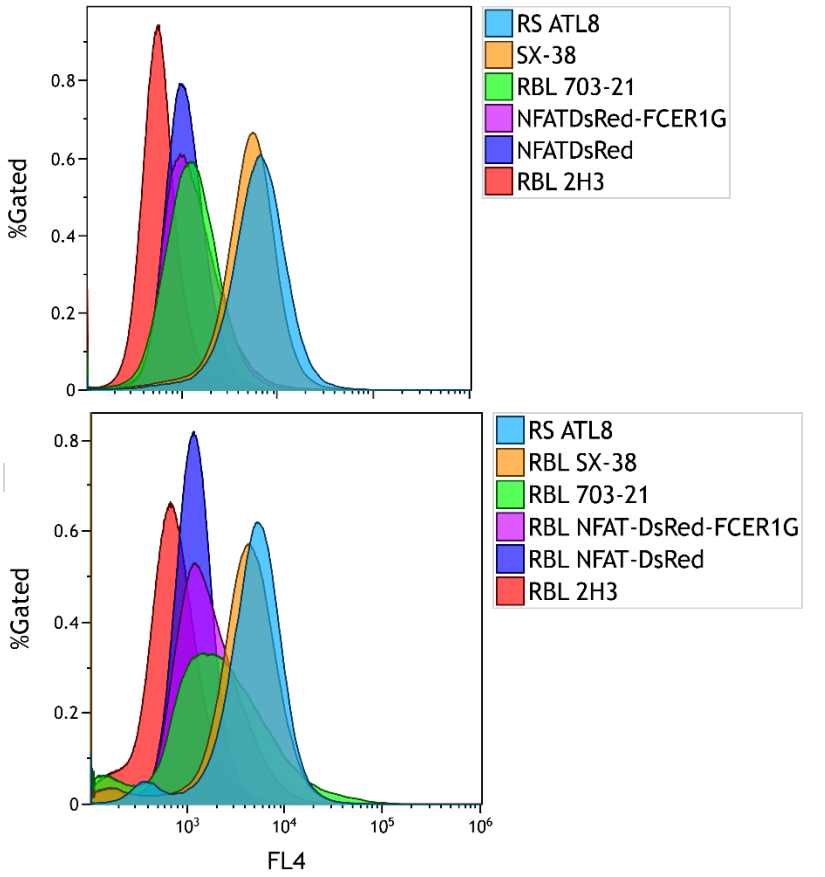


Figure 41. NFAT-DsRed FcεRIG cells treated with various concentrations of rat serum (1%, 2%, 5%, 7% and 10%) for 24 hours.

Appendix XI



Marker	X-Med	X-Mode
All	3,979.36	4,227.35
All	3,817.12	3,933.86
All	994.19	1,048.58
All	682.00	627.98
All	561.21	492.58
All	532.29	492.58



Marker	X-Med	X-Mode
All	5,034.39	5,949.85
All	4,216.72	104.86
All	1,940.90	104.86
All	1,474.56	104.86
All	1,158.77	104.86
All	705.95	104.86

Figure 42. Histograms of FcεRIα$_H$ surface expression in all RBL cell lines. The blue histograms show untransfected NFAT-DsRed cells compared to stably transfected cells with pBJ1 neo-hu FcεRI gamma (NFAT-DsRed-FCERIG) in purple and RS-ATL8 cells in light blue. All cells were stained with APC-labelled anti-human FcεRI α-chain monoclonal antibody (CRA-1).

Appendix XII

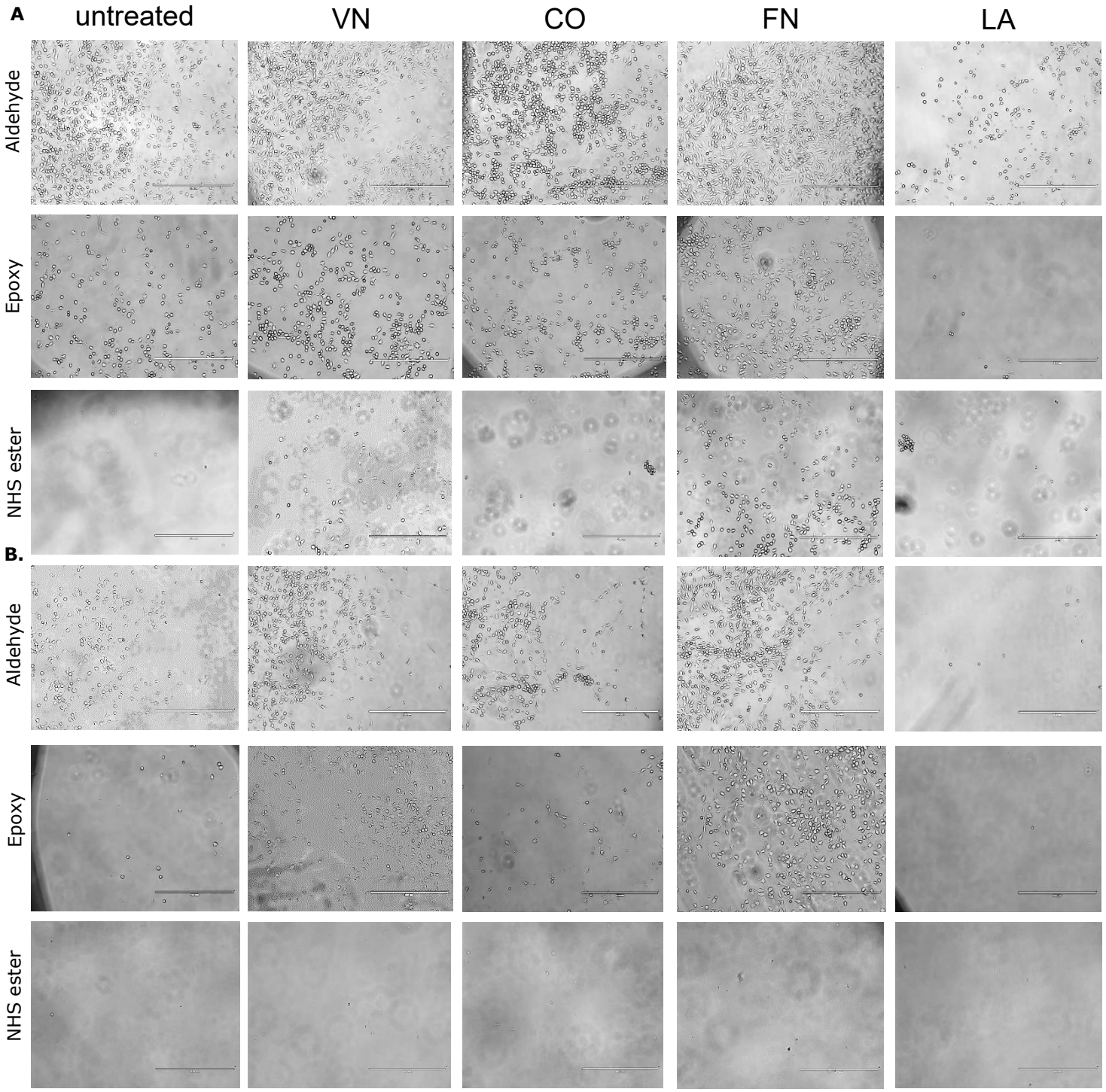


Figure 43. RBL 703-21/NFAT-DsRed cell attachment on aldehyde, epoxy and NHS ester-coated surfaces treated with vitronectin, collagen, fibronectin, laminin or no treatment, after **A**. 48 hours and **B**. 72 hours incubation. Images were captured using fluorescence light microscopy with a 10x objective lens. Images scale bar 400 µm.

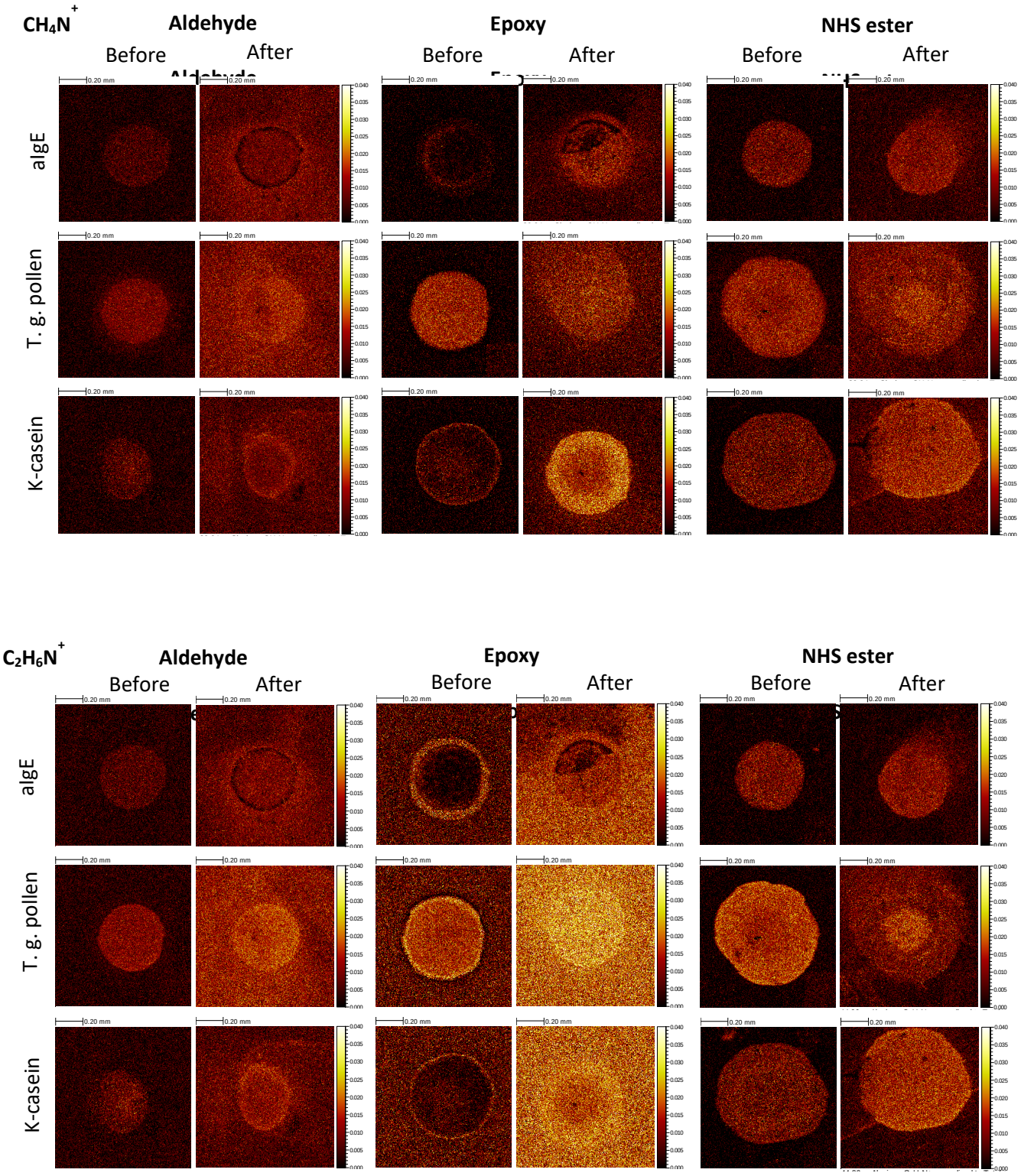


Figure 44. ToF-SIMS ion distribution maps for the characteristic glycine (*m/z* 30, CH_4N^+) and alanine (*m/z* 44 $C_2H_6N^+$) fragments on aldehyde, epoxy and NHS ester coated surfaces. Each image was normalised to the corresponding total ion map. Brighter colour corresponds to higher normalised ion intensity.

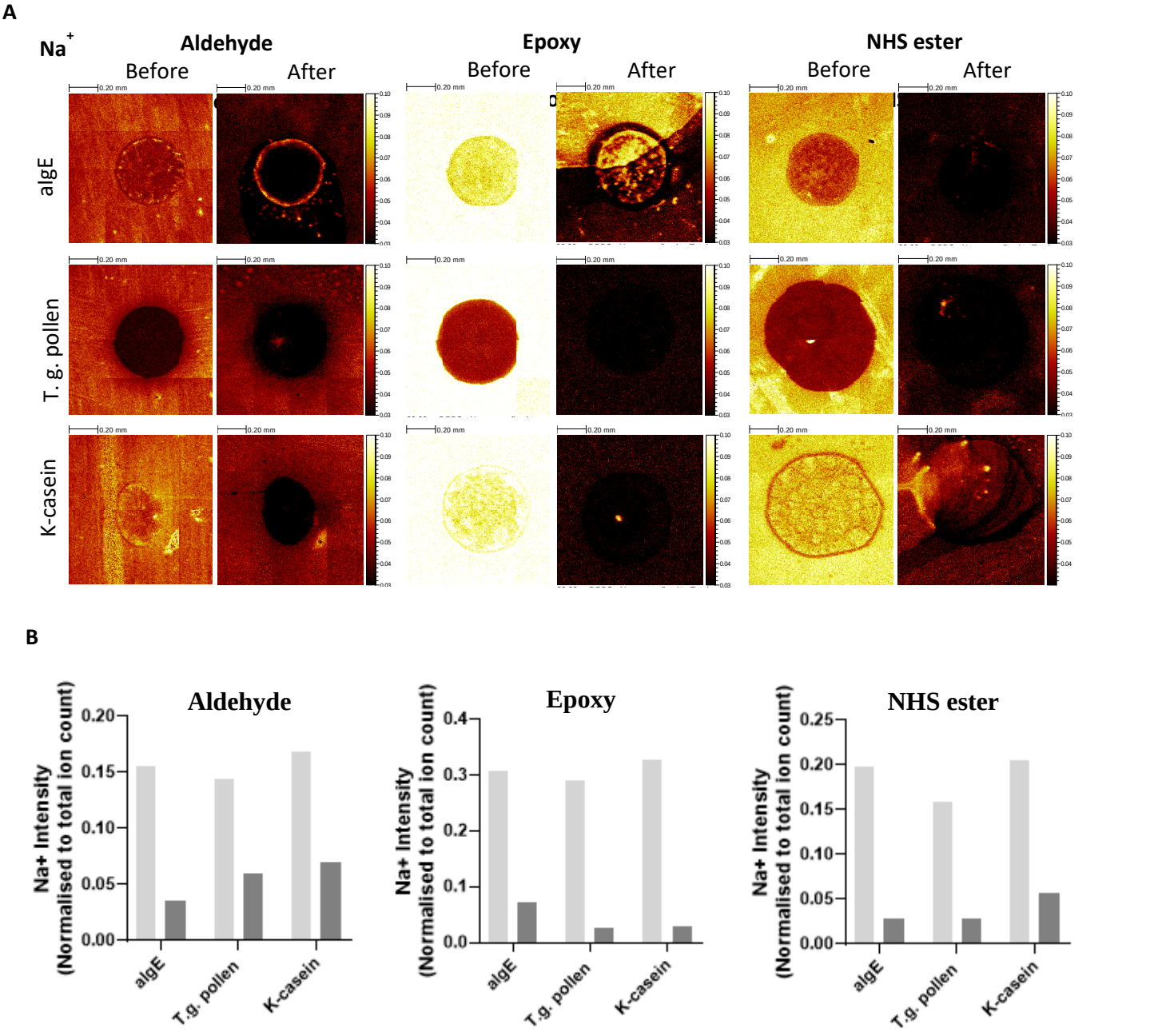


Figure 45. A. ToF-SIMS ion distribution maps for the characteristic sodium fragment (*m/z* 22.99 Na^+) on aldehyde, epoxy and NHS ester-coated surfaces. Each image was normalised to the corresponding total ion count. Brighter colour corresponds to higher normalised ion intensity. **B**. Statistic Plots displaying normalised sodium (Na^+) ion intensities of the three proteins (anti-human IgE, timothy grass pollen extract and k-casein) printed on aldehyde, epoxy and NHS ester-treated surfaces. Data presented derive from single ion intensity measurements.

Table 18. Patient serum specific-IgE profile determined by RAST test.

Appendix XIII

Component Results

Component	Your Value	Standard Range	Flag
Bermuda Grass Allergen	**92.50** kU/L	*<0.10 kU/L*	H
Perennial Rye Grass Allergen	**>100.00** kU/L	*<0.10 kU/L*	H
Oak Allergen	**64.70** kU/L	*<0.10 kU/L*	H
Olive Allergen	**81.10** kU/L	*<0.10 kU/L*	H
Russian Thistle Allergen	**29.80** kU/L	*<0.10 kU/L*	H
Sagebrush (Common) Allergen	**0.97** kU/L	*<0.10 kU/L*	H
Alternaria Tenuis	**79.40** kU/L	*<0.10 kU/L*	H
Aspergillus Fumigatus	**5.38** kU/L	*<0.10 kU/L*	H
Cladosp. Herbarum Allergen	**0.12** kU/L	*<0.10 kU/L*	H
Penicillium Notatum Allergen	**<0.10** kU/L	*<0.10 kU/L*	
Cat Dander Allergen	**5.93** kU/L	*<0.10 kU/L*	H
Dog Epithelium Allergen	**0.16** kU/L	*<0.10 kU/L*	H
Dermatophagoides Farinae Allergen	**27.10** kU/L	*<0.10 kU/L*	H
Dermatophagoides Pteronyssinus Allergen	**26.20** kU/L	*<0.10 kU/L*	H
Cockroach Allergen	**10.10** kU/L	*<0.10 kU/L*	H

Reactivity for Individual/Component Allergens
Class kU/L Allergen Reactivity

0 <0.10 Absent or ND
0 0.10 - 0.34 Very Low
I 0.35 - 0.69 Low
II 0.70 - 3.49 Moderate
III 3.50 - 17.49 High
IV 17.5 - 52.49 Very High
V 52.5 - 99.99 Very High
VI >=100 Very High