UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

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Assessing Basophil Activation in Allergic Disease by the Measurement of the Unique Marker Basogranulin: Release into cell supernatants and biological fluids, and alterations in intracellular and membrane expression

By

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

FACULTY OF MEDICINE

Infection, inflammation and immunity

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Assessing Basophil Activation in Allergic Disease by the Measurement of the Unique Marker Basogranulin: Release into cell supernatants and biological fluids, and alterations in intracellular and membrane expression

Mohammad O. Alzahrani

Allergic conditions affect increasing numbers of the population, are associated with considerable morbidity and in their most severe forms can be life-threatening. A prominent feature in allergic conditions is the activation of mast cells and basophils by allergen resulting in the release of inflammatory mediators. A unique product of basophils is basogranulin, a protein stored and released from the secretory granules on cell activation. Our aim has been to develop new assays for assessing basophil activation based on basogranulin measurements, and applying these assays for measuring basophil sensitivity in samples from allergic patients. In addition, we have investigated basogranulin in saliva and BAL fluid as a marker for basophil activation *in vivo*.

Basophils stimulated with fmlp, anti-IgE and grass pollen *in vitro* and the basogranulin released into supernatants was quantified by dot blotting. In addition, flow cytometric assays were developed for measuring alterations of intracellular and surface basogranulin expression following basophil activation *in vitro* with fmlp, anti-IgE anti-FcεRI or specific allergens. There was an apparent depletion in intracellular basogranulin stores in activated basophils when compared with that in non-stimulated basophils. The depletion of intracellular basogranulin was inversely associated with increased expression of CD63.

Surface basogranulin expression was barely detected in nonstimulated basophils but was increased following stimulation. Membrane expression of basogranulin was correlated with that of CD63 in nonstimulated basophils (0.829, $P< 0.005$, n=10), and in basophils stimulated with anti-IgE (r=0.877, $P< 0.001$, n=51), anti-FcεRI (r=0.680, $P< 0.0001$, n=20), or fmlp (r=0.914, P<

0.0001, n=9). When basophils were stimulated with extracts of various allergens including D. *pteronyssinus*, *D. farinae*, crab, shrimp and oyster increased basogranulin expression was observed in cases for where this was not detected for CD63. Alterations in intracellular and surface basogranulin expression were also visualised by confocal microscopy, and by this technique considerable heterogeneity in marker expression was observed between individual cells.

Basogranulin could be detected in saliva samples of peanut allergic children, suggesting a potential role in assessing the contribution of basophils in clinical disease. In BAL fluid from patients with atopic asthma basogranulin levels were higher than that from healthy subjects, but not from those with severe asthma.

Basogranulin measurement within basophils, on the cell membrane and in biological fluids represent novel approaches for assessing basophil activation and for establishing the contribution of basophils in clinical disease. They show promise as new means for the diagnosis of allergic sensitivity and confirmation of allergic reactions mediated by basophils.

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Presentations

1- Mohammad O Alzahrani, M Aref Kyyaly, Syed Hasan Arshad, Andrew F Walls, Abstract: Novel method for assessing basophil activation by measuring altered expression of membrane-bound and intracellular basogranulin stores (Oral presentation), the World Allergy Congress, 12-14 December 2019, Lyon, France.

2- Mohammad O Alzahrani, Shalini Chandel, M Aref Kyyaly, Syed Hasan Arshad and Andrew F Walls, Novel Method for Measuring Basophil Activation: A Test for Allergy Diagnosis Based on Expression of Basogranulin in Basophils, (Oral presentation), Southampton Medical and Health Research Conference, 6 -7 June 2018, Southampton, United Kingdom.

3- Mohammad O Alzahrani, Chloe Rose, Shalini Chandel, M Aref Kyyaly, S Hasan Arshad and Andrew F Walls, Basophil Activation Tests based on Surface Expression of Basogranulin and CD63 and the Release of Basogranulin and Histamine, (Poster Presentation) the EAACI Food Allergy Training Course “Prevention and Treatment of Food Allergy” 14 - 16 September 2017, Manchester, United Kingdom.

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Declaration of Authorship

I, Mohammad Alzahrani declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research. [Measuring Basophil Activation by Assessing Basogranulin Measurements: Novel Methods for Diagnosing Allergic Sensitivity, and for Confirming and Predicting Susceptibility to Severe Reactions]

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as presentations.

Signed: Mohammad Alzahrani

Date: 07.10.2020

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Firstly, I would like to plentifully thank my God, Allah the Almighty for providing me patience and strength to complete this work. Then, with big love and sincere appreciation I would like to thank my parents for their support and honest prayers every day. I really regret that I have had to be away from them to pursue my studies while their health has been poor, and that my father died before I completed this work.

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Abbreviations

ACE	angiotensin-converting enzyme
APC	allophycocyanin
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
CI	calcium ionophore
CRTH2	chemoattractant receptor-homologous molecule expressed on TH2 cells
CCR C-C	motif receptor
DAPI	49,6-Diamidino-2-phenylindole dihydrochloride
DBPCFC	double blind placebo-controlled food challenge
DMSO	dimethyl sulphoxide
FAHF-2	Food Allergy Herbal Formula-2
FITC	fluorescein isothiocyanate
fmlp	n-formyl-methionyl-leucyl-phenylalanine
GM-CSF	granulocyte macrophage colony-stimulating factor receptor
HRP	horseradish peroxidase
IL	interleukin
LT	leukotriene
mAb	monoclonal antibody
NGF	nerve growth factor
NK	natural killer cells
PE	phycoerythrin
PGD2	prostaglandin D2
PBS	phosphate buffer saline
SGM	standard growth medium
TMB	3,3’,5,5’-tetramethylbenzidine
MFI	Median florescence intensity

SBU	standardised biological unit
SDS	sodium dodecyl sulphate
SQ-U	standardised quality unit
g	relative centrifugal force

Chapter 1 Introduction

Allergy is a growing public health concern that compromises quality of life and is potentially fatal. Great strides have been made in laboratory diagnosis of allergic conditions, although there are major deficiencies in techniques currently available for investigation of allergic sensitivity and particularly to some foods. Moreover, there are unmet medical needs in establishing effective means for confirmation of the allergic nature of a reaction, and of identifying those at risk of serious reactions.

Though often overlooked, there is a growing appreciation of the roles of basophils in allergic conditions and examining the activation of these cells in response to allergens in vitro can provide useful information on allergic sensitivity and perhaps also on susceptibility to more severe reactions. The various methods currently available are generally poorly standardised and there is uncertainty over the most appropriate means to use. The advent of new tools has opened the way for novel approaches for investigation of basophil activation both in vitro and in clinical disease. There is a pressing need for evaluation of the different means for measuring basophil activation and potential for new diagnostic methods based on basophil activation to be developed for clinical use.

1.1 Allergy

The major role of the immune system is to protect the host against harmful bacteria, fungi, viruses and parasites by recognising pathogens, and distinguishing self from non-self [1]. However, the immune system can recognise and respond to innocuous molecules, generating undesirable reactions, such as in the allergic response [2]. The term *allergy* was first introduced by Clemens von Pirquet in 1906 from the Greek *allos*, meaning other or different, and *ergia*, meaning energy or action. He noted that the exposure of the body to a substance resulted in the production of antibodies that induced a change in subject-specific reactivity to the substance which he called ‘allergy’. He recognized that sensitisation to an allergen can occur, leading to responses on subsequent allergen exposure [3].

Allergy was defined by the Nomenclature Review Committee of the World Allergy Organization as “a hypersensitivity reaction initiated by proven or strongly suspected

immunological mechanisms" [4]. Hypersensitivity reactions were originally categorised by Gell and Coombs into four subtypes based on the type of immune response and the effector mechanism responsible for cell and tissue injury: type I, immediate or IgE mediated; type II, cytotoxic or IgG/IgM mediated; type III, IgG/IgM immune complex mediated; and type IV, delayed-type hypersensitivity or T-cell mediated (Table 1-1). The reaction involves sensitised T cells, which in response to antigen release lymphokines may also directly kill target cells The reaction involves sensitised T cells, which in response to antigen release lymphokines may also directly kill target cellsThe reaction involves sensitised T cells, which in response to antigen release lymphokines may also directly kill target cells [8].

Table 1-1Type I hypersensitivity reactions usually can be extremely rapid and dramatic and are mediated by IgE antibodies interacting with mast cells and basophils. There may be roles for eosinophils, platelets, and neutrophils in amplifying the response though mast cells and basophils represent the key cells in developing a reaction. Allergen can cross-link the IgE antibodies bound on high affinity receptors (FcεRI), leading to degranulation of the anchoring mast cells or basophils and release of a variety of mediators of inflammation. Type I reactions underlie allergic rhinitis, allergic asthma and many food or drug induced reactions and anaphylaxis [5].

Type II hypersensitivity, involves a cytotoxic reaction, with direct antibody-mediated cell interactions leading to the production of anaphylatoxin (C5a), the recruitment of polymorphonuclear leukocytes and tissue injury as a result of the release of hydrolytic neutrophil enzymes after their autolysis [6]. Type III hypersensitivity reactions, also termed immune complex hypersensitivity, are mediated by soluble antibodies of the IgG class, and sometimes of IgM, forming antibody-antigen complexes. When substantial quantities of such immune complexes are formed, and they can become deposited in tissues and result in a tissue reaction which is initiated by complement activation. This leads to mast cell degranulation, leukocyte chemotaxis and inflammatory reactions associated with the activation of these cells. After exposure to specific antigen, reactions may develop over a period of about three to ten hours after exposure to specific antigens.

Unlike types I, II, and III hypersensitivities that are mediated by antibodies, type IV is an immune response mediated by sensitised lymphocytes. The reaction occurs 48 to 72 hours after antigen exposure. Type IV reactions typically manifest as contact dermatitis in response to drugs, cosmetics and environmental chemicals to which the skin is often exposed [7]. The reaction involves sensitised T cells, which in response to antigen release lymphokines may also directly kill target cells [8].

Table 1-1. Gell and Coomb's classification for hypersensitivity.

Type of hypersensitivity	**I**	**II**	**III**	**IV**
Other characteristics	Immediate; anaphylactic	Cytotoxic	Immune complex	Delayed; cell mediated
Antibody	IgE	IgG, IgM	IgG, IgM	None
Antigen	Exogenous	Cell surface	Soluble	Tissues and organs
Response time	Seconds to 30 minutes	Minutes to hours	3 to 10 hours	24 to 72 hours
Effector mechanism	Mast cell and basophil activation	Complement fixation Phagocytes, NK cells (Fc receptor cells)	Complement Phagocytes	Macrophage activation, Cytotoxic lymphocytes, Eosinophil activation
Response appearance to antigen	Weal and flare	Lysis and necrosis	Erythema and oedema, necrosis	Erythema and induration
Histology	Degranulated mast cells; Cellular infiltrates including basophils and eosinophils	Antibody and complement	Complement and neutrophils	Monocytes and lymphocytes
Sensitivity transferred by	Antibody	Antibody	Antibody	Lymphoid T cells
Examples	Allergic asthma, hay fever	Erythroblastosis feotalis Good pasture's nephritis	Systemic lupus erythematosus, farmer's lung disease	Tuberculin test, poison ivy, granuloma

Adapted from Coombs and Gell [5],[9, 10].

The term allergy has become most commonly associated with conditions mediated by type I processes and henceforward the term allergy will be employed here where such reactions are involved. IgE-mediated reactions occur following primary sensitization, which is associated with the generation of specific IgE antibodies that become attached to high affinity IgE receptors (FcεRI) on mast cells and basophils [11].. When the allergen is re-introduced in an individual, who is sensitized, it can interact and stimulate these receptor- IgE molecules on sensitized mast cells and basophils. The cross-linking of receptors leads to the release of various inflammatory mediators. These mediators contribute to the development of a diverse range of symptoms manifested in allergic reactions. Activated mast cells or basophils release preformed and newly synthesized mediators, that contribute to clinical manifestations of allergy [12]. Increased vascular permeability, smooth-muscle contraction and mucus production may be provoked by mediators including histamine, leukotrienes and cytokines [13, 14] resulting in the typical symptoms of an allergic reaction (Table 1-2). Symptoms may occur alone or in combination and characteristically appear within minutes of ingestion.

Table 1-2. Symptoms of allergy.

Organ	**Symptoms**
Oral	Oedema and pruritus of lips and tongue, metallic taste.
Cutaneous	Pruritus, urticaria, flushing, angioedema.
Gastrointestinal	Nausea, abdominal pain, vomiting, diarrhoea.
Respiratory	Dysphagia, dysphonia and hoarseness, dry cough, pruritus and 'tightness' in throat, shortness of breath, 'tightness' in chest, wheezing, congestion, sneezing.
Cardiovascular	Hypotension, chest pain, syncope, dysrhythmia.

Adapted from [15],[16].

The prevalence of allergic reactions has increased in recent decades to be between 10 to 40% worldwide as it has reported by the World Allergy Organisation (WAO) [17]. This occurs not only in industrialized and developed countries but also increasingly in the low and middle-income countries [18]. Many efforts have been

directed to define the underlying mechanisms and predisposing factors that increase the allergic reactions. However, the underlying molecular and cellular processes are less established. Allergic diseases have a large social and economic impact that includes the costs of health care, lost work and school hours, and lower quality of life [18].

The complex physiopathology of allergic diseases may be influenced by genetic susceptibility, route of exposure, allergen dose, time of exposure, allergen structure, and co-exposure with environmental agents, infections or commensal bacteria [19]. Common allergens may include pollens, fungal spores, house dust mites, and animal products but can also include food, drugs, biological products, and insect venoms [20]. Dust mites and grass pollen have been reported to be the most common allergens responsible for IgE-mediated allergies [21-23]. The most common triggers of allergic rhinitis and asthma are pollen and house dust mites [24-26], while house dust mites has become regarded the most important source of persistent allergens, and grass pollen of seasonal allergens. As food allergy is a particular focus for the present studies, it will be discussed separately (*vide infra*).

Grasses are ubiquitous throughout the world. In places with a temperate climate, members of the *Pooideae* subfamily[27] like timothy grass (*Phleum pratense*, *Phl p*), orchard grass (*Dactylis glomerata*), perennial ryegrass (*Lolium perenne*), and bluegrass (*Poa pratensis*) are the most common pollen sources. Grasses have a pollinating season from May to August in Central Europe, peaking in June. In northern Europe, the grass pollen season starts later, while pollination lasts for a longer period in Mediterranean Europe. In subtropical and tropical regions of the world, grass pollen seasons can be perennial [28]. There are substantial geographic variations, grass pollen may be the most prevalent sensitizing pollen in Europe [29].

Allergy to house-dust mites is of considerable importance, with 45% to 85% of patients with asthma in the United Kingdom showing skin test reactivity to mites, as compared with 5% to 30% in the general population [30]. The most important mite species that have been recognized as significant sources of indoor allergens include *Dermatophagoides pteronyssinus*, *D. farinae* and *Blomia tropicalis* [31].

Food allergy

The US National Institutes of Allergy and Infectious Diseases (NIAID) defined food allergy as "an adverse immune response that occurs reproducibly on exposure to a given food and is distinct from other adverse responses to food, such as food intolerance, pharmacological reactions, and toxin-mediated reactions" [32]. This definition encompasses immune responses that are IgE mediated, non-IgE mediated, or a combination of both in agreement with other international guidelines [33-35]. However, a European Academy of Allergy and Clinical Immunology (EACCI) task force has suggested that any adverse reaction to food should be called food hypersensitivity, and once immunological mechanisms have been established, the appropriate term is food allergy [4]. Whenever the role of IgE is confirmed, it can be identified as IgE-mediated food allergy. They have suggested that other reactions, previously termed 'food intolerance' should be referred to as non-allergic food hypersensitivity, and severe, generalised allergic reactions to food classified as anaphylaxis.

Food allergy is rapidly becoming a cause for concern in the Western world due to its increasing prevalence and potentially serious outcomes [36]. However, there is uncertainty about the prevalence of food allergy because of the lack of high-quality evidence based on reliable diagnostic tests. Moreover, most studies have employed surrogate markers, such as food allergy or anaphylaxis hospitalisation rates [37-39], outpatient presentations for food allergy or population cohorts evaluated by clinical history together with allergen-specific IgE and/or challenge [40, 41]. There is a pressing need for a low cost and reliable method for diagnosis of food allergy. Approximately 12% of the European population have been reported to have food allergy or intolerance based on the European Community Respiratory Health Survey administered to 17,280 adults in fifteen countries [42]. In the UK, about 20% of adults thought that they had a food allergy or intolerance based on a population-based study of 7,500 subjects in the Wycombe Health Authority area and the same number of randomly-selected households nationwide was followed up by interviews of positive respondents from the Wycombe Health Authority area [43]. However, the prevalence of reactions to food in the population varied from 1.4 to 1.8% as determined by allergen challenge. In spite of the limitations including changing

health-seeking behaviour, dramatic increases in food mediated anaphylaxis have been reported between 1992 and 2012 in the England and with doubling of hospital admission rates from 1.2 to 2.4 per 100,000 population with the highest rates observed in those aged 0-4 years [44].

In a questionnaire-based study in a French population of over 33,000 members, the incidence of food allergy was found to be 3.24%, with the most commonly implicated foods including egg, milk, tree nut, peanut, crustaceans, fruit and vegetables [45]. In a similar study of French school children, almost 7% were reported on the basis of medical history to be food allergic [46].Of the affected children, there appeared to be a greater incidence of allergy in the age range of 6-10 years than in other age groups. The most commonly reported allergenic foods were peanut, cow's milk, egg, shellfish, tree nut and exotic fruits. In about a quarter of the children, the condition was outgrown, particularly so with cow's milk allergy.

Most allergenic foods are high in protein, for example, eggs in which the majority of allergic reactions are to ovalbumin, and shrimps for which reactions are caused by tropomyosin. Cross-reactivity, both between different food types and between foods and other substances, has also been found. This is likely to occur when there are similarities in the allergenic proteins of the two substances [47].

Symptoms of food allergy can be acute and take place within minutes to hours of ingesting the trigger food and can vary in severity from mild to life-threatening [48]. The severity of allergic reactions varies based on the amount of food ingested, co-ingestion of other foods, and preparation of the food. In addition, the severity of a reaction may be influenced by the stability of the allergen against digestion process and the permeability of the epithelial barrier [49]. The presence of other concomitant conditions, such as asthma or atopic dermatitis, may also influence severity [50]. The most common symptoms to have been reported in a questionnaire-based study involving 109 food-allergic members of the Anaphylaxis Campaign in the UK included oral and respiratory problems, abdominal pain, and cutaneous manifestations [51].

1.1.1 Diagnosis of allergic diseases

Approaches currently available for diagnosing allergic disease should include (1) diagnosis of allergic sensitivity, (2) confirmation that an allergic reaction has taken place, and (3) assessment of the risk of a serious reaction. These three approaches will be discussed separately below. There are instances when diagnostic methods can provide contradictory results, are inaccurate, or may even be unethical to perform. Overdiagnosis may lead to undesirable outcome including malnutrition in case of food allergy, avoidance of the drug of choice in drug allergy, and to psychological problems. On the other hand, underdiagnosis may leave the patients suffering unnecessarily and may result in growth disorders and lead to significant quality of life impairment.

1.1.1.1 Allergic sensitivity

Clinical history

The clinical history should include an accurate description of the adverse food event in order to determine accurately its clinical features. The main issues of the reaction to include in the clinical history involve a description of the specific symptoms, the timing of the reaction, and the allergen suspected of causing the reaction [34, 52, 53]. However, diagnosis based primarily on a patient's clinical history can be inaccurate [54] as the symptoms may be present also in other conditions. Although there is no surrogate for a good history, laboratory tests are often needed to support the diagnosis and to provide a means of objectively assessing the disease, its severity and its response to treatment. As will be covered below, the use of laboratory tests has indicated shortcomings in the accuracy of the diagnosis of allergic reactions based only on medical history.

Skin testing

The skin test is the most commonly applied diagnostic applied method for investigating allergic sensitivity, and involves administration of allergens epicutaneously or intracutaneously. The subsequent wheal-and-flare reaction induced by the activation of skin mast cells is compared with the response to negative and positive controls [55]. Intracutaneous testing is generally not favoured

as a first line method because this approach carries the risks of severe reactions [56, 57]. Skin prick tests can provide a rapid means to detect sensitization in allergic disorders [58].

Although skin testing is simple and inexpensive, it may not be practical to testing large numbers of allergens and findings may not consistently correlate with levels of serum specific IgE (*vide infra*). In addition, skin prick testing can also result is false positive reactions [59]. Furthermore, a negative skin prick test response does not rule out clinical reactivity [55, 59].

In food allergy, extracts of allergen employed in skin testing may not be subjected to processes of allergen degradation during passage through the gastrointestinal tract. The ability of proteins to resist conditions found in the gastrointestinal tract, and for instance to resist the actions of low pH, pepsin, bile acids, and trypsin is of significance to the ability of allergens to induce sensitization and, more importantly in the context of allergy testing, to trigger cell activation via the FcεRI receptor [55]. Moreover, utilizing commercial allergens extracts produced via thermal processing may destroy heat-labile proteins suggesting that heating might lead to unfolding and disruption of conformational epitopes in foods that might be consumed uncooked [60]. This may contribute to altered allergenicity, thereby generating false negative results [61].

Although, a National Institute of Allergy and Infectious Diseases' Expert Panel suggested that performing skin prick testing can support the identification of foods that may provoke IgE-mediated food-induced allergic reactions [53], it was concluded that the skin test alone cannot be considered diagnostic of food allergy and therefore cannot always be taken as an indication of clinical sensitivity to the offending allergen. Moreover, false negative skin prick test results can result from the intake of antihistamines by the patient and therefore anti-allergic drugs should be avoided before the skin test, and this may affect the patients' quality of life [55].

Allergen challenge

An allergen challenge test that involves directly exposing a subject to an allergen in the way of natural exposure, can represent a better means than skin testing for determining allergic sensitivity. *In vivo* allergen challenge may be used as a

secondary-level confirmatory test employed where there is discordance between clinical history and the results of other diagnostic tests (*vide infra*). However, allergen challenges are more difficult to perform in a reproducible manner than skin tests or blood tests (*vide infra*).

Although allergen challenge has come to be regarded as the most reliable test to diagnose allergic sensitivity, it requires expensive resources, highly skilled personnel, and carries the risk of causing an acute allergic reaction [12, 62, 63]. Interpretation of findings can also be difficult because end points can be subjective and affected by observer and patient bias. In addition, preparation for oral challenge can also lead to unnecessary dietary restrictions and there is anxiety associated with diagnostic ambiguity [64]. Thus, allergen challenge may be reserved for cases where the clinical history and the results of skin prick test and/or specific IgE (*vide infra*) do not confirm or exclude the diagnosis of allergy.

DBPCFC has been taken as the gold standard to which all other diagnostic assays are compared [56], it is still a diagnostic test, and like all diagnostic tests, it is neither 100% sensitive nor 100% specific [56]. False-negative and false-positive [56] reactions can also occur. Several studies in which DBPCFC were used to establish the diagnosis of food allergy revealed that only about 40% of patients' histories of food-induced allergic reactions can be verified [65, 66].

Measurement of allergen specific IgE

Assays to detect allergen-specific IgE are particularly useful as a safe method to identify and monitor allergic sensitivity when skin testing cannot be performed or when medications interfere with skin or challenge tests, although results are not available as quickly as with SPT. The amount of circulating allergen-specific IgE antibodies can be determined using the radioallergosorbent test (RAST) or more usually now by a fluorescent allergosorbent test (FAST) and total levels measured using the fluorescent enzymoimmunoassay (FEIA) in which IgE is quantified using kilo units per litre (kU/l) based on the World Health Organization Reference Standard with 1 unit equalling 2.42 ng of IgE [67]. The general principle of an allergen-specific assay is to detect IgE that will bind to allergen fixed on a solid surface. The assays are influenced by the amount and quality of allergen bound to the solid support, the

degree of non-specific IgE binding, the affinity of the IgE antibody, and the degree of blocking of allergen-specific IgE binding by allergen-specific IgG. Consequently, there are inconsistencies between levels of allergen-specific IgE measured using different methods and different reagents, making comparison between systems challenging [68].

Specific IgE testing measures both functional IgE (capable of binding to FcεRI on mast cells or basophils and activating them) and non-functional IgE. Therefore, specific IgE testing is not a definitive diagnostic test but titres of IgE indicate the likelihood (probability) of a true allergy. The probability curve is the product of a logistic regression analysis of the specific IgE titres calculated in accordance with the results of oral food challenge testing. Limitation of the probability curve is that the specific IgE titre does not predict the threshold dose of allergens or the severity of symptoms. The diagnostic performance, in terms of sensitivity and specificity, varies between different allergens, and the IgE titre must be evaluated based on appropriate knowledge of the allergen [12].

Specific IgE testing seems to have high sensitivity but poor specificity for diagnosis of allergy [12]. It has been estimated that up to 60% of individuals have detectable food specific IgE levels, and approximately 25% of individuals have detectable insect venom specific IgE levels. Most of these individuals are not clinically reactive meaning that they do not experience signs or symptoms when exposed to the allergen to which they are sensitised [69, 70]. This lack of a direct relationship between allergen specific IgE and clinical reactivity may be influenced not only by levels of allergen-specific IgE levels and the levels of total IgE, but also by the ratio of these two measurements that may determine the threshold and likelihood for cellular, and clinical reactivity by influencing FcεRI occupancy and density, and that multiallergen sensitization [71].

Another means for determining the presence of allergen-specific IgE has involved to use of humanized rat basophilic leukaemia (RBL) cells lines [72-74]. These cell lines have been used for analysis and for monitoring of specific allergen sensitization in patient serum and measuring cellular activation after challenge with the suspected allergens [75]. Among these cell lines, α/β/γ-transfected RBL cells have been sensitised with diluted patients' sera and degranulation is measured after the

addition of specific allergens through determination of β-hexosaminidase release. However, the release of β-hexosaminidase has been reported to be occasionally lower upon stimulation with less diluted serum than in stimulation with higher diluted serum. However, a critical problem with this cell line has been the cytotoxicity of certain human sera for the rat cells, requiring high dilutions and leading to insufficient sensitisation and subsequently false results [72, 75]. A new generation of reporter cell lines such as RS-ATL8 has allowed detection of allergen-specific IgE even when using serum dilutions as high as 1:100 [76].These cells are stably transfected with a reporter gene such as firefly luciferase and luciferase expression and activity monitored as a mean of measuring cell stimulation [55].

Basophil activation testing

Clinical reactivity of an allergen can be established by provoking the body *in vivo* or investigating the responsiveness of the cells responsible *in vitro*. As activation of basophils may be a prominent feature in allergic reactions, stimulation of these cells *in vitro* can provide important information on the nature of allergic sensitivity. Because basophils are more accessible than mast cells, they represent the cells of choice for *ex vivo* challenge. Basophil activation can be assessed by measurement of the release of mediators or through altered expression of membrane markers. Basophil activation tests have potential as a diagnostic method, that can allow simultaneous testing for sensitivity to several allergens implicated in a reaction, including aeroallergens, latex, Hymenoptera venom, and drugs [77] and food [78].

Activation of basophils is dependent not just on the amount of allergen- specific IgE but also on IgE epitope specificity, affinity, and clonality (i.e the number of different IgE clones binding one allergen molecule simultaneously) [79]. In addition, activation of basophil can be influenced by the allergen valency, concentration, the inter-epitope distance, minimum contact time between the allergen and the related membrane receptor bound specific IgE and the intrinsic basophil reactivity [80]. However, basophils in about 10 to 20% of subjects do not respond to IgE-dependent stimulation due to a defect in early signalling, particularly spleen tyrosine kinase phosphorylation [81].

Effective *in vitro* basophil activation tests initially focused on histamine and leukotriene release [82]. However, time-consuming and costly two-step methods involving cell incubation and quantification of mediators have hindered their clinical application. Basophil histamine release can be measured following addition of allergen to heparinised whole blood. Purification of basophils may improve the accuracy, though isolation of basophils is onerous. Measurement of basophil histamine release in response to allergens has been reported to be affected by poor reproducibility and its use has been restricted to research applications rather than in allergy diagnosis [32, 83].

Currently available immunoassays for measuring histamine release tend to be laborious, expensive, time-consuming, and poorly reproducible and require an indicator source of whole blood from an atopic subject to test basophil ex vivo allergen-induced histamine release [84]. In addition, histamine is labile and has short half-life and samples require special handling prior to processing [85]. Histamine assay has not been shown to be a reliable marker for predicting allergic reactions. Lau et al. have examined the utility of basophil histamine release test in the diagnosis of hen's egg allergen in patients 3.5 months to 12 years of age [86]. They have suggested that histamine release test was not an effective predictor of the outcome of oral food challenges in childhood egg allergy because the sensitivity of histamine release test was 64.7%, the specificity was 40.0%, and the efficiency was 55.6%.

As an alternative to measurements of extracellular histamine or its metabolites, fluorescently labelled diamine oxidase was proposed as a means to measure intracellular histamine at the single cell level [87] and monitor basophil responsiveness during immunotherapy [84]. This assay can potentially serve as a way of monitoring immunotherapy efficacy, but this approach is still in its infancy and its validity needs to be established.

In the late 1990s, the expression of a number of surface proteins including CD45, CD63 [88], CD69, and CD203c [89] were reported to be upregulated on basophils when these cells are activated by allergen [90-92]. The most widely used markers are CD63 and CD203c whose expression is measured by flow cytometry.

In flow cytometric basophil activation tests, the reaction of basophils to allergen as assessed in a test tube has been taken as an *in vitro* surrogate of allergen challenge *in vivo*. Non-sensitised subjects can more readily be used to establish the specificity of a response than would be possible with allergen challenge tests [93]. The functionality of blood basophils can be confirmed by stimulation with the peptide fmlp, anti-IgE or anti-FcεRI antibodies, and buffer alone can be used as negative control [94]. Basophil activation can be performed even when the subject receives anti-allergic treatment [95] with exception of immunosuppressants such as Cyclosporine A [96] and also systemic steroids [97] as they have been reported to have an inhibitory effect.

CD63 is a tetraspanin protein anchored in the basophilic granule membrane, and is expressed on the plasma membrane due to the fusion of these granules with the membrane after transcytoplasmic migration [98]. CD63 expression is upregulated following stimulation by allergen and expression reaches a maximum at 20 to 30 minutes [95]. Expression of CD63 has been established as an indirect measure of histamine granule fusion events and reflect histamine release upon activation [90, 99, 100] by IL-3, polyclonal anti-IgE, and allergen, as well as other degranulation stimuli [101, 102]. Although the expression of CD63 has been reported to be correlated with degranulation [101, 103], other studies have reported that the upregulation of surface-CD63 may be dissociated from histamine release. MacGlashan et al. compared flow-assisted phenotyping and quantification of extracellular histamine and reported that CD63 expression can be dissociated from mediator release [100, 104]. However, CD63 is not a specific marker of basophils and it can be expressed by other activated leukocytes and by activated platelets that may adhere to basophils [100, 105]. Upregulated expression of this molecule has been observed in several other cell types, such as neutrophils [106], dendritic cells [107], T cells [108], eosinophils [108], and platelets [88, 109]. This may limit its specificity as a marker for basophil activation. Therefore, there is a need for a basophil activation marker that is more specific and sensitive than CD63.

CD203c, another protein expressed by basophils, belongs to a family of ecto-nucleotide pyrophosphates/phosphodiesterases (E-NPPs). E-NPPs catalyse the cleavage of phosphodiester and phosphosulphate bonds of molecules, including

deoxynucleotides, NAD and nucleotide sugars [110]. CD203c has been reported to be an activation antigen on the surface of basophils and mast cells that is upregulated in response to IgE receptor cross-linking [111]. CD203c expression after activation of IgE-sensitized basophils with allergen or anti-IgE [112], is transient and more rapid than expression of CD63 [100]. Also, CD203c has not been found to correlate significantly with histamine release, raising the concerns about its validity as a marker for measuring basophil degranulation. The baseline expression of CD203c on basophils from nut-allergic subjects was found to be elevated in comparison to normal controls. However, it may decrease in the nut-allergic subjects after receiving omalizumab [113]. Furthermore, CD203c expression may be increased on circulating basophils from asthmatic subjects following an exacerbation [114]. This can be an indication of basophil activation *in vivo*. It appears that these CD203c high expressing basophils are slowly releasing bioactive amines and/or cytokines from their granules in a mode similar to spontaneous histamine release. In contrast, CD63 expression was not observed on basophils from either group of subjects [115]. This may suggest that CD63 expression is linked more with anaphylactic-type degranulation, whereas CD203c is associated with so-called piecemeal degranulation [100, 116].

Santos et al., have reported that the basophils of allergic patients typically display a dose-dependent expression of activation markers, such as CD63 or CD203c, whereas the basophils of peanut sensitized-tolerant patients do not express or have a much lower expression of activation markers after stimulation with allergen [117]. The underlying mechanism may involve IgG4 antibodies and possibly peanut-specific antibodies of other isotypes that block IgE either by inhibiting co-cross-linking of IgE and IgG receptors or by competing with IgE for binding to the peanut allergens [118]. The difference in upregulation of basophil activation markers in response to allergen between allergic and non-allergic patients may form the basis of the use of the basophil activation test to diagnose food allergy [12].

Basophil activation in the diagnosis of different food allergies including pollen-food syndromes has been assessed in various studies. The sensitivity of basophil activation tests in diagnosis of food allergy has been reported to range from 77% to 98%, and the specificity 75 to 100% [78, 119, 120]. The utility of basophil activation test has been evaluated in the diagnosis of 104 children (43 peanut allergic and 61

peanut tolerant). About 60% had a positive skin prick test or specific IgE to peanut in consistent with other allergy tests performed [117]. Also, in the study of egg oral immunotherapy conducted by Burks et al., there was a correlation between basophil suppression measured by CD63 and clinical desensitization involving skin prick test and immunoglobulin levels, but not with long lasting clinical tolerance [121]. Similarly, in two separate studies, basophil reactivity has been assessed in 14 subjects treated with a Chinese herbal medicine (Food Allergy Herbal Formula-2) [122], and in five milk-allergic subjects treated with omalizumab. CD63 expression has been reported to be decreased in response to *in vitro* stimulation after treatment suggesting that basophil activations may serve as a means to predict allergy resolution.

Basophil activation markers including CD63 and CD203c have been employed in assessing the natural resolution of food allergies that are becoming increasingly prevalent, such as cow's milk allergy, and in determining when the food in question can safely be reintroduced in the diet [120].

The basophil activation test has been applied to monitor clinical responses to immunotherapy of food allergy including peanut [123, 124], milk [125] and egg [121, 126], where basophil reactivity to the respective food allergens has been reported to be diminished during treatment. Ocmant and colleagues evaluated the clinical relevance of basophil activation tests for peanut or egg allergy diagnosis using flow cytometric analysis of CD63 expression or CD203c upregulation on basophils, and they reported that neither conventional tests nor basophil activation tests are sensitive and specific enough to predict food allergy accurately [119, 127]. The corresponding performances of basophil activation testing in allergic, sensitized and controls applied to egg allergy diagnosis were 88.9%, 62.5% and 77.8% for the sensitivity and 100%, 96.4% and 96.4% for the specificity.

1.1.1.2 Confirmation of an allergic reaction

Confirmation that an allergic reaction has taken place can be challenging. The diagnosis of severe reactions may be established primarily on clinical history [128-130] and can sometimes be supported by laboratory tests for example, measurement of histamine concentrations in plasma, or of total tryptase

concentrations in serum or plasma although these tests have fundamental limitations [131]. Plasma histamine levels can be elevated five to ten minutes after the onset of symptoms. However, such levels are rapidly metabolised and usually return to normal within 60 minutes after the onset of the reaction. Therefore, the blood sample must be obtained within minutes for histamine assay to a few hours for tryptase assay after onset of symptoms [83], although, in some cases with anaphylaxis, a serum tryptase may be elevated well beyond this four hour interval [132]. However, this seems impossible in the many patients who experience anaphylaxis in public settings and arrive in the emergency department sometime later with resolving symptoms [133]. This may explain why plasma tryptase should be obtained within four hours after an anaphylactic episode.

Although, serum tryptase may be the most widely used marker for confirming an allergic reaction, there is no consensus on the minimal increase in serum tryptase indicative of mast cell activation-related event [134]. Nevertheless, many centres consider the normal range of tryptase as being below 11.4 ng/ml [135], though some healthy individuals have been reported with serum tryptase level exceeding 20 or even 25 ng/ml [136, 137]. Elevated tryptase can be detected usually within 15 to 30 minutes after an allergen challenge, and declines with an approximate half-life of 2 hours [83].

Tryptase may give high false positive value [138] or false negative values in confirmed reaction such as food-induced anaphylaxis [48, 139]. Tryptase has been observed to be elevated in some, but not the majority of cases of food allergic anaphylactic reactions [131, 140, 141]. Tryptase levels have been found within normal levels in patients with food-induced anaphylaxis even when symptoms occur during food challenges in which blood samples for tryptase measurement are obtained promptly at the onset of symptoms [142]. Furthermore, in a study that included ninety seven adults presenting to an emergency department with clinically confirmed anaphylaxis, only 21% had elevated serum tryptase levels [131]. Similarly, no elevations in the levels of tryptase have been found in two patients whose serum was available in a study included six fatal and seven near-fatal food-associated severe anaphylaxis [139]. A possible explanation for this is that mast cells such as those present in the respiratory epithelium, alveolar wall, and small intestinal

mucosa may express less tryptase than others such as the skin, heart and perivascular tissue. Moreover, tryptase clearance from the circulation of some subjects may be more rapid than in others [133]. In addition, it can be speculated that small increases in protease may be due to that tryptase entering the gut lumen rather than in the circulation and/or that reaction was mainly driven by basophils (which are tryptase-deficient cells) rather than mast cells [143].

Serum carboxypeptidase A3 (CPA3) levels have also been reported to be elevated in those with a clinical diagnosis of anaphylaxis but not in the serum of adult healthy blood subjects or individuals with a diagnosis of asthma of another IgE-mediated allergic disease. The serum levels of tryptase and of CPA3 after anaphylaxis do not necessarily correlate [133]. CPA3 levels seems to remain elevated longer than the tryptase levels. Furthermore, CPA3 serum levels have also been reported to be present in individuals with anaphylaxis where elevations in total serum tryptase levels were absent [133]. Abadalkareem et al., reported that "measurement of baseline levels of tryptase and CPA3 may be of value in identifying patients at greater risk of severe allergic reactions to drugs and the lack of a strong correlation between levels of these markers would argue for a need to measure both" [144].

1.1.1.3 Predicting the susceptibility to severe allergic reactions

The prediction of severity of allergic reactions represents an area of unmet medical need and the National Institute for Health and Clinical Excellence Guidelines have recommended further research in this area [145]. Allergic reactions may range from simple irritating symptoms to life threatening reactions such as anaphylaxis [146, 147]. Severe allergic reactions may occur following exposure to allergens from a variety of sources including foods, insect venom, drugs and others [147]. However, foods are involved in a significant number and often a majority of severe allergic cases [16, 148, 149], and patients who have experienced a reaction to food of any severity are at risk of subsequent severe reactions [150]. Nonetheless, there is no widely accepted reliable means to predict the severity of subsequent reactions in each patient. There is a need for laboratory tests to distinguish between individuals who are sensitized to allergens known to trigger severe reaction but are not at increased risk of severe reaction on exposure to these allergens, and those who are

not only sensitized but also at increased risk of developing symptoms of severe reaction on exposure, and of possible fatality.

In hospital-based studies of patients with anaphylaxis, foods accounted for 25 to 60% of all anaphylactic reactions in which the cause had been established with reasonable certainty [151-153]. Anaphylaxis is characterized by the sudden onset and rapid progression of multiple signs and by the simultaneous involvement of more than one organ system. Other reactions that may be life threatening, such as, severe asthma or angioedema are therefore included in the term 'severe allergic reactions' [147].

In terms of the importance of the history of reaction predicting future severity, studies based on questionnaire of self-reported allergic subjects where logistic regression was used to identify associated factors, have suggested a relationship between a history of anaphylaxis or severe symptoms and the risk of anaphylaxis upon subsequent exposure [154, 155]. Although severe reactions may follow reactions classed as severe [50], mild reactions can also be followed by severe reactions [156]. However, number of studies found contradictory results where the severity of previous attacks appeared to be an unreliable prognostic tool as the majority of reported case fatalities have a history of only mild allergic reactions [157].

Wainstein et al. have shown no association between previous reactions and severity of the reaction during food challenge in a group of 21 children with anaphylaxis who reacted following oral food challenges. Some 78% of the children who completed the oral food challenge, developed anaphylaxis. Moreover, among the 21 children developed anaphylaxis, in only 3 cases was anaphylaxis the initial reaction suggesting that the history of anaphylaxis was not predictive of anaphylaxis on challenge [158]. Flinterman et al. also found no association between the severity of reaction in the history and severity of reaction during double-blind placebo-controlled food challenges or with the eliciting dose [159]. Moreover, a poor correlation exists between reactions reported in the community and the reactions elicited in low dose DBPCFCs [160]. The need for an assay to predict the severity of future food allergic reactions has still to be addressed.

Although, skin tests or allergen-specific IgE measurements are often used in establishing allergic sensitivity, they have not been reported to be associated with the severity of reactions. There have been cases where sensitized individuals do not develop any symptoms after exposure to the relevant allergen [69, 70, 161]. This discordance is not well understood, nor is it fully understood why individuals with negative allergen skin tests and undetectable allergen specific IgE levels develop severe or even fatal reactions to the antigen to which they are sensitised [69, 162]. In a study of 51 anaphylactic deaths from insect stings, the level of specific IgE antibodies against venoms was not found to be predictive of the severity of anaphylactic reaction. Furthermore, IgE antibody was not detected in 10% of sera from sting-related deaths sera, and levels from 0.35 to 0.65 ng/mL were only found in 24% [162].

The severity of a reaction cannot be accurately predicted by the severity of past reactions or by specific IgE levels or size of a skin prick test wheal [163]. Investigations of the relationship between skin prick test data or specific IgE measurements with susceptibility to severe reaction have encountered with mixed results [164]. In a study performed on twenty four subjects challenged to nine possible food allergens, neither skin prick tests or specific IgE were found to be useful for predicting severity of reaction [165]. Similarly, in a five- year retrospective study performed on 983 children who underwent oral food challenge to egg, milk and peanut, skin prick tests and specific IgE concentrations were found to be independently associated with both failed oral food challenge and anaphylaxis [166]. Similar findings have been reported in a study of 1094 patients in whom the most severe reaction was to peanut and tree nut [167].

Activation-linked cell surface membrane antigens on basophils are increasingly used in research and in practice, in order to explore the biology of basophils, their role in various pathologic reactions, and to determine their responses to allergens in allergic disorders. Although, great efforts have been made to investigate the basophils in diagnosing allergic reactions, our knowledge is still limited on the diagnostic potential of basophils in allergic conditions.

1.2 Basophils

Basophils arise and develop in the bone marrow from haematopoietic pluripotent CD34+ stem cells, and like other granulocytes, are released into the circulation as mature cells [168, 169]. Basophils complete their maturation in the bone marrow, circulate in the peripheral blood in a steady state, and migrate into peripheral tissues in various pathological settings, including allergic reactions. The life span of a mature basophil is estimated at two to three days [170]. They are the least abundant of the granulocytes accounting for less than one percent of circulating leukocytes [171]. The constant presence of basophils in peripheral blood is assumed to be a result of continuing development and replenishment of these cells from bone marrow-resident progenitors [172]. However, peripheral blood basophil counts may increase in allergic diseases and in response to parasitic infections [173] and some forms of leukaemia such as chronic myeloid leukaemia (CML)[174].

Basophils are not normally present in the tissues of healthy subjects [175]. However, they have the capacity to bind to the vascular endothelium, and to transmigrate through the endothelial layer into tissues [176-178] and accumulate in affected areas [177-179]. An accumulation of basophils has been found in the inflamed tissue of several allergic diseases including allergic rhinitis, severe asthma [180-185], contact dermatitis [179], and atopic dermatitis [186]. These findings indicate that basophils may contribute in a major way to the pathogenesis of allergic diseases through their production of mediators capable of influencing allergic responses.

Basophils have been found to express several adhesion molecule receptors that are likely be involved in binding to the endothelium [187]. These receptors include CCR1, CCR2, CCR3, CCR5, CXCR1, CXCR2, CXCR4, and CRTH2 [188-194]. In addition, it has been suggested that chemokines such as CCL2, CCL5 and CCL11 may play a key role in basophil migration through inducing trans-endothelial migration and directional movement [195, 196]. Basophils also express integrins that interact with different ligands on the endothelium such as VCAM-1, fibronectin and ICAM-1-3 [195, 196].

The clinical relevance of basophil migration is currently unclear, and it is not clear where or when basophil activation and degranulation occur during a severe reaction. Findings that IgE-mediated basophil migration might occur without degranulation

[197] [198], and that migration out of the circulation may occur at the onset of symptoms [199], are consistent with the idea that basophils migrate to sites of allergen exposure where activation and degranulation occurs, thereby contributing to clinical manifestations. Basophil migration may also occur as a protective response, preventing their activation and degranulation in the circulation, therefore limiting systemic degranulation and protecting against severe reactions [200, 201].

Basophils and mast cells have similarities in several features including their ability to stain metachromatically with certain basic dyes, store histamine, express the high-affinity IgE receptor (FcεRI) on the membrane and be susceptible to activation and mediator release (Table 1-3). However, these two cell types demonstrate fundamental differences in their anatomical localisation, life span, and ability to proliferate.

Table 1-3. Major features of mast cells and basophils (Adapted from [202-204]).

Feature	Basophils	Mast cells
Origin	Haematopoietic stem cells (CD34+)	Haematopoietic stem cells (CD34+)
Site of maturation	Bone marrow	Connective tissues
Lifespan	Days	Months
Primary location	Intravascular circulation	Tissues
Size	7-11 µm	6-12 µm
Nucleus	Segmented	Oval or round
Granules	Larger and fewer compared with mast cells and more heterogenous	Smaller and more numerous compared with basophils

1.2.1 Mechanism of basophil activation

Like mast cells, basophils become activated by antigen crosslinking of IgE by allergen leads to aggregation of FcεRI, which can induce degranulation and the release of preformed granule mediators (Table 1-4). Furthermore, IgE dependent activation of basophils can induce *de novo* production and release mediators such as LTC_4, in

addition to cytokines such as IL-4 and IL-13 (which may be secreted preformed).However, basophils can be activated through non-IgE dependent processes by binding by inflammatory mediators such as complement factors C3a and C5a, major basic protein, platelet-activating factor (PAF) and chemokines [205]. Through release of these mediators and cytokines, basophils may play a crucial role in the pathogenesis of IgE-mediated allergic inflammation, and have been taken to be a practical and convenient surrogate for mast cell activation when investigating allergen sensitization. There is particular need for elucidation of the roles of basophils in severe allergic reactions, as these cells may be involved to a greater extent than previously thought.

1.2.1.1 Secretion of mediators of allergic inflammation

Mediators of mast cells and basophils encompass both preformed mediators stored inside the granules in an active form and mediators synthesized *de novo* (Table 1 5). The preformed mediators include histamine, proteoglycans and proteases whereas leukotrienes and prostaglandins are synthesized upon cellular activation. Cytokines may be stored in the granules as well as synthesized upon cellular activation.

Preformed mediators

Histamine

Histamine is the main biogenic amine and is synthesized from the amino acid histidine and stored within secretory granules of both mast cells and basophils. Histamine is released upon IgE dependent activation by human mast cells and basophils [206, 207], and is then rapidly metabolized by conversion into methyl histamine or by oxidative deamination into imidazole acetaldehyde. These are both excreted in the urine [208] and its rapid degradation and clearance makes its measurement in the blood problematic [85]. Histamine induces vasodilation, vascular permeability, and smooth muscle contraction in tissues via histamine receptors (H1 to H4) [209].

Table 1-4. Major mediators of basophil and mast cell activation.

Products		Basophils	Mast Cells
Preformed	Histamine	+	+
	Chondroitin sulphates	+	+
	Heparin	-	+
	Tryptase	- /+*	+
	Chymase	-	+
	B-hexosaminidase	+	+
	Basogranulin	+	-
	2D7	+	-
Newly generated	LTC_4	+	+
	PDG_2	-	+
	PAF	-	+
Pre-formed and newly generated	IL-4	+	-
	IL-3	+	+
	IL-5	-	+
	IL-13	+	+
	GM-CSF	-	+
	TNF-α	-	+

*Some basophils express small amounts of tryptase with quantities less than 1% of those found in mast cells [210-212].

Basophils have been suggested to be the source of most of the histamine found in normal human peripheral blood [213]. Due to its storage in secretory basophil granules in substantial quantities and release during immunological activation, histamine release has been used as a biomarker of basophil activation whether in plasma [131], serum, and in *ex vivo* stimulation tests [214, 215]. Moreover, histamine can be found in several body fluids, and been reported to be increased in

plasma of patients with atopic dermatitis or chronic urticaria [216, 217], and in bronchoalveolar lavage from patients with allergic asthma. However, there is uncertainty regarding the origin of histamine detected in these fluids. There is a need for a better marker to convincingly demonstrate the major source of such mediators.

Basophil-specific proteins

Only a small number of proteins have been identified to be specific for basophils including 2D7 and basogranulin antigen. The 2D7 antigen is localized mainly in the secretory granules of basophils using the monoclonal antibody 2D7 [218]. However, two bands reacting with 2D7 antibody have been observed with molecular masses of 76 and 72 kDa on Western blotting of separated basophil lysate. The function of 2D7 antigen has not been identified. Antibody 2D7 antibody has been used to identify basophils in tissues specimens [181, 218, 219], and in suspension by flow cytometry [211]. However, staining intensity of these cells is reduced due to the release of the 2D7 antigen in activated basophils. Basogranulin is described in a separate section with more details (*vide infra*).

Tryptase

Tryptase is recognized as a mast cell specific marker [115]. Basophils are the only other cell type identified to contain tryptase, but the levels in basophils may be negligible, and represent just 1% of the quantity found in mast cells [210-212]. Tryptase is a serine protease and primarily produced and stored in mast cells and stored in the secretory granules as an active enzyme in a complex stabilised by heparin. When dissociated from heparin, tryptase rapidly degrades into its monomeric form and loses enzymatic activity [83]. The molecular weight of tryptase is 134 kDa and there are approximately 10 pg per lung mast cell and up to 35 pg per skin mast cell [220].

Although several forms of tryptase have been described including α, β, γ, δ and ε, only the α and β variants are detected by the immunoassays currently available [83]. Mature β-tryptase is proteolytically active as a homotetramer, whereas mature α-tryptase is not, despite being 93% identical in amino acid sequence. The tetrameric structure of β-tryptase with the active site of each of the four monomers positioned towards the inner face of a central pore [221] makes it resistant to inactivation by

most biological inhibitors of serine proteases [222]. The exact mechanism for the regulation of tryptase activity is unclear.

Tryptase has been reported to have the ability to cleave certain extracellular substrates such as vasoactive intestinal peptide [223], calcitonin gene-related peptide [224], fibronectin [225] and kininogens [226]. During inflammation, tryptase can stimulate the release of granulocyte chemoattractant IL-8, and upregulate expression of intracellular adhesion molecule 1 (ICAM 1) on epithelial cells [227]. It induces the expression of IL-1β, which may be important for the recruitment of inflammatory cells to sites of mast cell activation [228]. Moreover, the release of tryptase from activated mast cells may stimulate secretion from adjacent mast cells, and consequently amplify the reaction [229]. Thus, tryptase may play an important role in allergic diseases by intensifying the responses of mast cells to allergens and other stimuli.

Chymase

Chymase is a chymotrypsin-like serine protease often described as being specific for mast cells. It is released from these cells in a macromolecular complex with heparin and distinct from complexes containing tryptase [230]. Chymase can mediate the conversion of angiotensin I into angiotensin II [231, 232], generate fibronectin and transforming growth factor-β from extracellular matrix [233, 234], that has been associated in the pathogenesis of tissue fibrosis and wound healing [235, 236]. Chymase can also degrade lipoproteins, thereby promoting macrophage foam cell formation [237]. However, chymase may also occasionally act in pathological conditions in which there is migration and stimulation of mast cells due to cardiovascular tissue damage [238]. Chymase may participate in multiple inflammatory responses in the vasculature, including blood pressure regulation [239] and plaque instability [240]. Chymase has been also found to induce eosinophil migration [241], which was mediated through the extracellular signal-regulated kinase pathway [242], and stimulates mucin secretion from bronchial epithelial cells.

Carboxypeptidase A (CPA3)

CPA3 has been reported to have a molecular weight of 30 to 35 kDa and has optimal activity at neutral to basic of a pH 7 to 9. While the exact contribution of CPA3 to

allergy is unclear [243], this protease plays a role in regulating innate immunity responses, including the degradation of harmful substances, such as the vasoconstrictive factor endothelin 1 and snake venom toxins [244].

Proteoglycans

Chondroitin sulphate and heparin proteoglycans are recognized to bind histamine, neutral proteases, and CPA3 primarily by ionic interactions and, therefore, contribute to the packaging and storage of these molecules in the cells' cytoplasmic granules [201]. Chondroitin sulphate is released on activation of both basophils and mast cells though a means for its detection has not been developed for measuring the activation of basophils or mast cells.

Heparin is a proteoglycan produced by mast cells and human lung mast cells contain approximately 2.4 to 7.8 micrograms of heparin per million cells [245]. Heparin has been reported as a potent chemoattractant for neutrophils, which may increase vascular permeability through a heparin-initiated bradykinin formation mechanism [246]. Heparin plays an essential role in promoting the storage of other granule-contained compounds, including bioactive monoamines and different mast cell-specific proteases, and regulating the enzymatic activities of mast cell proteases [247]. However, measurement of heparin in biological fluid has not been examined in the diagnosis of allergy.

Newly generated mediators

Lipid mediators are arachidonic acid-derived proinflammatory and vasoactive mediators which are synthesized *de novo* in activated mast cells and basophils. Among these, the newly generated mediators PGD2 and PAF which can be can be released by mast cells in response to IgE-mediated activation [248]. They are formed from catalytic conversion of arachidonic acid by the action of cytosolic phospholipase A2 on membrane phospholipids [249]. PGD_2 is not produced by basophils, but it can also be produced by eosinophils, other immune cells such as Th2 cells and dendritic cells and by non-haematopoietic tissues such as brain, heart, lungs, and kidney [250]. The production of PGD_2 by other sources limits its usefulness as biomarker of only mast cell activation.

PAF is a potent phospholipid-derived mediator released by mast cells and basophils and also several types of cells, including, monocytes, macrophages, platelets, eosinophils, endothelial cells, and neutrophils, in response to various stimuli [251]. PAF is a potent bronchoconstrictor, and can induce intense vasodilation and oedema formation and is involved in platelet aggregation as well as activation of neutrophils, eosinophils and platelets [252]. PAF is rapidly metabolised into its inactive metabolite, lyso-PAF, by the enzyme PAF acetylhydrolase [253]. PAF has a short half-life of few minutes making its measurement problematic.

Mediators that are both preformed and newly generated

Some cytokines may be secreted, performed as well as being newly generated on cellular activation. LTC_4, LTD_4 and LTE_4 are released from mast cells, basophils, myeloid dendritic cells, eosinophils and macrophages [254]. However, they can be synthesized rapidly upon activation. They may mediate various inflammatory responses [255]. LTC_4 seems to be an important mediator produced through the arachidonic acid pathway in the plasma membrane of mast cells and basophils via 5-lipoxygenase, which promotes processes of inflammation, including eosinophil migration, increased vascular permeability and bronchoconstriction [256].

Cytokines, such as IL-4 [257], IL-6, vascular endothelial growth factor [258], IL-13, and TNF [259], can be released without degranulation. The cytokines may act as potent pro-inflammatory factors to contribute to the pathogenesis of allergy [260]. For example, the interactions of eotaxin, regulated upon activation normal T cell expressed and secreted (RANTES) and major basic protein (MCP-1) with CCR3 are responsible for the recruitment of basophils, eosinophils and mast cells [261]. Basophils have been found to release substantial quantities of IL-4 [262, 263]and IL-13 [264, 265].

1.2.2 Membrane markers for basophil activation

Currently, CD203c and CD63 represent the most prominent markers of basophil activation. The clinical utility of basophil activation in the diagnosis of allergy was discussed above (*vide supra*). Other cell surface antigens that have been described as activation-linked markers and are detectable on resting blood basophils include aminopeptidase N (CD13) [266, 267], the lysosomal membrane antigens LAMP-1

(CD107a), LAMP-2 (CD107b), and endolyn (CD164). These markers can also be expressed by other cells and this lack of specificity has limited their usefulness as markers for measuring basophil activation. However, combinations of some of these markers may improve the accuracy of assessment of basophil activation [268, 269].

A specific antigen on the surface of human basophils has been identified by an antibody termed Bsp1 [270]. However, this antibody has not shown to exhibit sufficient sensitivity for immunohistochemical identification of basophils, and the corresponding antigen is not likely to be released from activated basophil, though expression of Bsp-1 antigen has been reported to not upregulate but decrease after basophil activation [266]. Another epitope expressed on the surface of human resting basophils and is recognised by an IgM monoclonal antibody termed 212H6 [168]. However, this antigen is found also on epithelial cells in tonsils as well as keratinocytes in the skin. Also, 212H6 antigen has not been shown to be upregulated on basophil activation and therefore its value has been limited for identification of these cells for investigation of the activation.

1.2.3 Basogranulin

Basogranulin is a unique basophil granule protein localised within granules of the basophil (Figure 1-1) that is recognized by the specific monoclonal antibody BB1 [271]. This product can be released from basophils stimulated with anti-IgE, and IgE independent stimuli including fmlp, calcium ionophore A23187 and complement component C5a [272]. Basogranulin has also been reported to be expressed on the surface of nonstimulated basophils, and increased after stimulation. It has been characterized as a highly basic macromolecular protein of 5,000 kDa, and has been resolved by gel filtration chromatography from a 210 kDa supramolecular complex containing tryptase [273-275]. However, the kinetics and regulation of basogranulin secretion have been little studied.

Table 1-5. Basophil and mast cell activation markers.

Marker	Basophils	Mast cells
CD13	+	?
CD63 (LAMP-3)	+	+
CD107a (LAMP-1)	?	+
CD107b (LAMP-2)	±	?
CD164	+	?
CD203c	+	+
Basogranulin	?	-
2D7	+	-

+ present
± weakly present
- absent
? unknown

Our knowledge about basogranulin is limited and the BB1 antibody has been employed primarily in studies involving the identification and enumeration of basophils that infiltrate into tissues involving nasal mucosa of patients with hay fever [276] [276-278] in inflammatory skin diseases [186]. However, infiltrative basophils numbers might be underestimated since basogranulin is a granule-associated protein and it might not be possible to detect some of the degranulated basophils in tissues. It remains to be determined to what extent developing new basogranulin-based assays can provide better means for measuring basophil activation and extend our knowledge of basophil activation processes. For instance, it is not known if measuring basogranulin release into cell supernatants or body fluids, assessing upregulation of surface basogranulin or downregulation pg internal stores may provide a means for investigating basophil activation.

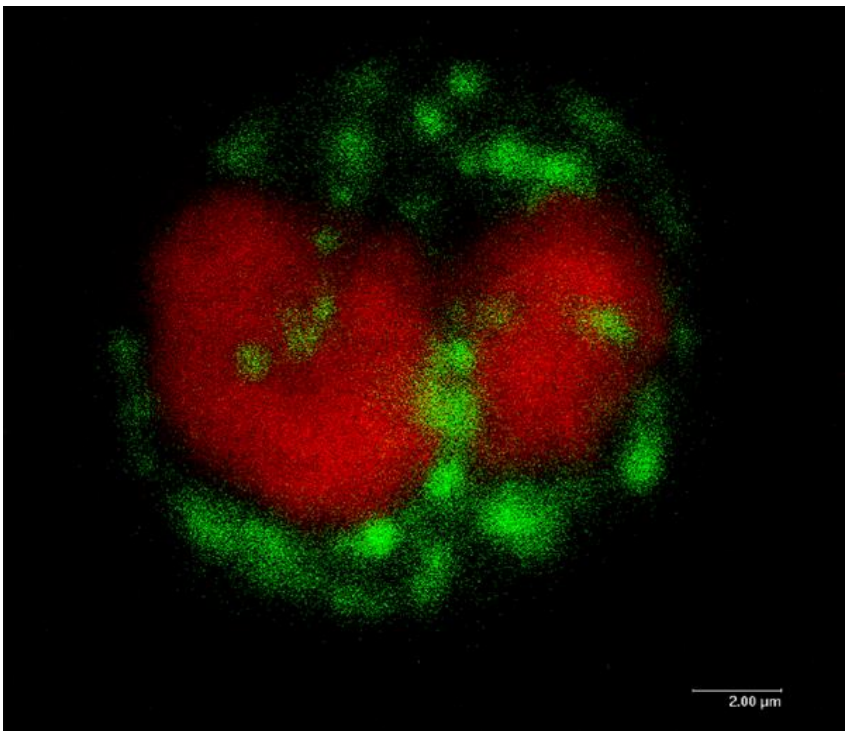


Figure 1-1. Confocal microscopic image of basophils stained with fluorescently labelled BB1 visualised by confocal laser-scanning microscopy. The green colour represents intracellular basogranulin within granules in a resting basophil. Nucleus was indicated by red DAPI. Figure was provided by A. F. Walls and Akinori Mochizuki, University of Southampton.

Aims and objectives

We hypothesise that basophils play key roles in mediating allergic reactions, and that their unique product basogranulin can be employed as a valuable diagnostic marker for assessing basophil activation in allergic diseases. Our aims have been to:

1) Develop better means for assessing basophil activation based on basogranulin measurement, whether extracellular, intracellular or membrane associated.

2) Apply the newly developed assays to assess specific allergic sensitivity to various allergens.

3) Investigate basogranulin levels in body fluids as means for assess the extent of basophil activation in clinical disease.

In order to achieve these aims, our objectives were to:

1) Develop and evaluate dot blotting and ELISA procedures to determine basogranulin levels in cell supernatants, and apply flow cytometric and confocal microscopy methods to study cellular distribution of basogranulin following basophil activation.

2) Apply the flow cytometric assays to determine extracellular or pericellular basogranulin following allergen-induced basophil activation.

3) Assess the potential to measure basogranulin levels in body fluids applying dot blotting procedures to biological fluids from patients with allergic disease and healthy subjects.

Chapter 2 Materials and Methods

2.1 Sampling, materials and equipment

2.1.1 Ethics approval and subject recruitment

Healthy subjects were recruited in this study and samples were collected after written informed consent was obtained (Approval number. 05 / Q1702 /9), from the Southampton and South West Hampshire Local Research Ethics Committee) for developing a new basophil activation test. All the subjects were healthy at the day of sample collection. In addition, volunteers with history of allergy to grass pollen or dust mites were recruited to take part in these studies. Subjects with grass pollen allergy had a history of symptoms consistent with this condition. Also, patients with allergy to grass pollen, dust mites and with food allergy were recruited in Allergy Clinic at Southampton General Hospital to take part in this study (Approval No. 16/LO/2244).

2.1.2 Sample collection

Blood

Blood was withdrawn from the antecubital vein into heparin or potassium ethylene diamine tetra acetic acid (K-EDTA) coated tubes using a butterfly needle with a tube built in holder.

Bronchoalveolar lavage (BAL) fluids and saliva

BAL stored at - 80 °C, which had been collected for previous studies (n=18) [279]. Another batch of samples from earlier studies involved BAL samples from normal healthy subjects (n=10), atopic athematic (n= 20) and severe asthmatic (n=5) subjects. Saliva for 83 peanut allergic subjects who were challenged with peanut provided by Dr. Paul Turner, National Heart and Lung Institute, Imperial College of London. All the samples were stored at -80 ^{o}C until the time of analysis. Saliva samples were collected before and an hour, two hours after the challenge.

2.1.3 Materials

Antibody	Clone	Supplier
PE anti-human CD193 (CCR3)	5E8	Biolegend, UK
FITC anti-human CD63	H5C6	
PerCP anti-human CD203c	NP4D6	
APC-conjugated mouse IgG2a isotype control	eBM2a	Invitrogen by Thermofisher Scientific, San Diego, USA
FITC Mouse IgG1, κ Isotype control (FC) antibody	MOPC-21	
PE Mouse IgG2b, κ Isotype control antibody	MG2b-57	
PerCP mouse IgG1, k isotype control antibody	MOPC-21	
Combined basophil staining kit Flow CAST B-CCR-SR PE- conjugated anti-CCR3 and FITC conjugated anti-CD63 antibodies	Unknown	Bühlmann laboratories, Schönenbuch, Switzerland
Anti-mouse Ig, k/negative control compensation particles set	Unknown	BD Biosciences, Erembodegem, Belgium
Rabbit polyclonal anti mouse immunoglobulin conjugated with ExtrAvidin- horseradish peroxidase	NA	Dako, Glostrup, Denmark

N -hydroxysuccinimidyl 6- (biotin amido) hexanoate, glycine, allophycocyanin sodium azide, bovine serum albumin (BSA), Hydrogen peroxide (H_2O_2), Chemiluminescent horseradish peroxidase (HRP) substrate, 0.45 µm pore size polyvinylidene fluoride (PVDF) membrane , ExtrAvidin-horseradish peroxidase conjugate (all were obtained from Millipore Merck, UK), APC fluorochrome conjugation kit (Abcam, UK), HRP substrate containing 0.1 mg/ml of 3,3',5,5'-tetramethylbenzidine (TMB), Sulphuric acid (Fisher Scientific, UK).Pre-cast 4-12% SDS-PAGE gels (Sigma Aldrich, Gillingham, UK). TruPAGE DTT Sample Reducer solution (Sigma Aldrich, UK), protein marker (Precision Plus Protein Standard) (Bio Rad, UK). **Buffers:** PBS (Gibco). Stimulation buffer containing IL-3 (Bühlmann laboratories, Schönenbuch, Switzerland). RoboSep buffer (StemCell Technologies, Vancouver, Canada), Permeabilisation and fixation buffer Cytofix/Cytoperm solution (BD Biosciences, San Jose, USA), Perm/Wash buffer

(BD Biosciences, San Jose, USA). **In house made buffers:** Tyrode's buffer (Southampton), binding buffer (20 mM sodium phosphate with ammonium sulphate 0.8 M, pH 7.5), elution buffer (20 mM sodium phosphate, pH 7.5), regeneration buffer (20 mM sodium phosphate, pH 7.5 with 30% isopropanol). One ml HiTrap™ IgM purification column (GH Healthcare, Buckinghamshire). Fraction collector (Bio-Rad, UK). Ammonium sulphate (Sigma Aldrich). Running buffer (0.02 M disodium phosphate, pH 7.5), dialysis buffer (phosphate buffer pH 7.4 (prepared in our lab), neutralisation buffer 2 M Tris, pH 8.0 Elution buffer 0.1 M glycine pH 2.7. 5 ml HiTrap protein G HP column (GE Healthcare, Pollards Wood). **Erythrocyte depletion buffer**: Hetasep (Stemcell Technologies, Vancouver, Canada), Lymphoprep (Stemcell Technologies, Vancouver, Canada). Lysis buffer 10x (Bühlmann laboratories, Schönenbuch, Switzerland), Phosflow™ Lyse/Fix Buffer 5x, (BD Biosciences, San Diego, USA).

Cell culture: Standard growth medium (SGM) employed contained Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), 1% non-essential amino acids (Sigma Aldrich), 3% hybridoma enhancing medium (Sigma Aldrich), 15% heat inactivated foetal calf serum (FCS), 0.001 M sodium pyruvate, 0.002 M L-glutamine final concentration (Gibco), 60 µg/ml penicillin, and 100 µg/ml streptomycin sulphate. **Basophil purification kit:** Immuno-magnetic basophil enrichment kit (EasySep; Stemcell Technologies, Vancouver, Canada). **Stains/ dyes**: Trypan blue (StemCell Technologies, Vancouver, Canada). The following materials were obtained from Sigma Aldrich unless indicated otherwise. Alcian blue made up of 1% Alcian (Sigma, UK), diluted in 60% ethanol and 3.7% hydrochloric acid. Coomassie blue protein assay (Bio-Rad), ɣ- bovine globulin blue (heat shock fraction, protease free, essentially globulin free Bovine serum albumin (BSA) Coomassie brilliant blue dye reagent (Bio-Rad), DAPI stain was kindly provide by Bio imaging unit at Southampton University.

Miscellaneous: Triton X-100, nonyl deoxycholic acid, sodium dodecyl sulphate (SDS), Tween-20, DMSO, ethanol and methanol were obtained from Merck, Southampton, UK.

Flasks, tubes, plates: potassium ethylene diamine tetra acetic acid (K-EDTA) coated tubes, or heparin (BD, San Jose, USA) Corning™, 5 ml sterile round-bottom

polystyrene tubes, with snap cap (Fisher Scientific, UK), 96 well polystyrene V-shape plates (Sigma Aldrich), 14 ml polystyrene round-bottom tube (Corning, Liversedge). 75-cm2 tissue culture flasks (Merck, UK). Cover glass system 8 chamber slides (Ibidi, Thisle Scientific, UK). 96-well cell culture sterile V-bottom polystyrene plate (Corning Life Sciences, New York, USA). 96-well polypropylene plate (StarLab, UK). High binding 96-well plate (Greiner, Germany). **Stimuli:** anti-IgE goat anti-human IgE antibody (Bio-Rad). Solutions included anti-FcεRI and Fmlp with neither known concentrations nor clone number (Bühlmann laboratories, Schönenbuch, Switzerland), fmlp (Sigma Aldrich, UK). Allergen extract: almond (Prunus dulcis), hazelnut (Corylus avellana), Peanut (Arachis hypogaea), shrimp, *D. farinae*, *D. pteronyssinus*, crab, oyster, shellfish 1:20 w/v (ALK, Hørsholm, Denmark), both Brazil nut and grass mix (Allergopharma GmbH, Reinbeck, Germany). Recombinant Human IL-3 (rhIL-3) (PeproTech, UK).

Equipment

Humidified atmosphere incubator (NuAire Laboratory, Plymouth, USA), pump (Bio-Rad). ELISA plate reader (Molecular Devices Thermo Max, Molecular Devices, San Jose, CA, USA). Microplate shaker (Denley Wellwarm1, UK), plate shaker (Platform Rocker, Stuart Scientific, UK). Magnetic stirrer (IKA, Oxford, UK), Leica TCS-SP8 confocal microscope on a Leica DMI-8 inverted microscope frame (Leica microsystems, Wetzlar, Germany), benchtop centrifuge (Falcon, MSE, UK). Micro-centrifuge (Thermo Scientific, Germany), ultra-centrifuged (Beckman Coulter, Indianapolis, Indiana, USA), Low-speed orbital shaker (Orbit™ LS, UK), a 96 well dot blot microfiltration apparatus (Bio-Dot, Bio-Rad Laboratories, California, USA). FACSCalibur flow cytometer (BD, Becton Dickinson).

Software

Imaging system: Dotblot software (SynGene, Cambridge, United Kingdom) supported by bio-imaging system (Chemi Genius) and supporting and BioRad Chemidoc software. **Confocal microscopy:** Leica LAS-X software (Leica microsystems, Wetzlar, Germany). **ELISA:** (SoftMax Pro® GXP Software, San Jose, CA 95134 USA). **Flow cytometry:** CellQuest™ software (Becton-Dickinson, San Jose, CA, USA).

Statistical analysis software: Microsoft Excel 2010 and Graph pad prism 8. (Prism 8 Statistics software; GraphPad Software, San Diego, CA, USA).

2.2 Measuring basogranulin release in basophil supernatants following cell activation

2.2.1 Basophil purification

Sufficient numbers of pure, functionally unimpaired basophils were required for basophil studies. Basophil purification from blood has in past posed a challenge, though the availability of commercial purification kits has significantly improved the process of purification. Purification procedures exploit properties of basophils which distinguish them from those of other cell types such as their localisation in blood, density, and the presence or absence of surface markers. In this the present study, purified basophils were employed for developing novel assays for monitoring basophil activation, though basophil activation was also investigated using mixed cell population (*vide infra*).

2.2.2 Preparation of a leukocyte-rich fraction from peripheral whole blood

The tendency of aggregated erythrocytes to settle faster than cells has provided the basis of a method to deplete these cells from a suspension of mixed blood cells. Erythrocyte aggregation agent Hetasep was employed to increase the erythrocyte sedimentation rate by increasing the effective size of the cells through formation of aggregates or rouleaux, while the nucleated cells remain suspended in the supernatant. Leukocyte-rich plasma was prepared from peripheral blood samples with Hetasep (Stemcell Technologies) according to the manufacturer's protocol with slight modifications. In brief, blood that was anticoagulated with EDTA or heparin was mixed with five parts of Hetasep solution, split into 14 ml tubes and immediately centrifuged at 110g for six min at 22°C with brake off. This resulted in an initial separation of red blood cells from the leukocyte-containing upper phase. For further gravity sedimentation of the erythrocytes, tubes were allowed to stand for 10-15 minutes to improve recovery of the nucleated cells.

Upper phases containing leukocyte-rich supernatant were carefully harvested, pooled, and washed once in 50 ml tubes with at least a four-fold volume of chilled

purification RoboSep buffer, and subjected to a low-acceleration centrifugation at a speed of 450g for 10 min at room temperature to deplete non-adherent platelets. Platelets were removed by centrifuging at 120g for 10 minutes at room temperature (15 – 25°C) with brake off. The supernatant was carefully removed, and the cell pellet was resuspended in 0.5 to 1 ml purification buffer and transferred into a 14 ml polystyrene round-bottom tube for further purification using negative selection immunomagnetic cell separation.

2.2.3 Preparation of erythrocyte-lysed basophils

Various preparations of nucleated blood cells were made using ammonium chloride as a hypotonic erythrocyte lysing solution. Erythrocyte lysing solution (10X) was made using ammonium chloride (NH_4Cl, 8.02 g), sodium hydrogen carbonate ($NaHCO_3$, 0.84 g), EDTA (0.37 g) and made up to 100 ml with distilled water. A working erythrocyte depletion buffer 1X ammonium chloride solution was made with a 1/10 dilution of stock solution (10X) with distilled water and left at room temperature until use. One ml of anticoagulated blood was mixed with 19 ml of ammonium chloride erythrocytes lysing solution in a 50 ml tube and kept for 10 minutes in a spiral roller at 22 ^{o}C. Tubes were then centrifuged at 450g for 10 minutes. Supernatants were decanted, and the pellet washed twice in PBS pH 7.4 and resuspended in Tyrode's buffer for 20 minutes before addition of stimuli of basophil activation.

2.2.4 Basophil purification by negative immunomagnetic selection

Basophils were purified from freshly nucleated cells suspended in purification medium using immuno-magnetic negative selection procedure according to the manufacturer's protocol. Briefly 50 µl of cocktail of monoclonal antibodies to cell-surface antigens: CD2, CD3, CD14, CD15, CD16, CD19, CD24, CD34, CD36, CD45RA, CD56 and glycophorin A were added per 5 X 10^7/ml leukocytes. This was mixed and incubated at 22°C for 10 minutes. After 10 minutes 100 µl of magnetic nanoparticles was added to the antibody–cell suspension incubated for 10 minutes at 22°C. The suspension was subsequently topped up to 5 to 10 ml with purification buffer. The tube was then placed into the magnetic tube and incubated for 10 minutes. Unlabeled basophils were poured off with a single motion into a new tube, and this was itself placed again into the magnetic field and incubated for a further 10

minutes. Finally, it was poured off into a new 14 ml propylene centrifuge tube and centrifuged at 450g for 6 minutes. The supernatant was decanted, and the numbers and purities of basophils were determined.

2.2.5 Cell count and determination viability and purity

Cell viability was determined by the trypan blue exclusion test as described elsewhere [280]. The principle is that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not, and the percentage of living cells may be taken to represent the viability of the cell preparation. In addition, cell recovery was determined using alcian blue staining of cells both before and after magnetic sorting. Alcian blue allowed rapid basophil-specific staining in a haemocytometer, as described by Gilbert and Ornstein [281].

For direct counting, blood was diluted of 1:10. The percentage of purity was calculated as nucleated cells stained metachromatically against the total of both stained and unstained nucleated cells.

2.2.6 Extraction of basogranulin from basophils

Basophils were lysed using several detergents to extract basogranulin as described elsewhere [282]. The detergents examined included Triton X-100, nonyl deoxycholic acid, SDS, all of which were added as 1% in PBS. PBS only and high purity water were also used. Basophils were suspended in a volume of 500 µl detergent solution and mixed with 1.5 ml and left for 3 minutes on the roller at room temperature. It was then centrifuged at 2.4 x 10^3g for 5 minutes at 4 ^{o}C, and the supernatant was transferred to new Eppendorf tubes on ice for further analysis. LAD2 cells were also lysed by 1% of Triton X-100. Protein concentrations for some treatments was determined by Bradford assay using BSA for the standard. More details about the procedures of Bradford assay is described later (*vide infra*).

2.2.6.1 Preparation of basogranulin standards

LAD2 cell hybridoma cell culture was maintained in SGM, as described above, in a humidified incubator with 5% carbon dioxide at 37 ^{o}C. LAD2 cells were concentrated and washed three times in neutral PBS pH 7.4. The pellet was then lysed by 1% Triton X-100 and vigorous vortexing for 2 to 3 minutes. The lysate was subsequently

centrifuged at 400g at 4 ^{o}C and the supernatant was carefully harvested and immediately stored at - 80 ^{o}C until use for further experiments. The total number of cells lysed per ml of the original cell suspension was used as basis of quantitation. LAD2 cell lysate was also employed an alternative source of basogranulin primarily in assessment the reliability of the dot blot assay for basogranulin and as a positive control. Basogranulin extraction was assessed by measuring basogranulin using monoclonal antibody BB1 in dot blotting assay.

2.2.7 Generation and purification of basogranulin specific antibodies

In this section, it was essential to expand the options of measuring basogranulin using specific antibodies in addition to BB1 antibody. Procedures involved antibodies production from hybridoma cells, purification and labelling.

2.2.7.1 Production of basogranulin specific monoclonal antibody BB1

Monoclonal antibody producing hybridoma cells stored at –80 ^{o}C were retrieved and cultured in SGM in a humidified atmosphere incubator of 5% carbon dioxide at 37 ^{o}C. The SGM employed contained DMEM, 1% non-essential amino acids, 2-3% hybridoma enhancing medium, 1% (v/v) non-essential amino-acids, 1% (v/v) sodium pyruvate, 1% (v/v) penicillin-streptomycin-glutamine, and 15% (v/v) foetal bovine serum. Growth of hybridoma cells in culture was monitored medium added to the cells every three days. BB1 antibody containing supernatant was harvested and stored at -20 ^{o}C until use. The new yield of hybridoma cells were maintained in 10% of DMSO in SGM stored at -80 for 24 hours before transferring to liquid nitrogen. In addition, a total of 37 clones of monoclonal antibodies, which were developed in our laboratory against purified basogranulin extracted from purified basophil lysate, were tested for developing assay for measuring basogranulin.

Purification of basogranulin specific antibody

BB1 antibody was purified from hybridoma cell culture supernatant using ammonium sulphate precipitation followed by a thiophilic affinity-based chromatography. Ammonium sulphate was used for initial concentration and precipitation of antibody from hybridoma supernatant. The process involved addition of ammonium sulphate (0.02 M) to antibody containing supernatant and it

was then maintained on magnetic stirrer overnight at 4 ^{o}C. The mixture was ultra-centrifuged at 3000g for 30 min at 4°C. Supernatant was removed and the pellet was re-suspended in running buffer (0.02 M disodium phosphate, pH 7.5). It was then dialysed against phosphate buffer pH 7.4 for 3 hours at 4 ^{o}C before it was loaded into a pre-washed 5 ml HiTrap protein G HP column at 0.5 ml/min at room temperature. The column was washed by running buffer and eluted with 0.1 M glycine pH 2.7 at a rate of 1ml/min using a pump. The eluted fractions were immediately neutralised in 2 M Tris, pH 8.0. After complete elution process, the column was re-equilibrated with 30 ml PBS pH 7.4 and stored with 20% ethanol at 4 ^{o}C.

For IgM-rich culture supernatants, the pH was maintained by adding 1 M Tris-HCl pH 8.0. The antibodies were precipitated by slowly adding a saturated solution of ammonium sulphate to the supernatant until 50% final saturation was reached, followed by slow stirring on a magnetic stirrer overnight at 4 ^{o}C. The solution was ultra-centrifuged at 3000g for 30 min at 4°C, supernatant decanted and the pellet was re-suspended and dissolved five times in binding buffer (0.1 M phosphate buffer, pH 7.5) before loading onto the column.

The column was prewashed by binding buffer (20 mM sodium phosphate with ammonium sulphate 0.8 M, pH 7.5) followed by elution buffer (20 mM sodium phosphate, pH 7.5) and finally regeneration buffer (20 mM sodium phosphate, pH 7.5 with 30% isopropanol) five column volumes each. The solution was loaded onto a prepared one ml HiTrap IgM purification column at a rate of 1 ml/min as above. Unbound material was removed by washing the column with fifteen column volumes of binding buffer, and the basogranulin antibodies were eluted by application of twelve column volumes of elution buffer. The eluted fractions of one ml were collected using fraction collector and stored at 4 ^{o}C until further analysis. After the elution process completed, the column was regenerated using seven column volumes of regeneration buffer, re-equilibrated with five column volumes of binding buffer followed by five column volumes of 20% ethanol and stored at 4 ^{o}C.

Confirmation of efficiency of antibody purification

Protein assay

The elution fraction profile was constructed by estimating protein concentration in each fraction using Coomassie blue protein assay. As the standard, ɣ- bovine globulin was serially diluted in a mixture of elution and neutralisation buffers to concentrations ranging between 25 - 1000 µg/ml. Volumes of 10 µl of standard or samples were pipetted into a 96-well plate (Greiner Bio-one, Stonehouse, UK) and 90 µl of a diluted (1/5) Coomassie dye reagent was added. The plate was incubated at 22 °C for 5 minutes and the optical density was then measured at a wavelength of 595 nm using an ELISA plate reader. Data was analysed automatically using computer programme SoftMax Pro® GXP Software and a histogram was constructed using Microsoft Excel 2010. It was necessary for fractions with concentrations beyond the upper limit of the standard to be diluted and re-measured using the same standard. Fractions with high protein concentration were pooled and dialysed against PBS, pH 7.4. The final concentration of the antibody was determined as described above.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The presence of heavy and light chains from antibody in highly concentrated fractions was assessed using pre-cast 4-12% SDS-PAGE gels according to manufacturer's protocol (Sigma Aldrich)Briefly, a 15 µl aliquot of each sample was loaded in a specified well. The samples included antibody supernatant as starting material, high and low protein concentration fractions, flow through, and washing fractions. Each of the sample was prepared by mixing 10 µl sample with 3.5 µl sample buffer and 1.5 µl of DTT reducing solution. A 2.5 µl of protein marker was used as a for molecular weight determination.

All samples were prepared as above and loaded into the wells of the precast gel that was fitted into the electrophoresis chamber. The tank chamber was filled with 500 ml running buffer diluted with high purity water according to the manufacturer's protocol. A volume of 500 µl of antioxidant 4 M sodium bisulphite antioxidant was added to the tank chamber to prevent sample re-oxidation. The antioxidant sodium bisulphate was added in order to maintain the proteins in a reduced state during the

run. The built-in electrode of the tank was connected to the electricity source and the voltage was set to run at 100 V for 90 min. The device was switched off after 90 min and the gel was removed. The gel was then placed in 100 ml ultrapure water in a glass container and microwaved at 950 watts for one minute to reduce the staining time. It was then placed on an orbital shaker for two minutes and the water discarded, and this step was repeated three times to get rid of the SDS-PAGE buffer and salts which may interfere with the binding of the Coomassie blue dye to the protein. The gel was then immersed in about 30 ml of Coomassie blue dye and microwaved for 45 seconds until the solution almost boiled. The gel was removed and placed on an orbital shaker for 10 min. The gel was then washed with 100 ml of ultrapure water for 10 min on an orbital shaker. It was then incubated at room temperature with 20 ml of 20% NaCl (w/v) If the gel overnight in the stain, add 2 mL of 20% NaCl (w/v) in water for every 20 mL of stain. This procedure will not affect sensitivity. The stained gel was visualised using a bio-imaging system.

Validity of antibody was confirmed by assessing the ability of the antibody to bind to basogranulin in purified basophil lysate. This was performed by assessing the detection of basogranulin in using different concentrations of lysates of purified basophils by dot blotting and by ELISA.

Western blotting

The electrophoresed proteins were transferred to a nitrocellulose membrane. The membrane was washed with PBS and non-specific binding was blocked for one hour with 5% (w/v) non-fat dry milk blotting grade blocker in Tris-buffered saline with tween 0.05% (v/v). The membrane was washed three times with Tris-buffered saline with tween 0.05% for five minutes each and incubated with goat anti-mouse IgM (µ-chain specific) conjugated to HRP for ninety minutes at 22 ^{o}C. The reaction was developed by adding TMB at 22 ^{o}C and visualised using an imaging system.

2.2.8 Antibody biotinylation

One millilitre of antibody (5 mg/ml) was incubated with 20 µl of 25 mg/ml N - hydroxysuccinimidyl 6- (biotinamido) hexanoate for 2 hours at room temperature. The reaction was stopped by the addition of 12 mg glycine, and the biotinylated antibodies were dialyzed extensively against PBS at 4°C.

2.2.9 Fluorochrome conjugation of antibody

Purified antibody was directly conjugated with APC fluorochrome conjugation kit according to the manufacturer's protocol (Abcam, UK). APC has an excitation at 650 nm and an emission at 670 nm. Briefly, 100 µl of APC modifier reagent to each was mixed with one ml of 2.5 mg/ml purified antibody in PBS pH 7.2. The antibody mixture was resuspended by mixing with a lyophilised APC conjugation material and incubated for 3 hours in a dark place at room temperature (20-25°C). The mixture followed by gently mixing with 100 µl of APC quencher. The mixture was aliquoted in sodium azide 0.02% was added to preserve mixture components and stored at 4°C until use.

2.2.9.1 Confirming of validity of BB1 in flow cytometry assays

Specificity of BB1 in detection basogranulin by flow cytometry was investigated. Eosinophils were selected as a potential example of cells that BB1 may cross-react with. Fluorochrome conjugated antibodies including BB1-APC, anti-CCR3-PEand anti-CD63-FITC were added to 100 µl of whole blood. Eosinophils were gated by flow cytometry as high expression CCR3 and high scatter characteristics whereas basophils were gated as high expression CCR3 and low side scatter. The expression of basogranulin and CD63 for basophils and eosinophils were measured by flow cytometry and the data was analysed as described later (*vide infra*).

2.3 Basophil stimulation

Basogranulin release was measured in purpose of monitoring basophil activation *in vitro*. However, basogranulin release was also measured in saliva and BAL by dot blotting for monitoring basophil activation *ex vivo* (*vide infra*). Investigation of basogranulin release was performed with fresh anticoagulated whole blood obtained from healthy volunteers, and patients with a history of grass pollen allergy. Basophil was stimulated with anti-FcεRI antibody or fmlp served positive controls whereas stimulation buffer acted as a negative control.

Purified basophils were incubated with Tyrode's buffer (pH 7.4) for 30 minutes before stimulation was conducted. In a 96 well polystyrene V-shape plate, a 180 µl of whole blood or purified basophils were incubated with 20 µl of stimuli with different

concentrations including; anti-FcεRI or anti-IgE monoclonal antibody and fmlp, and recombinant cocktail of Phleum allergens; and Tyrode's buffer or release buffer for spontaneous release; for 20 minutes at 37 ^{o}C humidified chamber with 5% carbon dioxide. Freezing and thawing and vortexing was used to lyse the basophils to determine the total basogranulin content of the cells. The activation was then terminated by placing the plate on icy water for 1-2 minutes, and the plate was centrifuged at 800 g, at 4 ^{o}C with brake off. The supernatant was then carefully transferred to a new polypropylene 96-well plates or 1.5 ml Eppendorf tubes on ice and stored immediately at -80 ^{o}C until use.

2.3.1 Basogranulin dot blotting assay

A volume of 100 µl of samples; supernatant, saliva, or BAL fluids was transferred to a 96-well dot blot microfiltration apparatus that contained a preactivated PVDF membrane with methanol and left for 40 minutes to seep through by gravity. However, it was necessary assist the seeping through of samples by applying gentle tap water flow-assisted vacuuming. This was carried out by linking the dot-blotting device to a running tap water driven pressure pump to apply a partial vacuum. With saliva samples, vacuum was applied using an electric pressure pump.

The membrane was removed and washed twice in PBS containing Tween-20 (1%) on low speed orbital shaker with speed of 25 rpm. It was then blocked for 2 hours at room temperature with PBS containing 0.1% Tween 20, 5% BSA, 0.3% H_2O_2, and 0.1% NaN_3 on low speed orbital shaker. This was followed by incubation overnight with BB1 antibody supernatant diluted 1/5 in PBS containing BSA 3% at 4 ^{o}C. The membrane was then washed three times and incubated with rabbit polyclonal anti-mouse immunoglobulin with ExtrAvidin- horseradish peroxidase conjugate for 90 minutes at room temperature. This was followed by washing six times as mentioned above and the signal was developed by chemiluminescent HRP substrate. Spots were visualized, and the intensity of signals was quantified with a bio-imaging system and supporting software.

2.3.2 measuring basogranulin by ELISA

ELISA is considered as a robust method for detecting and quantifying a specific protein. BB1 antibody other potential antibodies were employed for developing

ELISA for basogranulin. Basogranulin is either detected by indirect or sandwich ELISA. Sandwich ELISA requires a pair of specific antibodies that are mutually compatible i.e. bind to different epitopes expressed on basogranulin. Optimisation of each component becomes a critical step to improve the performance of the assay. This also involved extraction of basogranulin from basophils and establishing the appropriate dilution of samples for each assay. The main objective of the present section was to test the binding activity of available antibodies for basogranulin and to optimise assay conditions.

2.3.2.1 ELISA using BB1 antibody

Indirect ELISA

A 50 µl aliquot of a serial dilutions of purified basophil lysate (6×10^5 /ml) was pipetted into the wells of a high binding 96-well plate and incubated at 22 ^oC for 90 minutes. Non-specific binding was eliminated by blocking the wells with PBS containing 5% of BSA for 2 hours at 22 ^oC. The wells were washed and then incubated for 90 minutes at 22 ^oC with 50 µl of BB1, biotinylated BB1 antibody or various concentrations including (2.5, 1.25, and 0.63 µg/ml). Other antibodies were also screened for basogranulin detection. After washing away unbound biotinylated antibody, 50 µl of 1/1000 or 1/2000 (v/v) ExtrAvidin- horseradish peroxidase conjugate was pipetted to the wells and incubated for 60 minutes at 22 ^oC. The wells were again washed, and the reaction was developed by applying 50 µl of HRP substrate containing 0.1 mg/ml of TMB. The reaction was stopped by addition of 50 µl of 2 M sulphuric acid per well. The intensity of the colour was measured at 450-595 nm. Other monoclonal antibodies were also tested for measuring basogranulin in basophil lysate.

Sandwich ELISA

A high binding 96-well plate was coated with 2 µg/ml BB1 antibody as described. Different dilutions of purified basophil lysate (6×10^3 /ml) were pipetted into the wells and incubated at 22 ^oC for 90 minutes. Non-specific binding was eliminated by blocking the wells with PBS containing 5% of BSA for 2 hours at 22 ^oC. The wells were washed and then incubated for 90 minutes at 22 ^oC with 50 µl of different

concentrations biotinylated BB1 antibody at concentrations including 2.5, 1.25, and 0.63 µg/ml. After washing away unbound biotinylated antibody, 50 µl of ExtrAvidin-horseradish peroxidase conjugate diluted 1/1000 or 1/2000 (v/v) was pipetted into the wells and incubated for 60 minutes at 22 ^{o}C. The wells were again washed, and the reaction was developed by applying 50 µl of HRP substrate containing 0.1 mg/ml of TMB. The reaction was stopped with 2 M sulphuric acid. The intensity of the colour was measured at 450-595 nm. Fractions of hybridoma cell supernatant containing murine monoclonal antibodies against basogranulin, which had previously been developed in our lab, were screened using enzyme linked immunosorbent assay. The antibodies that showed high signals in indirect ELISA were further refined using sandwich ELISA as described above in indirect ELISA except that plates were coated with monoclonal antibody BB1 (2 µg/ml).

2.3.3 Measuring intracellular basogranulin expression by flow cytometry

Flow cytometry is considered a robust method for measuring marker expression on a cellular level. In addition, it is automated, allowing electronic selection of the analysed populations and visualisation, in contrast to basophil staining and counting or mediator release measurement (histamine and basogranulin release). Moreover, flow cytometry enables to combine analysis of surface markers with a simultaneous study of intracellular molecules such as basogranulin. A modification of the basic immunofluorescent staining and flow cytometric analysis protocol was used for the simultaneous analysis of basophil surface molecules and intracellular antigens at the single-cell level by flow cytometry. In this protocol, fixation was followed by permeabilisation resulting in the creation of pores in the cell membrane that require the continuous presence of the permeabilisation buffer during all subsequent steps. In order to ensure that there where a sufficient number of basophils, the volume of blood was increased by up to five or ten folds (250- 500 µl). However, it was essential to increase the number of washing steps 5-10 washes. Basophils were stimulated with fmlp, anti-FcεRI or allergen or with stimulation buffer alone for 15 minutes at 37 ^{o}C, and simultaneously stained for identification (CCR3) and activation markers (CD63). Depletion of erythrocytes and fixation of basophils was then achieved in a single step by mixing the blood vigorously with a pre-warmed Phosflow Lyse/Fix buffer at 37°C followed by incubation in a 37°C water bath for 10 min. The

tube was then centrifuged at 500g for 8 min, the supernatant aspirated, and the cells washed the cells once with FACS buffer. The cell pellet was dispersed by vortexing and permeabilised by adding the cold permeabilisation reagent slowly while vortexing, and then incubated at 4°C for 30 min. The cells were then centrifuged again and washed twice and resuspended with cold permeabilisation reagent. The cells were stained with appropriate BB1 conjugated APC for 30 min at 4°C. Cells were washed and prepare for flow cytometric analysis. Corresponding levels of surface basogranulin were also measured in non-permeabilised cells following activation for purposes of controlling comparison.

Optimisation of cell fixation and permeabilisation

Human anti-CD63 (FITC) and anti-CCR3 (PE) stained purified basophils were fixed and permeabilised by saponin containing buffer (Cytofix/Cytoperm) according to the manufacturer's protocol. Briefly, basophils were thoroughly resuspended in 250 µl of Cytofix/Cytoperm buffer and incubated for 20 min at 4°C. Basophils were then washed twice in a Perm/Wash buffer. The influence of fixation and permeabilisation on basophil gating characteristic including CCR3 expression and SSC was described later.

Flow cytometric data acquisition and analysis

Flow cytometric analysis was performed using a two-laser flow cytometer. Cytometric acquisition was performed with both argon laser (blue-green excitation light) with wavelength 488 nm and red diode lasers with wavelength 633 nm to detect forward scatter, side scatter and the three fluorochromes FITC, PE and APC. The gates were the same for all the tests conducted on an individual analysis. CCR3-staining and side scatter were applied to gate out at least 500-1000 basophils that expressed a high density of surface-bound CCR3. However, in some cases it was necessary to correct the gating of basophils by manually adjusting the basophil gating to where the basophils shown as the identification marker CCR3 expression and side scatter vary between individual and can be affected by some treatments (e.g. fixation and permeabilisation). Approximately 500 to 1000 basophils were acquired for each analysis. Basophils were gated by forward scatter and side scatter to eliminate debris or minor contaminating cells.

Flow cytometric characterization of basophils relied on a combination of side scatter, BB1, anti-CCR3 and anti-CD63. The analysis of the acquired data was performed with CellQuest software according to the manufacturer's protocol. Briefly, a gated region 1 (R1) was set by including the entire basophil population CCR3 positive with low position side scatter (SSC low) where eosinophils located on the high right side were excluded due to their SSC high position(Figure 2-1). Results are expressed as percentage (activated) positive basophils for basogranulin and CD63 or mean florescent intensity of each of them. The percentage of basogranulin positive basophils was calculated by comparing brightly fluorescent APC-BB1 positive cells to the total amount of basophilic cells gated in R1. The percentage of CD63 positive cells was calculated by comparing brightly fluorescent FITC-CD63 positive cells to the total amount of basophils gated in R1. The degree of basophil activation was expressed as the percentage of BB1+ and/ or CD63+ basophils above the threshold set for the negative control (

Figure 2-2). MFI for each fluorochrome-labelled monoclonal antibody was calculated automatically with the cytometer software by averaging the total fluorescence of the marker in the basophil gate.

Correct compensation settings for these fluorochromes were performed using beads and also purified basophils. Fluorescence minus one (FMO) and basogranulin staining with and without permeabilisation was used to set a marker between basogranulin positive and negative cells as described by Herzenberg et al. [283].

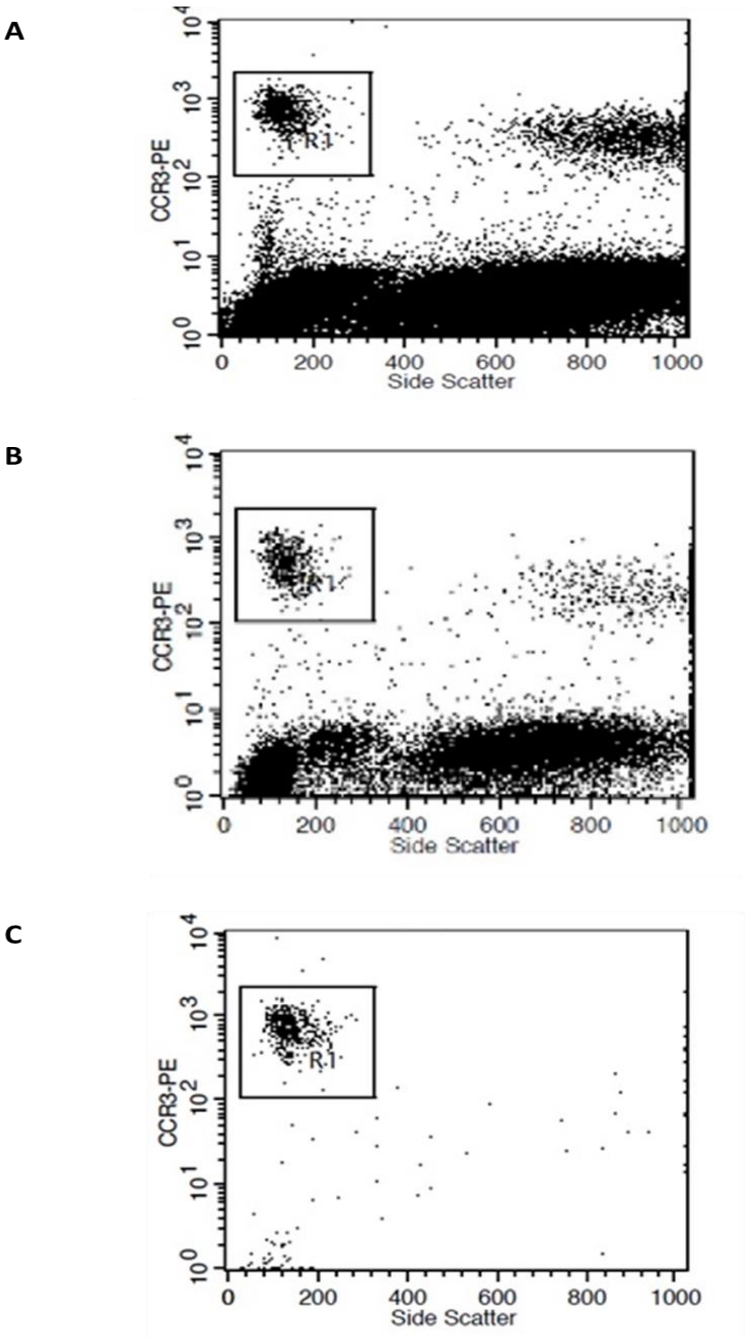


Figure 2-1. Selection of basophil populations by flow cytometric analysis of different preparations. Basophils were gated according to side scatter characteristics (SSC) and anti-CCR3 labelling basophils in (A) whole blood, (B) whole blood leukocytes fraction by erythrocytes depletion solution and (C) basophils purified by negative selection immunomagnetic method.

2.3.4 Measuring surface basogranulin expression by flow cytometry

This method was based on manufacturer's protocol of measuring basophil activation (Bühlmann laboratories, Schönenbuch, Switzerland) with some optimisation. A volume (50 µl) of anticoagulated blood or purified basophils was resuspended in 100 µl of stimulation buffer and immunostained with 20 µl of staining reagent containing anti-CD63 (FITC) and anti-CCR3 (PE) and 100 µl BB1 (1.25 µg/ml). Tubes were incubated for 15 minutes at 37 °C in a water bath. Erythrocytes from whole blood were lysed for 10 minutes with 2 mL of lysing solution at room temperature.

Samples were then centrifuged at 500g for 5 minutes to remove supernatant and washed with 2 ml of FACS buffer. The cell pellet was then resuspended in 300 µl of washing buffer and gently shaken prior to flow cytometric analysis. Cells were washed and resuspended in Perm/Wash buffer if permeabilisation was intended.

The expression of surface basogranulin in purified basophils was investigated as described for detection of intracellular basogranulin except that cells were not permeabilised. This was to allow detection of basogranulin exclusively on the surface membrane of basophils, and not intracellular basogranulin. Cells were washed twice in saponin free washing buffer prior to staining with BB1. Basophils in whole blood were also stained with different concentrations of BB1-APC whether prior or post erythrocytes lysis. The cells were then washed twice and resuspended in 300 µl of washing buffer.

Measuring basogranulin and CD63 expression

Fixed and permeabilised basophils were thoroughly resuspended in 50 µl of APC-conjugated BB1 at concentration of 2.5 X $\times 10^{-5}$ to 2.5 mg/ml or an irrelevant APC-conjugated mouse IgG2a isotype control at a concentration of 0.2 $\times 10^{-3}$ to 0.2 mg/ml. Tubes were then incubated at 4°C for 30 minutes in a dark place. Cells were washed and resuspended with washing buffer prior to flow cytometric analysis. The association between surface or intracellular basogranulin and CD63 expression was analysed by means of Pearson's correlation test, or Spearman's test. P values less than 0.05 were considered significant.

Effect of blood storage on the reactivity of basophils

The effect of blood storage on basophil reactivity in whole blood to different stimuli was assessed by measuring the upregulation of CD63 using flow cytometry. Basophil activation from one subject was conducted 4 and 24 hours following blood collection. Basophils were stimulated with a range of concentration of house dust mite *D. pteronyssinus* (0.0001-100 SQ-U/ml). Approximately 500 basophils were acquired for each analysis. The difference between the levels of CD63 expression at two time points (4 and 24 hours) was determined.

Investigation of increased expression of surface basogranulin in activated basophils

The expression of basogranulin on the surface of activated basophils was investigated by exposing basophils to different concentrations of exogenous basogranulin which was obtained from a lysate of highly purified basophils. Briefly, basophils were incubated with buffer containing basogranulin prepared in different dilutions of purified basophil lysates (10^6) at different time points (15, 30 or 45 minutes) in a 5% humidified incubator at 37 °C. Subsequent procedures were performed as described above for the flow cytometric assays (*vide supra*).

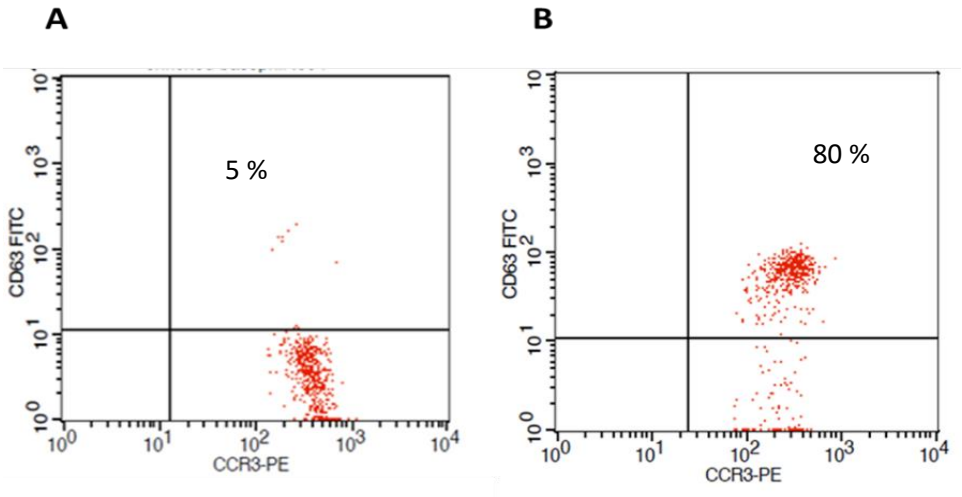


Figure 2-2. Detection of CD63 negative and positive basophils. Negative fluorescent FITC-CD63 for (A) (unstimulated) in lower right and (B) positive (stimulated) basophils in upper right region is counted to the total number of basophils gated in R1 as shown in Figure 2-1. Approximately 500- 1000 basophils were acquired for each analysis.

2.3.5 Measuring basogranulin, CD63 and CCR3 in basophils

Basogranulin expression was measured by flow cytometry by assessing both relative expression and MFI of basogranulin, CD63 and CCR3 in nonstimulated basophils and basophils stimulated with anti-IgE, anti-FcεRI, fmlp in various time points. Percentage of double positive (basogranulin+ CD63+), basogranulin positive (basogranulin+ CD63-), CD63 positive (basogranulin- CD63+) or double negative (basogranulin- CD63-) basophils were quantified by flow cytometry.

the potential effects of IL-3 on basogranulin expression was assessed in basophils which were incubated with a serial dilution of IL-3 (5-200 ng/ml) at 37 °C and simultaneously stimulated with anti-FcεRI or not only buffer as a control for 5 and 20 minutes. Stopping reaction and lysing erythrocytes were performed as describes above. The effect of IL-3 was assessed by both quantifying expression and MFI of basogranulin and CD63 and CCR3 using flow cytometry.

Effect of type of anticoagulant on measuring basophil activation

The effect of two common anticoagulants EDTA and heparin on basophil activation was investigated by monitoring the expression and MFI of surface basogranulin and CD63 in response to stimulation by fmlp and anti-FcεRI and also in nonstimulated basophils. In addition, the extent to which staining the cells before or after stimulation influences the measurements of basogranulin and CD63 was also assessed in blood which was anticoagulated by EDTA or heparin.

2.3.6 Visualisation and localisation of basogranulin in activated basophils and LAD2 cells

Basophils

The subcellular expression of basogranulin was examined using confocal laser scanning microscopy on purified basophils. Basophils were first purified from 100 ml of EDTA-anticoagulated blood using human basophil enrichment kit as described above. Purified basophils of with total of 1×10^6 were resuspended in stimulation buffer and incubated on a roller for 30 minutes to revive the cells and the number was adjusted to 5×10^5/ ml. In a 1.8 ml Eppendorf tube, 2×10^5 of basophils in 400 µl in were aliquoted per tube and stimulated with addition of 200 µl of fmlp solution

(for 20 minutes) or anti-FcεRI (5, 10, 15, or 30 minutes) in 37 ^{o}C and 5% CO_2. Fmlp and anti-FcεRI were provided without known concentrations (see materials section). The reaction was stopped by placing the tubes in icy water for 2 minutes and simultaneously the cells were fixed and permeabilised by addition of 200 µl of Fix/Perm buffer for 30 minutes at 4 ^{o}C. This was an essential step to disperse the cells prior to fixation. Dispersing the cells involved flicking the tubes and gentle vortexing to avoid cell clumping. Non-specific binding was eliminated by addition of 200 µl of Perm/Wash buffer containing 5% of BSA to each tube with 600 µl of the cell suspension and kept at 22 ^{o}C for an hour. The tubes were centrifuged at 3.2 $\times 10^3$ g for 5 minutes and the cells were subsequently resuspended in 500 µl Perm/Wash buffer that contains 5 µg/ml of APC-conjugated BB1 antibody, 5 µl of anti-CD63-FITC and anti-CCR3-PE, or isotypes and incubated at 4 ^{o}C for an hour. Subsequently, cells were washed as described above and a 200 µl of cell suspension of each tube was applied into an 8 chamber slide pre-coated with 0.5% Alcian blue aqueous solution. The slide was centrifuged for 300 g with brake off for 5 minutes. A 200 µl of DAPI stain 1 mg/ml was applied per well and kept for 15 minutes at 4 ^{o}C, and subsequently the cells were washed as described before, and kept in Perm/Wash buffer until imaging. Images were captured using laser scanning confocal microscope with x63 glycerol objective lens operated through Leica LAS-X software.

LAD2 cells

LAD2 cells were washed and resuspended in PBS. A suspension of 2 x 10^5 of cells/200 µl in each well was applied into an 8-chamber slide which had been pre-coated with 0.5% Alcian blue aqueous solution. This step was followed by centrifugation for 300 g or 5 minutes. Subsequently, the supernatant was removed by gently aspirating from one corner of the well. The cells were then fixed and permeabilised by addition of 200 µl permeabilisation and fixation buffer. Non-specific binding was eliminated by applying 1% of BSA for 60 min at 22 ^{o}C on a shaker. The cells were stained with range of concentrations of APC-labelled BB1 for 60 min at 22 ^{o}C on the shaker. After a couple of washes as stated above, DAPI stain was applied at 1 mg/ml (1:500) for 15 minutes. The slide was then washed and kept in PBS until imaging. Images were captured using laser scanning confocal microscope with x63 glycerol objective lens.

2.4 Assessment of basogranulin as a marker for basophil activation in clinical disease

2.4.1 Basogranulin measurement in BAL and saliva

Basogranulin levels were measured in 83 saliva from peanut allergic patients before and after peanut oral challenge, and also in BAL fluids from healthy, atopic, and severe asthma subjects. Saliva was diluted with PBS containing reducing agent DTT (52 mM) and kept on orbital shaker (25 rpm) for 10 minutes on ice. A 100 µl aliquot of the sample was employed in the dot blotting as described above.

2.4.2 Investigation of assay interference

, A standard curve for basogranulin was prepared by diluting purified basophil lysate in normal saline that was serially diluted and transferred to PVDF membrane. Also, a pre-tested BAL sample from healthy subject with no-detectable basogranulin was spiked by adding an equal volume of basogranulin standard to the BAL sample. In addition, two pre-tested BAL samples obtained from atopic asthmatic patients with high basogranulin concentration was diluted in normal saline in two-fold dilution. The linearity of all samples was investigated by interpolating the concentration. The concentration of added standard computed should correspond to that determined from the standard curve alone in the absence of interference. The lower limit of quantification of basogranulin was determined on basogranulin standard (purified basophil lysate) according to the FDA bioanalytical validation method guidelines [284].

Statistical analysis:

Assumption if normality of distribution was investigated using Shapiro-Walk test in which a p-value of .05 or more is considered significant. The difference between groups was calculated using t-test where a p value less 0.05 is considered significant.

Chapter 3 Development of basogranulin-based assays for measuring basophil activation based on basogranulin measurement

In the present chapter are presented the results of preparatory work for development of the assays for determining basogranulin release or expression, including basophil purification, basogranulin extraction, monoclonal antibody screening and purification. Then, is presented the development and optimisation of assays for measuring basophil activation (a) development of assays for measuring basogranulin release in supernatants from experimentally activated basophils, examining both purified and non-purified cell preparations using dot blotting, (b) development of ELISA for measuring basogranulin release as a more sensitive means for measuring basogranulin, and (c) development of basogranulin based flow cytometric assays for assessing alterations of intracellular and surface basogranulin expression following basophil activation *in vitro*.

Purification of basophils

Application of a negative immunoselection procedure resulted in isolation of basophils with a purity of 62.1 ± 20%, (mean + SD, n=20) (

Table 3-1). The number of basophils recovered ranged from 0.1×10^6 to 2.9×10^6. Mean basophil recovery was 51.8% as determined by alcian blue staining method (also see Figure 6-2). Application of Trypan blue exclusion test indicated mean viability of 96 ± 4.5 (mean ± SD). There was no apparent association between basophil purity, recovery, and viability observed before and after the negative immunoselection step.

Basogranulin extraction from purified basophils

Addition of detergents allowed more effective extraction of basogranulin from basophils and LAD2 cells as determined by dot blotting assay (Figure 3-1). Use water or PBS without any detergent allowed weak extraction of basogranulin. Of the detergents investigated, maximal extraction of basogranulin was found following

treatment with 1% SDS or deoxycholic acid. However, employing of SDS was associated with massive diffusion with the spots occupying greater areas on the dot blot membrane. This diffusion led to interference with adjacent spots and consequently made results difficult to interpret.

Treatment of basophils with 1% deoxycholic acid resulted in well-defined spots , and allowed basogranulin to be extracted up to concentration of 85% as efficiently as with the same concentration of SDS. Triton X-100 was the least effective of the three detergents employed to extract basogranulin from basophils, although it appeared more effective with LAD2 cells. The methods considered best for basogranulin involved use of SDS and deoxycholic acid.

Table 3-1. Immunomagnetic purification of peripheral blood basophils.

Experiment	purity (%)		Total number of purified basophils (× 10^6)	Recovery (%)	Viability (%)
	Partial separation	immunomagnetic enrichment			
1	1.1	92	0.85	59	95
2	3.2	89	2.9	61	91
3	2.1	85	1.6	74	92
4	1.4	51	2.2	55	99
5	1.1	37	0.21	63	97
6	2.3	63	1.1	38	96
7	1.3	71	2.8	81	100
8	0.5	63	1.3	41	99
9	2.7	85	0.41	46	96
10	2.3	23	2.3	52	94
11	1.8	66	1.6	57	96
12	0.75	45	0.3	21	99
13	0.2	27	0.1	35	95
14	0.4	55	0.8	30	100
15	1	66	0.5	65	99
16	1.5	70	1.1	72	98
17	1.2	65	0.7	41	90
18	0.8	85	1.1	60	94
19	0.3	70	0.1	30	80
20	0.6	35	0.2	55	96
Mean	**1.3**	**62**	**1.1**	**52**	**95**
STD	**0.85**	**20**	**0.86**	**15.7**	**4.5**

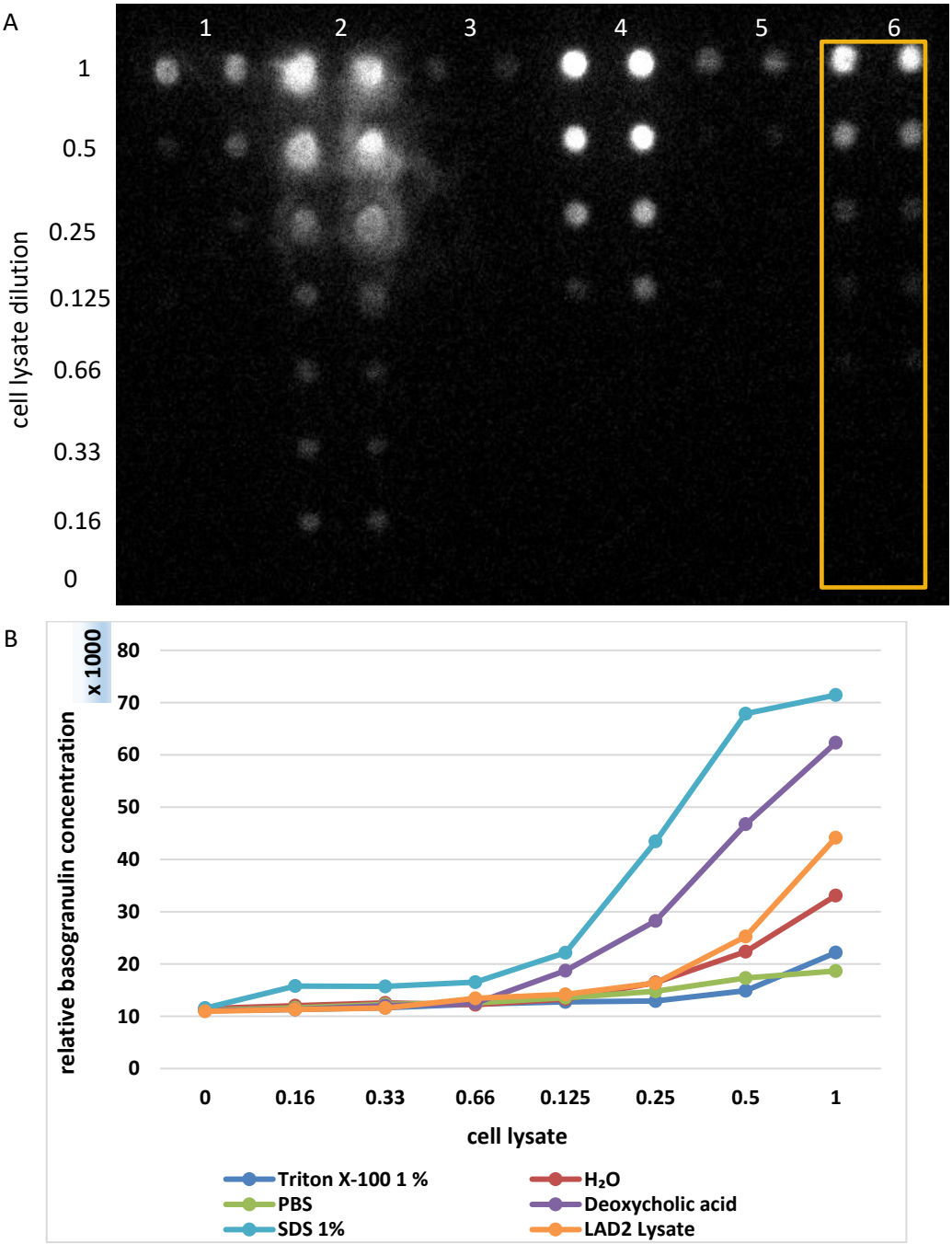


Figure 3-1. Effect of detergents on detection of basogranulin in serially diluted lysates of basophils and LAD2 cells. A) Dot blotting assay of basogranulin extracted from purified basophils (4×10^4) using (1) H_2O, (2) PBS with 1% SDS, (3) PBS alone, (4) 1% deoxycholic acid in PBS, (5) 1% Triton X-100 in PBS. LAD2 cells (1.8×10^3) lysed by in 1% Triton X-100 (6). B) Expression of basogranulin levels analysed by a bio-imaging (Chemi Genius).

3.1 Measuring basogranulin release in cell supernatant

3.1.1 Basogranulin release assay from erythrocyte-depleted basophils

Basogranulin release could be detected in supernatants of erythrocyte depleted basophils upon stimulation (Figure 3-2). Treatment of basophils with fmlp at dilution from 0.001- 0.1 µM induced basogranulin release in a bell-shaped manner in three of the subjects though the optimal concentration of fmlp varied between subjets. In addition, the extent of basogranulin release was variable between the subjects.

3.1.2 Basogranulin release from basophils in whole blood, and partially, purified basophils

Dot blotting proved impractical for measuring basogranulin in whole blood as the membrane could be readily blocked by the cells or macromolecules of the plasma. However, basogranulin release could be measured in whole blood cells after plasma was removed, and measurements compared with nucleated cell preparations and purified basophils. Basogranulin release was measured in supernatants of stimulated and unstimulated whole blood cells (suspended in PBS), and leukocyte- rich preparation with basophil purity of 2%, and purified basophils (85% of the total cells) (Figure 3-3).

Treatment of purified basophils with fmlp at concentrations of 0.003-10 µM induced basogranulin release in a bell-shaped concentration response manner. An optimal fmlp concentration of 0.1 µM provoked release of about a quarter of the total cellular basogranulin.

Although, basogranulin release from the whole blood cell preparation stimulated with 0.003 µM fmlp could not be detected, the extent of basogranulin release was similar to that with purified basophils with a maximal release with fmlp at a concentration of 1 µM (with release of 23% of the total cellular basogranulin). Stimulation of the leukocyte-rich preparation with fmlp induced basogranulin release in a concentration dependant manner with a trend for the response curve to be shifted to the right (Figure 3-3 A).

Treatment of basophils with anti-IgE antibody at concentrations of 0.003 to 10% provoked the release of basogranulin in a concentration dependent manner in all

three cell preparations, although a bell-shaped concentration response curve was only observed only with the purified basophils (maximal release at a concentration of anti-IgE antibody of 0.1%, for which release represented about 37% of the total cell content of basogranulin). Maximal IgE-dependent release of basogranulin from basophils in whole blood was observed with 0.3% anti-IgE antibody in leukocyte-rich preparations was 10% anti-IgE antibody (Figure 3-3 B).

Figure 3-3 C shows the release of basogranulin in response to various stimuli and with different cells purities. Treatment of basophils with *D. pteronyssinus* at concentrations of 0.001 to 10 SQ-U/ml induced basogranulin release in a concentration dependent manner in all three cell preparations. There was a bell-shaped dose response in the basogranulin release from purified basophils induced by *D. pteronyssinus* with maximal release of 31% of the total cellular basogranulin, corresponding to a concentration of *D. pteronyssinus* at 0.3 SQ-U/ml. Maximal release of basogranulin from basophils in whole blood represented 20% of the total cellular basogranulin, corresponding 10 SQ-U/ml of *D. pteronyssinus*. In, leukocyte-rich preparations, maximal release for basogranulin was 44% with corresponding concentration of 1 SQ-U/ml of *D. pteronyssinus*. In whole blood and leukocyte-rich preparations, stimulation with *D. pteronyssinus* shifted the concentration response curve to the right.

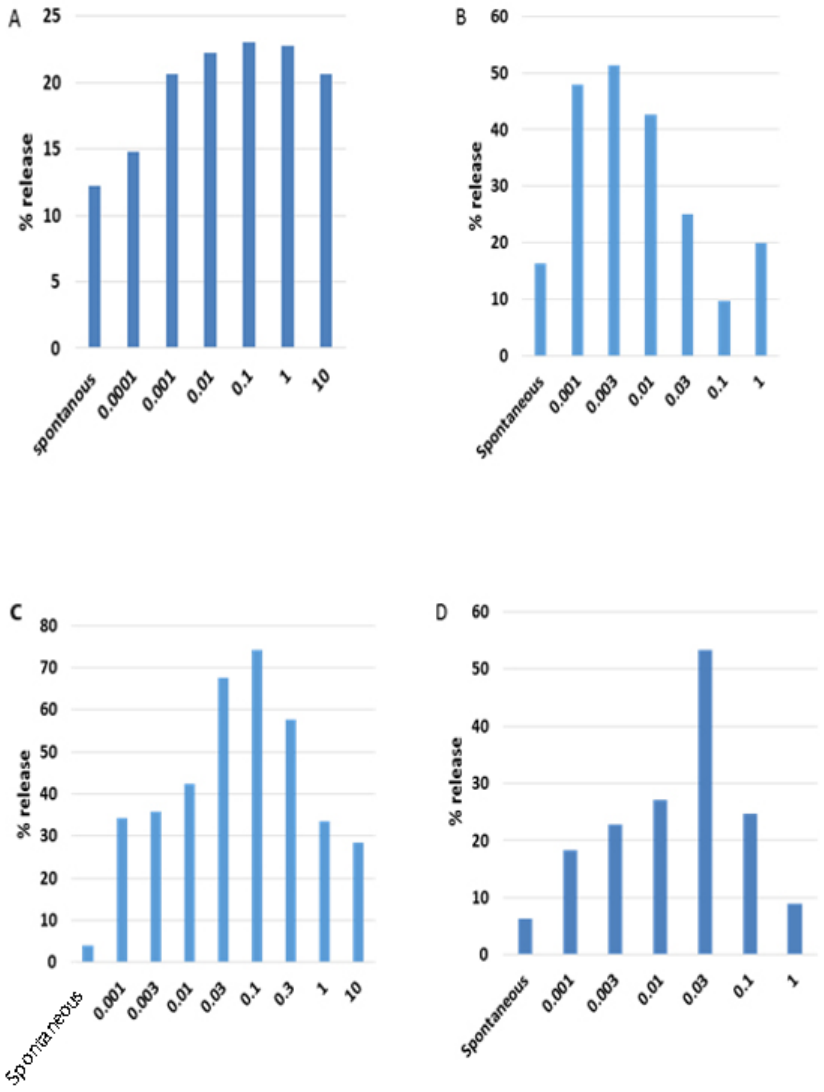
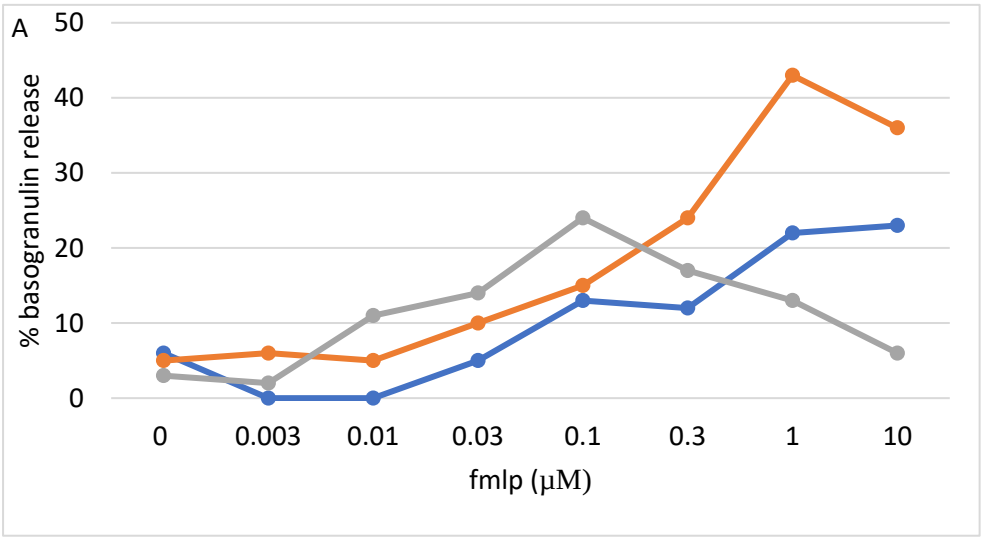
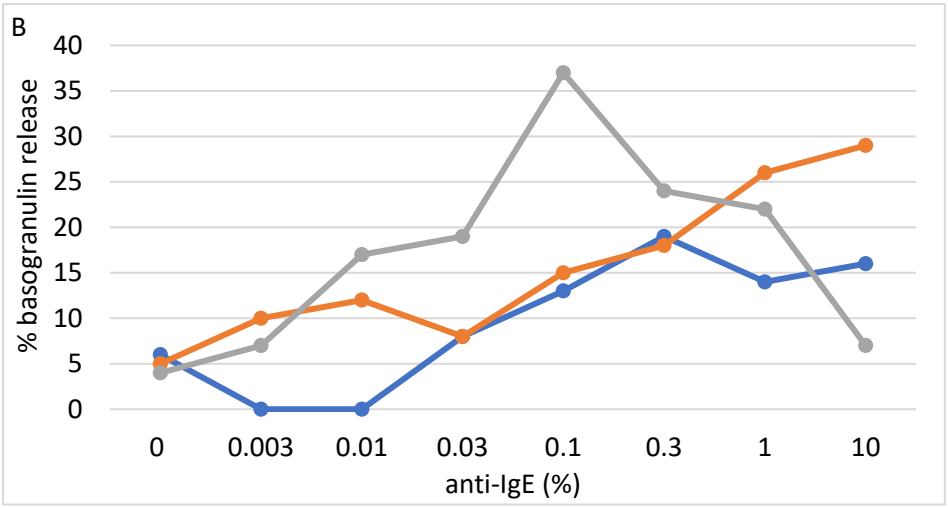


Figure 3-2. Basogranulin release from erythrocyte depleted basophils from four subjects (A,B,C and D) induced by a range of concentration of peptide fmlp (µM). Spontaneous release represented that from nonstimulated basophils. Data are expressed as the percentage release of total cell associated basogranulin. Mean of triplicate determinations are shown.


A
% basogranulin release
50
40
30
20
10
0
0
0.003
0.01
0.03
0.1
0.3
1
10
fmlp (µM)


B
% basogranulin release
40
35
30
25
20
15
10
5
0
0
0.003
0.01
0.03
0.1
0.3
1
10
anti-IgE (%)

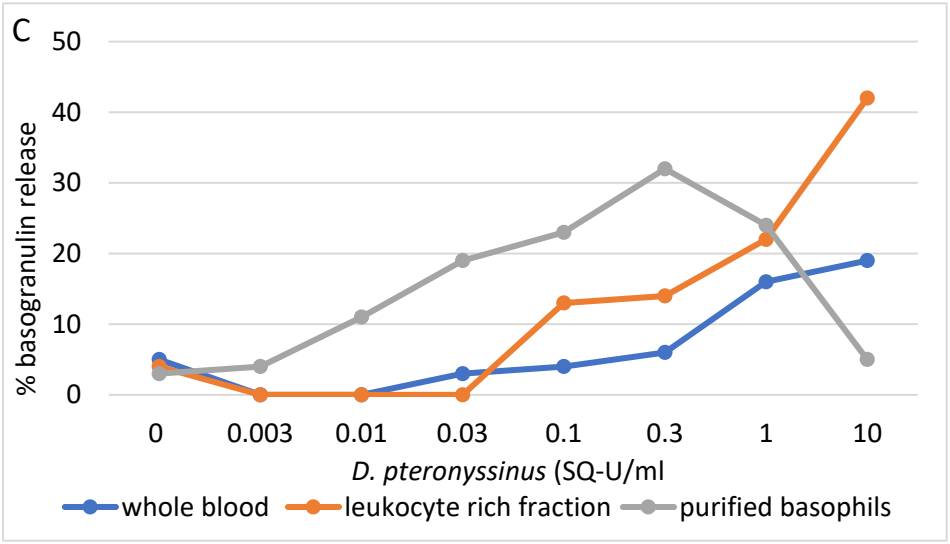
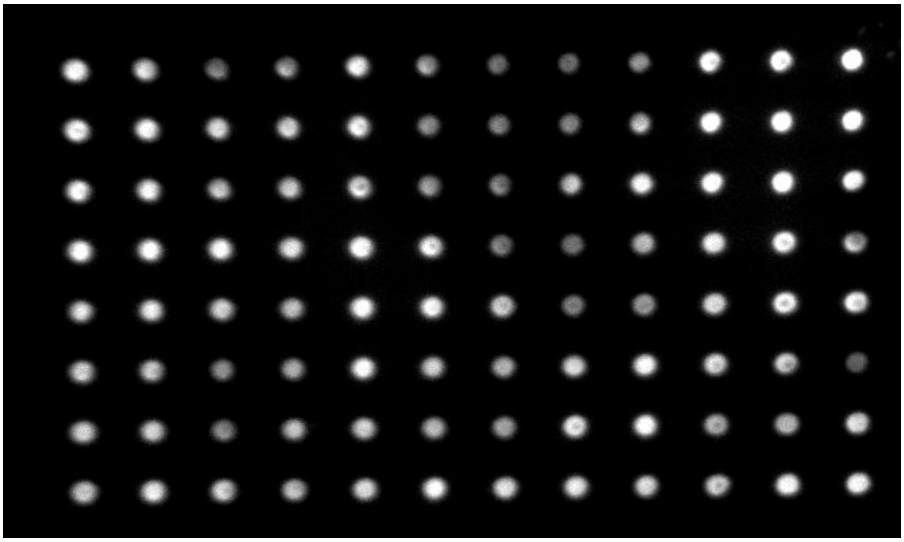

C
% basogranulin release
50
40
30
20
10
0
0
0.003
0.01
0.03
0.1
0.3
1
10
D. pteronyssinus (SQ-U/ml
whole blood
leukocyte rich fraction
purified basophils

Figure 3-3. Basogranulin release from basophils of cell preparations of different purity as analysed by dot blotting and with BB1. Basogranulin release from basophils in whole blood cells suspended in PBS (without plasma), separated leukocyte rich fraction, and purified basophils (basophil purity of 85%) induced by (A) fmlp (0.003-10 μM), (B) anti-IgE (0.003-10 %) and (C)*D. Pteronyssinus* (0.0.001-10 SQ-U/ml). Spontaneous release represents from unstimulated basophils. Data are expressed as the percentage release relative to total cell content of basogranulin.

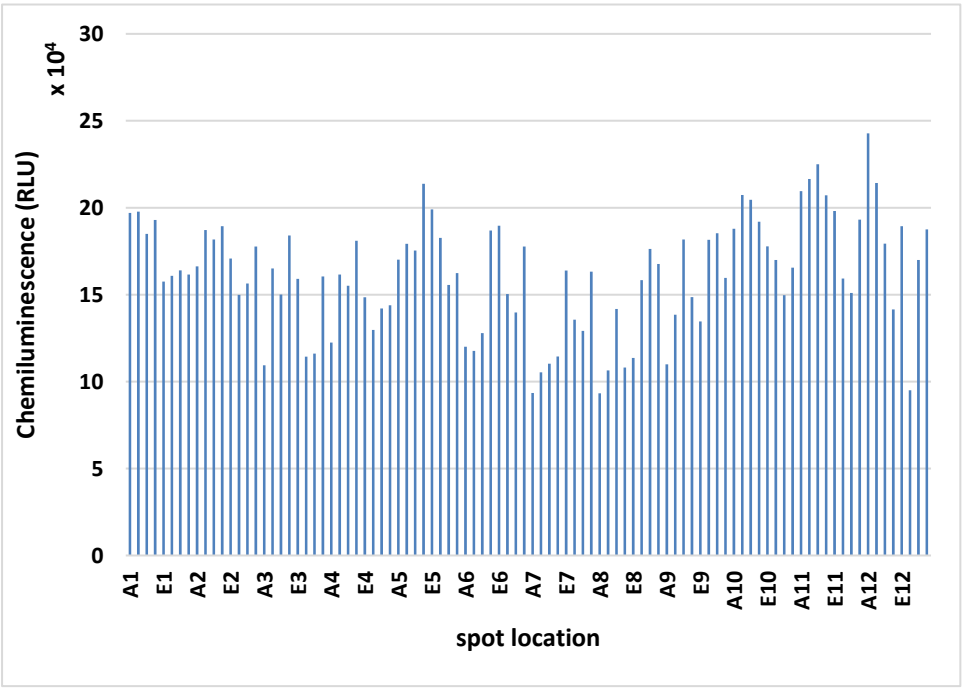
Assessing of variability in measuring basogranulin by dot botting

There was considerable variation in signal strength for the same concentration of basogranulin as measured by dot blotting procedure (Figure 3-4). The intra-assay coefficient of variation was high at 20% (with a mean of 161 $\times 10^3$ for 96 determinations). On account of the dot blotting procedure being affected by high variability, low sensitivity and being time-consuming, ELISA was considered as an alternative assay.

A



B



Statistical features	Min	Max	Median	Mean	STD ±
Relative intensity unit (× 10^3)	93	242	163	161	20

Figure 3-4. Variability of dot blotting in basogranulin assay. A) PVDF membrane was plotted with 100 µl per well of LAD2 lysate. The intensity of signals was quantified using bio-imaging system and supporting software (Chemi Genius). B). Line graph representation of signal intensity for each dot.

3.2 Developing ELISA assay for measuring basogranulin release

Purification of BB1

BB1 antibody was successfully purified from monoclonal BB1 antibody-rich hybridoma cells culture supernatant using a 5 HiTrap G protein column. About one litre of BB1 culture supernatant was concentrated to a volume of about 12 ml, with a concentration of 10 mg/ml as determined by Bradford assay using ɣ- bovine globulin for the standard.BB1 antibody was eluted by 0.1 M glycine at rate of 1 ml/minute in a single peak (the presence of bands with molecular weights of 50 and 25 kDa was confirmed on a Coomassie blue dye stained SDS-PAGE (Figure 3-5). These correspond to the expected molecular weights of mouse IgG heavy and light chains respectively. Bands above 50 kDa may represent aggregates.

Screening new specific monoclonal antibodies for basogranulin

Tremendous effort was expended to find a new basogranulin specific monoclonal antibody by screening antibody-rich hybridoma cell supernatants of 37 clones that had been selected previously for their potential to bind to a partially purified preparation of basogranulin by ELISA [282]. Screening of supernatants of clones by indirect ELISA with purified basophil lysates revealed some with strong signals, and for some no signals at all (data not shown). Of 37 preparations of antibody culture supernatant investigated, there were 21 which were investigated further. Antibody supernatants from three hybridoma clones (designated clones 14, 24 and 25) were purified and protein concentrations were determined as described above. Data for evaluation of potential of these antibodies in developing ELISA for measuring basogranulin will be presented later.

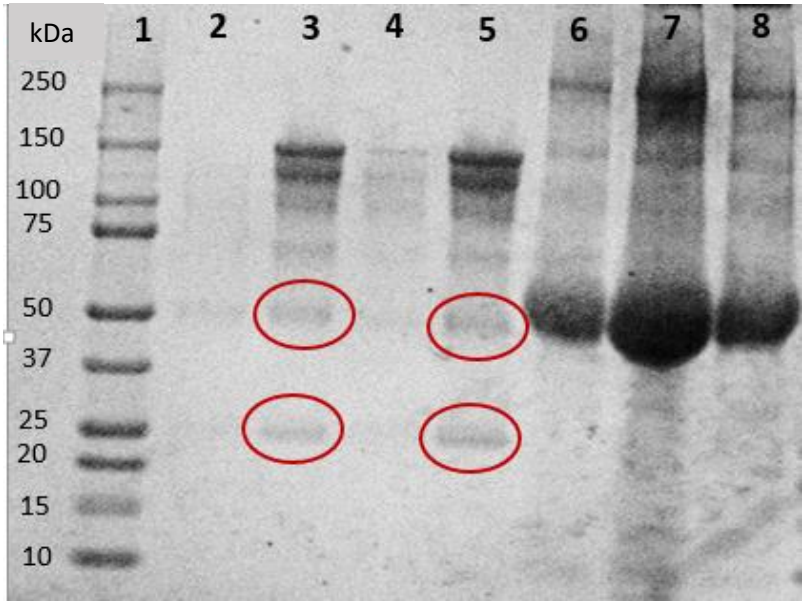


Figure 3-5. Coomassie blue stained SDS-PAGE (4-12% gradient) gel for preparations of BB1 monoclonal antibody purified using HiTrap protein G column. Protein electrophoresis Lanes (1) is protein standard markers, (2 and 4) are low protein concentration fractions, (3 and 5) are high protein concentration fractions, (6) washing, (7) flow through, and (8) starting materials.

3.2.1 Indirect ELISA

Basogranulin was detected by BB1 in indirect ELISA with plates coated with lysates of purified basophils and in a concentration dependant (Figure 3-6). The detection of basogranulin was not affected by the concentration of BB1 monoclonal antibody when added neat or in serial dilutions down to 1/16. Data with other potential basogranulin specific antibodies are shown In Figure 3-7 and in the Appendix (Figure 6-4).

3.2.2 Sandwich ELISA for potential basogranulin monoclonal antibodies.

Attempts to develop a sandwich ELISA for basogranulin with BB1 as a capture antibody and biotinylated BB1 for detection were unsuccessful (data not shown). Other potential basogranulin specific monoclonal antibodies were tested by sandwich ELISA for which BB1 was the capture antibody. Detecting antibodies investigated included those from clone numbers 5, 14, 15, 24 and 25 which were selected based their performance in indirect ELISA (Figure 3-8) and (Figure 6-5 in Appendix).

Reduction of non-specific binding in basogranulin sandwich ELISA

Diluting basophil lysate in PBS without addition of BSA as a carrier improved detection of basogranulin using antibody from clones 14 and 25 but slightly less so for antibody 24 (Figure 3-9). Addition of goat serum in a blocking buffer reduced detection of basogranulin by all of these antibodies (clones 14, 24 and 25) (Figure 3-10).In contrast, using a blocking buffer containing BSA enhanced the detection of basogranulin particularly with antibody from clone 25. However, non-specific binding was constantly high for antibodies from clones 14 and 24 than that of 25 (Figure 3-9). Although, the newly developed basogranulin ELISA showed some encouraging data, the assay overall involved shortcomings in particular with specificity. Thus, other methods were investigated as means for measuring basogranulin (*vide infra*).

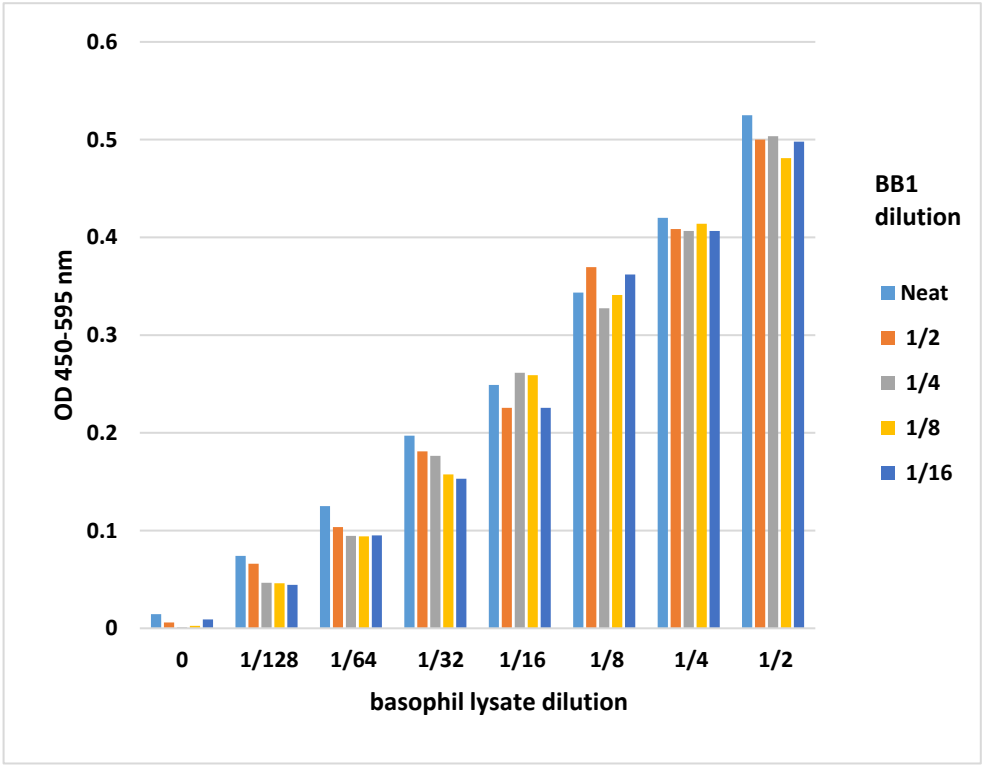


Figure 3-6. Optical densities for an indirect ELISA for basogranulin with BB1. Microplates were coated with various dilutions of a lysate of partially purified basophils, and with dilutions of antibody BB1(in hybridoma cell supernatant) was employed to detect basogranulin. Mean of duplicate determinations are shown.

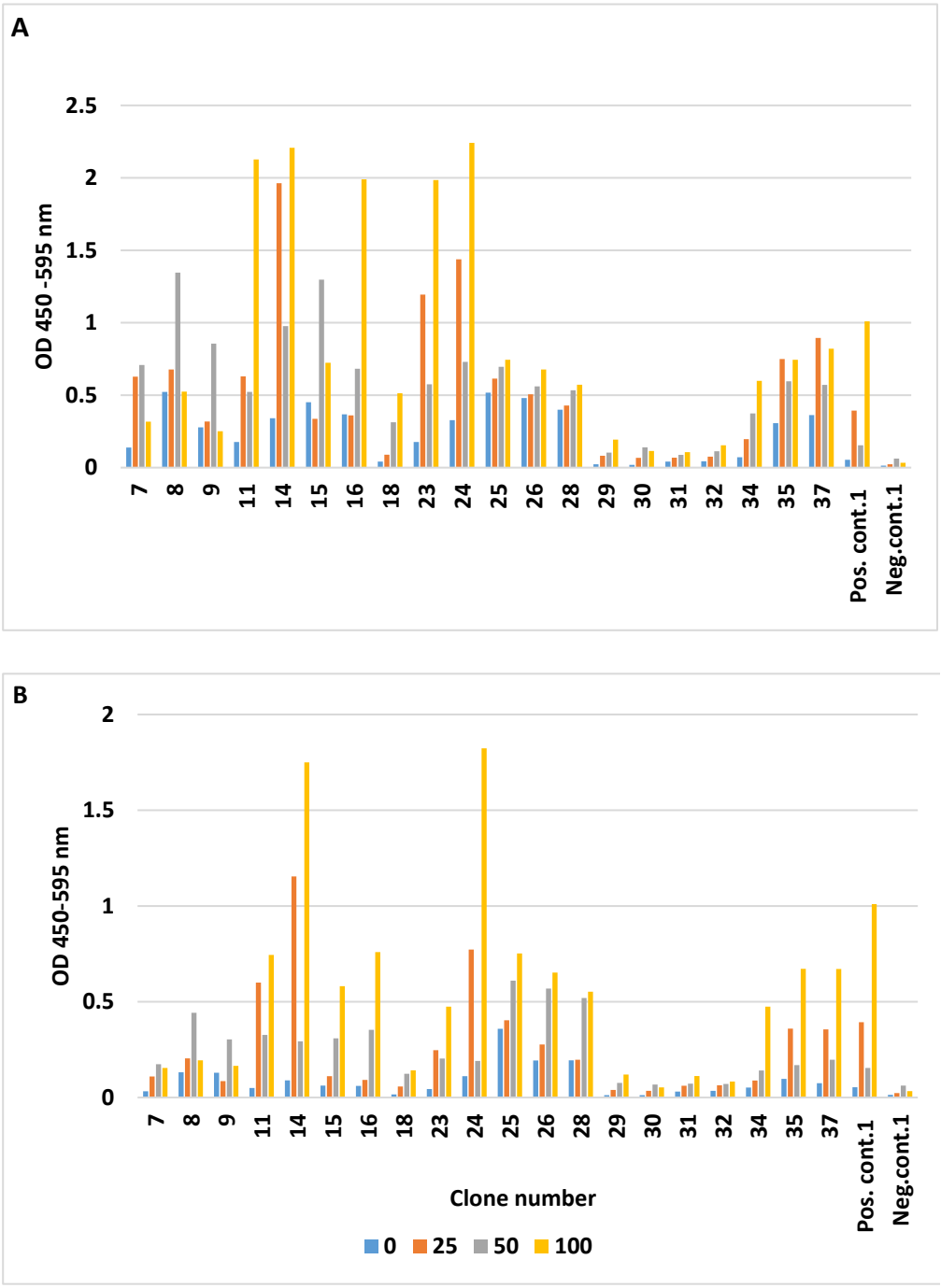


Figure 3-7. Indirect ELISA screening of new antibodies specific for basogranulin. Detection of basogranulin in a lysate of purified basophils coated on microliter plates by hybridoma cell antibody rich supernatants diluted (A) 1/5 and (B) 1/ 20. BB1 was used as a positive control and the dilution buffer PBS with BSA (3%) was a negative control. Mean of duplicate determinations are shown.

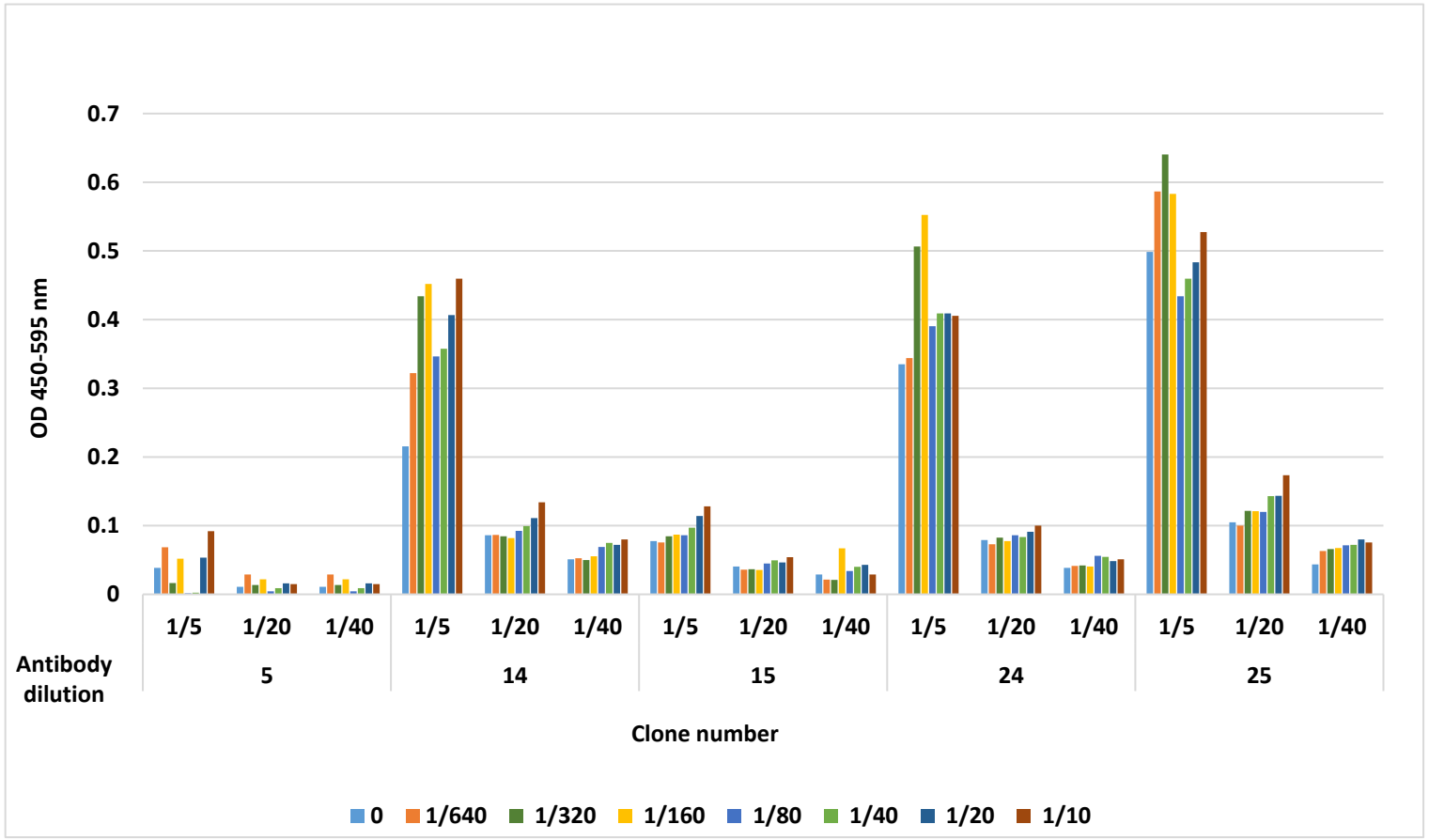


Figure 3-8. Sandwich ELISA for basogranulin in a lysate of purified basophils (10^5 cells/ml). Purified BB1 was the capture antibody (2 µg/ml) and new specific antibodies from clones 5, 14, 15, 24 and 25 were employed for detection (hybridoma culture supernatant diluted 1/5. 1/20 and 1/40). Mean of duplicate determinations are shown.

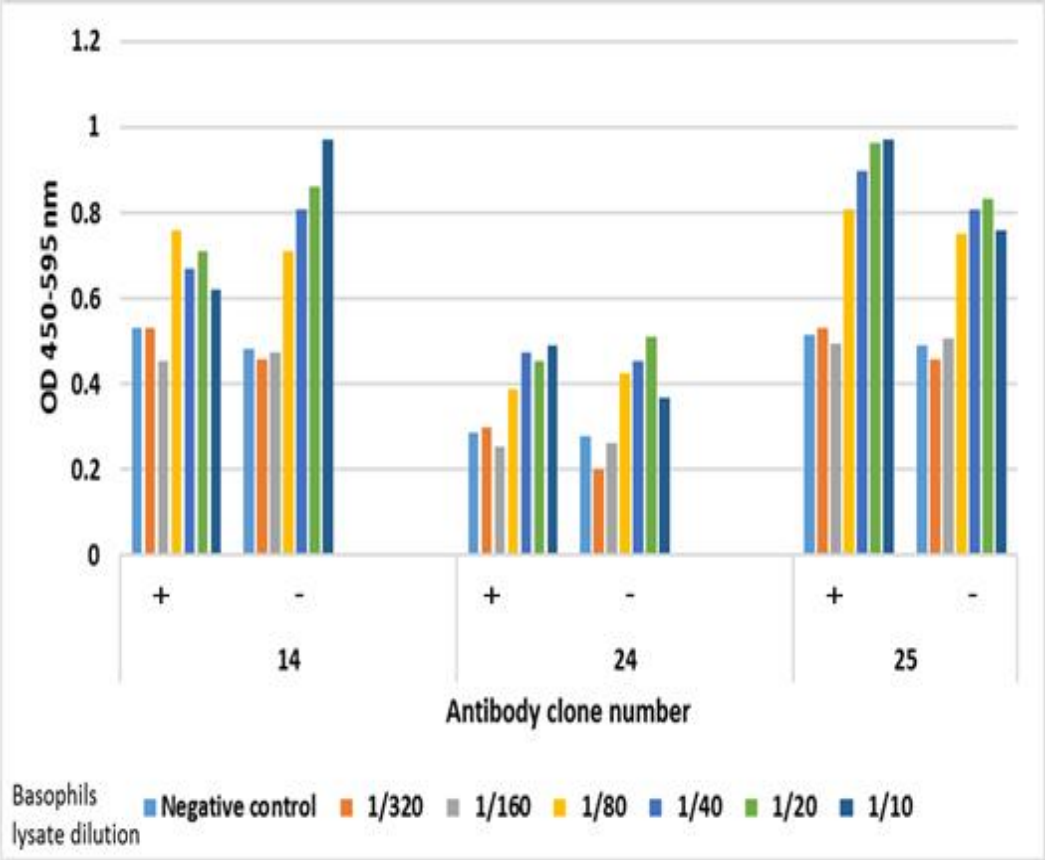


Figure 3-9. Effect of adding bovine serum albumin in buffer used for diluting the basophil lysate on the detection of basogranulin by potential new antibodies in sandwich ELISA. Purified basophils lysate (6×10^3 cells /ml) diluted in PBS with (**+**) or without (**-**) BSA (1%) was applied in serial dilution into BB1 pre- coated plate (2 µg/ml). Basogranulin was detected by three antibody supernatants (clones 14, 24 and 25). Mean of duplicate determinations are shown.

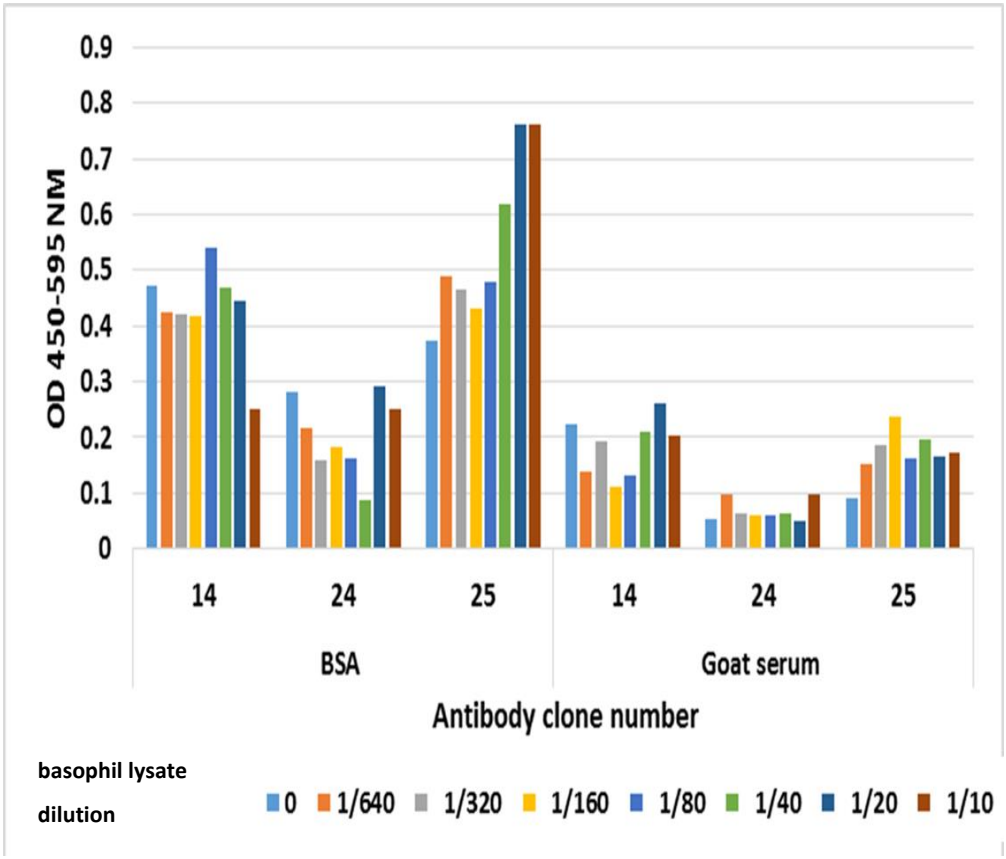


Figure 3-10. Effect of using BSA or goat serum blocking solution on the detection of basogranulin in basophil lysate using sandwich ELISA. A purified basophil (6×10^3 /ml) lysate diluted with PBS was applied in serial dilution from 1/10 to 1/620 into a pre-coated 96 well with BB1 (2 µg/ml). Antibodies from three clones were used as detecting antibodies (14,24 and 25). Negative control contained only dilution buffer. Mean of duplicate determinations are shown.

3.3 Measuring intracellular basogranulin expression by flow cytometry

A flow cytometric assay for measuring intracellular basogranulin was developed with BB1 conjugated to fluorochrome. A fluorochrome labelled isotype control IgG2a antibody did not bind to any intracellular component indicating little non-specific binding was eliminated. Selection of basophils was based on expression on CCR3 and granularity on side scatter (Figure 3-11 A). Basogranulin positive basophils were analysed within the total gated basophil population (CCR3 positive cells. Nonstimulated basophils had large quantities of intracellular basogranulin. and stimulation of basophils resulted in depletion (Figure 3-11 B).

Intracellular basogranulin could be detected in permeabilised basophils in whole blood using flow cytometry with fluorescently-labelled BB1. Intracellular basogranulin stores were depleted in basophils stimulated with anti-FcεRI (Figure 3-12 A and B) .Surface expression of basogranulin as well as CD63 was increased in non-permeabilised cells (Figure 3-12 B and E) were increased The levels of expression of surface basogranulin was relatively low in some basophil preparations , and these intracellular basogranulin stores did not decrease after stimulation (Figure 3-12 C and F).

Expression of CD63 was reduced by some 55% following permeabilisation compared with non-permeabilised cells (Figure 3-12 C and D). Intracellular basogranulin was predominantly detected in resting non-stimulated basophils using flow cytometry. Levels were much lower in basophils stimulated with peptide fmlp, or with anti-IgE or allergen. Overall, the apparent depletion in quantities of intracellular basogranulin was concentration-dependent and inversely correlated associated with apparent upregulation of surface basogranulin and CD63. Fmlp stimulation of basophils resulted in an apparent 20% depletion of intracellular basogranulin that was associated with an upregulation of CD63 by almost the same amount (23%) for the same concentration of 10 µM (Figure 3-13 A). There was a dramatic reduction of intracellular basogranulin levels in basophils stimulated with anti-IgE, some 30% lower at an IgE concentration of 0.1%. At this concentration of anti-IgE, surface marker CD63 was upregulated by about 80% (Figure 3-13 B). The extent to which there was a reduction in intracellular basogranulin levels was similar to that observed following stimulation with

grass pollen was similar at the highest concentration tested (10 SBU), though in that case CD63 was upregulated by less than 20%.

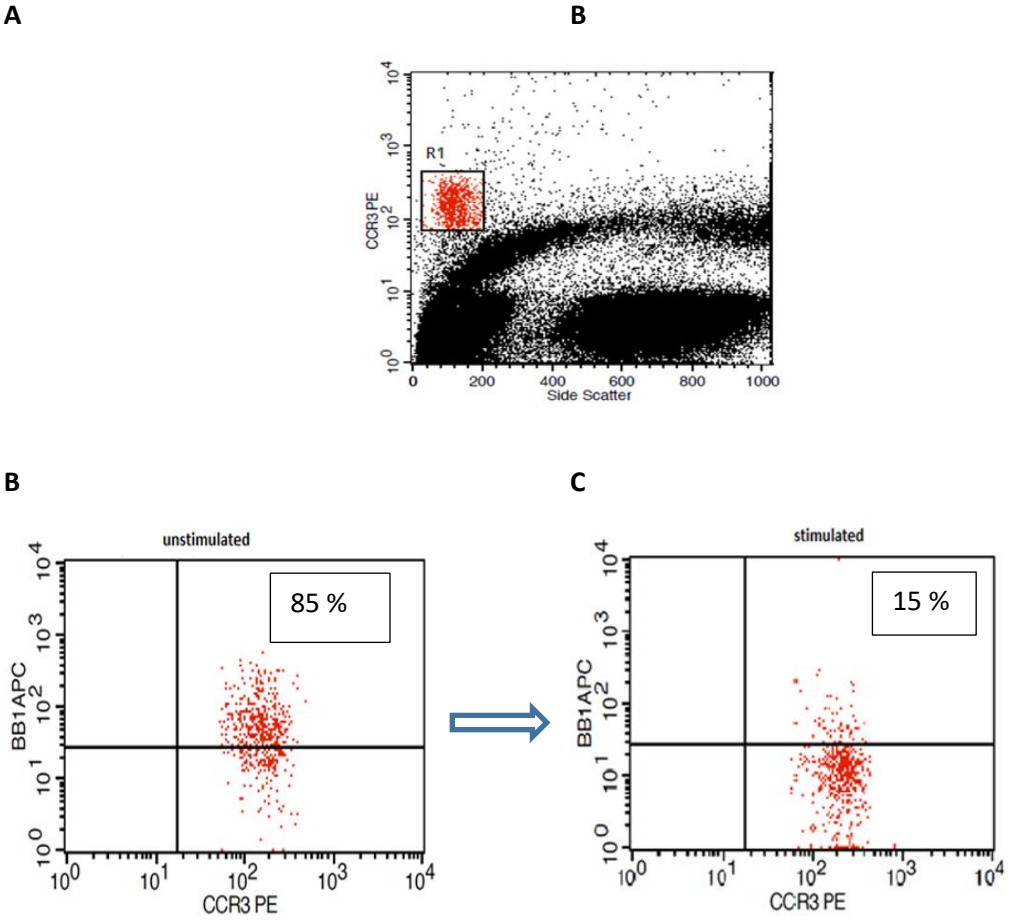


Figure 3-11. Depletion of intracellular basogranulin in permeabilised basophils using fluorochrome labelled BB1 antibody. A) Gated basophils (red dots) are indicated (low side scatter characteristics on the y-axis and high CCR3 expression on the x-axis). B) Unstimulated basophils containing high quantities of intracellular basogranulin are (upper right region). C) Depletion of Intracellular basogranulin stores in activated basophils (right panel) in comparison to that in nonstimulated basophils (left panel). Data is shown for a representative experiment.

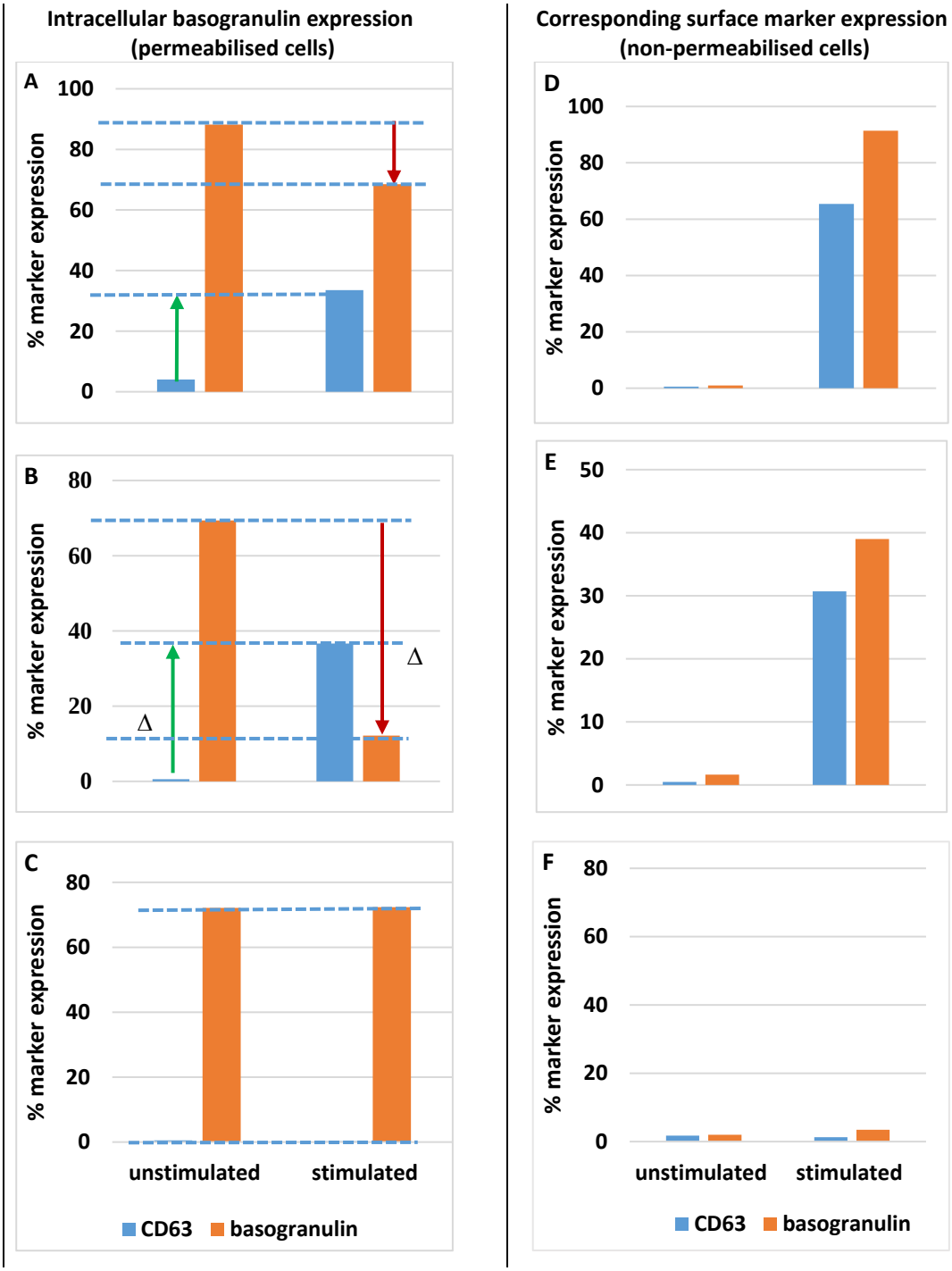


Figure 3-12. Measurement of intracellular basogranulin by flow cytometry in non-purified basophils stimulated with anti-FcεRI. The depletion of intracellular basogranulin (Δ) in basophils (red arrow) and increased expression of surface CD63 (Δ) (green arrow) in permeabilised basophils in three independent experiments (A,B,C). Expression of surface basogranulin and CD63 in corresponding nonpermeabilised basophils was shown in (D,E,F). The percentage of marker expression was calculated relative to that in basophils gated as described above (see Section 2.3.3)

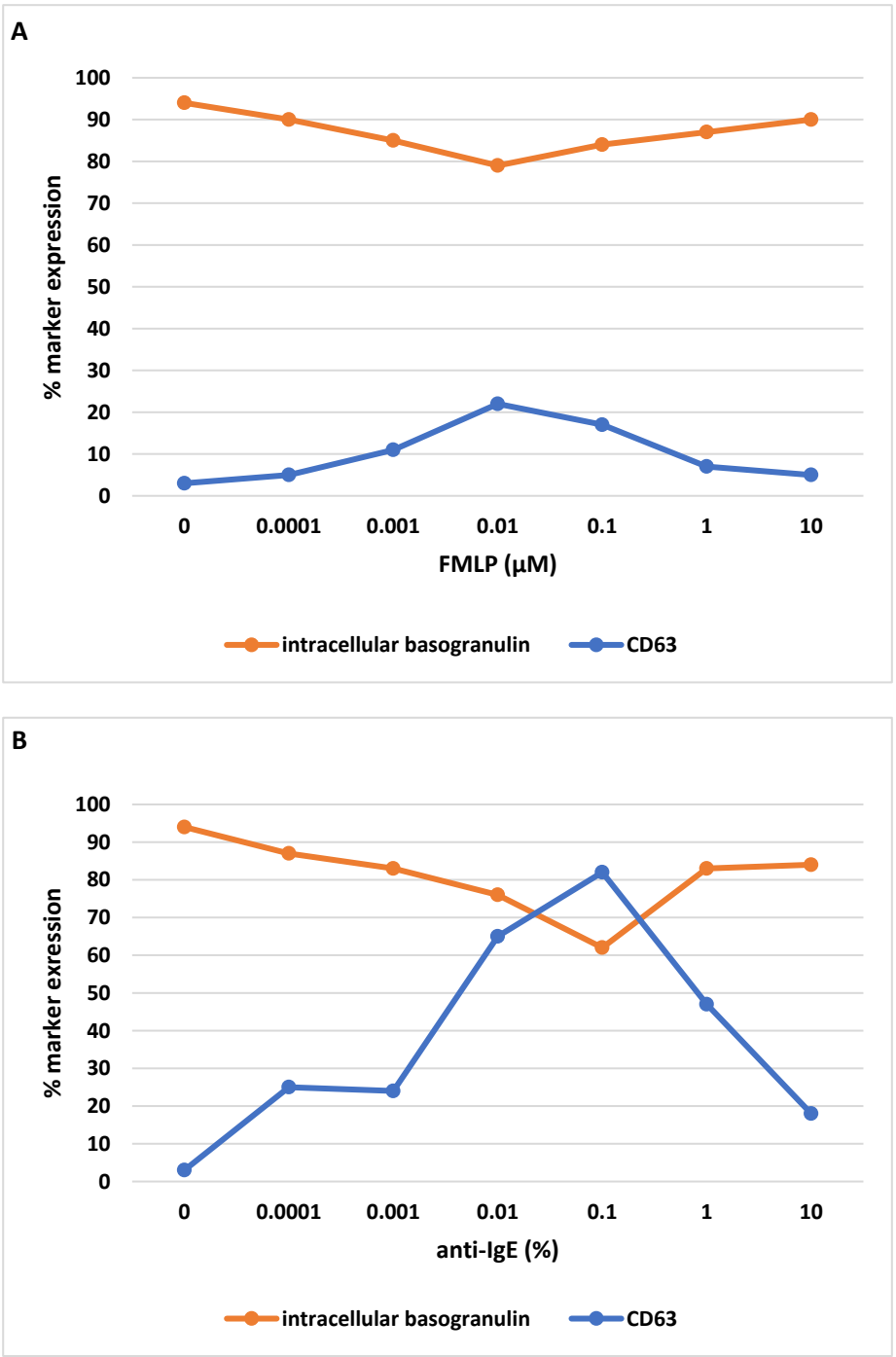


Figure 3-13. Depletion of intracellular basogranulin expression associated with basophil activation. Intracellular basogranulin expression and surface activation marker (CD63) was measured in purified basophils stimulated with (A) fmlp and (B) anti-IgE. All preparations were stained using APC-conjugated BB1 after fixation and permeabilisation. Data was acquired by flow cytometry from one donor.

Measuring intracellular basogranulin expression required basophil permeabilisation, but this process occasionally seemed to have an effect on basophil gating by flow cytometry (Figure 3-14). Non-permeabilised basophils showed high expression of CCR3 with high intensity of CCR3 staining of 2500 to 3200 arbitrary units and low side scatter of less than 200 arbitrary units. However, permeabilisation resulted in diminished intensity of CCR3 which was associated with a two-fold increase of side scatter. In some cases, permeabilisation resulted in disappearance in CCR3 staining. Yet, increasing the number of cell washes led to a massive loss in total cell number (data not shown). Further optimisation involved reducing the number of washes to improve the yield of numbers of basophils recovered by flow cytometry (*vide supra*).

Depletion of intracellular basogranulin stores was assessed by measuring the percentage of expression or by MFI of basogranulin in flow cytometry (Figure 3-15). The intracellular basogranulin expression was highest in non-stimulated basophils for which about 90% of basophils expressed basogranulin (Figure 3-15 A). Stimulation of basophils with low concentration of anti-IgE (10^{-8} to 10^{-6} μg/ml) did not induce depletion of intracellular basogranulin, and simultaneously surface CD63 was invariably found to be low in such cells. Depletion of intracellular basogranulin by about 10% was initially induced by an anti-IgE concentration of 10^{-5} μg/ml, whereas surface CD63 increased by more 50%. With increasing concentrations of anti-IgE, intracellular basogranulin expression appeared to be progressively depleted in basophils with maximal depletion of about 30% at with anti-IgE at 10^{-2} μg/ml. There was a trend for intracellular basogranulin expression was found to be slightly greater with higher concentrations of anti-IgE but not as much as for non-stimulated cells. In contrast, surface CD63 expression was progressively greater with increasing concentrations of anti-IgE up to 10^{-2} μg/ml and thereafter decreased or stabilised with further increases in anti-IgE concentration.

The MFI of intracellular basogranulin appeared to be reduced to a greater extent than observed for percentage expression, with maximal depletion of 65% at a concentration of 10^{-2} μg/ml anti-IgE (Figure 3-15 B). The MFI of surface CD63 increased with increasing concentrations of anti-IgE becoming maximal (97%) at 10^{-1} μg/ml (Figure 3-15 C). The response curve subsequently decreased with higher anti-IgE concentrations (10^{-1} -10 μg/ml) but did not return to baseline.

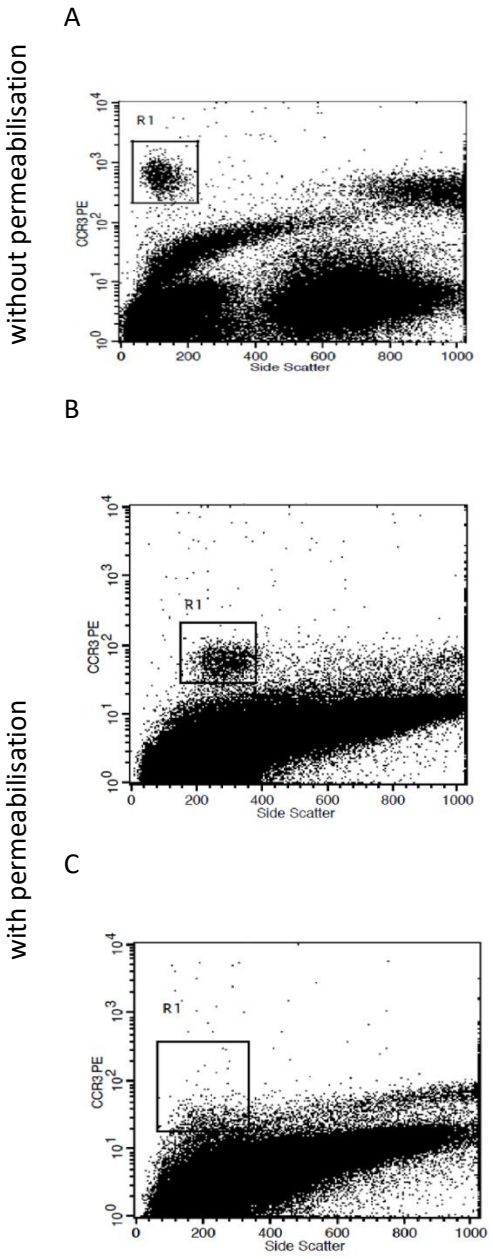


Figure 3-14. The effect of cell permeabilisation on side scatter and intensity of CCR3 staining in basophils measured by flow cytometry. Gating basophils in A) non-permeabilised basophils, and (B and C) permeabilised basophils. Permeabilisation resulted in changes in the intensity of CCR3 staining and the side scatter as indicated in B) or even complete loss of CCR3 staining.

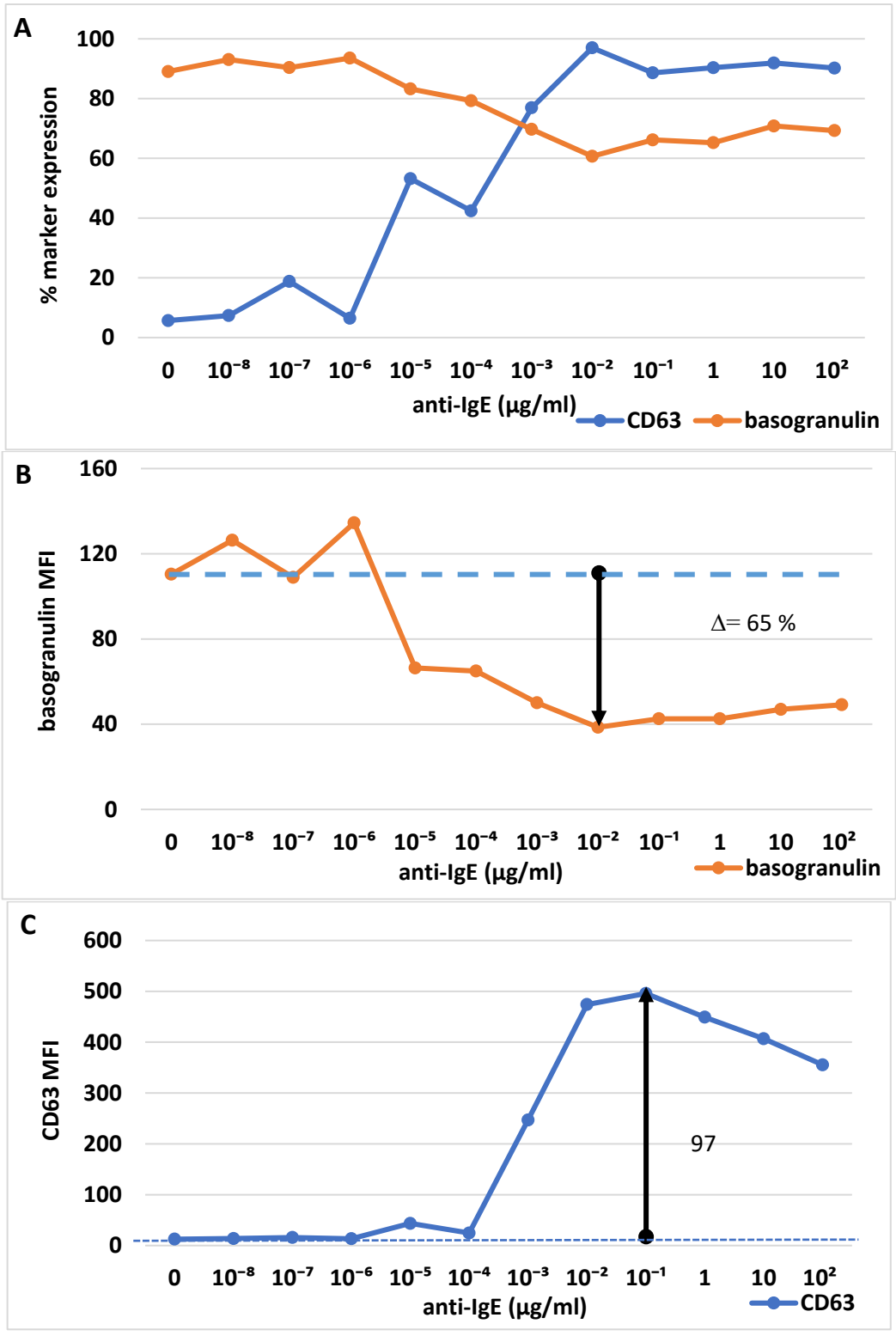


Figure 3-15. Concentration-dependent responses of anti-IgE stimulation on intracellular basogranulin levels in basophils. (A) Intracellular basogranulin depletion and increased expression of surface CD63 was analysed in basophils stimulated with different concentration of anti-IgE (10^{-8}-10^{2} µg/ml) or only buffer as a negative control by flow cytometry. Corresponding mean fluorescence intensity of (B) basogranulin and (C) CD63. The percentage of marker expression was calculated as the difference between the expression of the marker following stimulation compared to the negative control (nonstimulated basophils).

3.3.1 Association of intracellular basogranulin and surface CD63 expression

Stimulating basophils with anti-IgE, anti-FcεRI or grass pollen, induced basogranulin depletion in a concentration-dependent manner, which corresponded to increases in expression of surface CD63 (Figure 3-16). Nonstimulated basophils had high levels of their intracellular basogranulin stores whereas surface CD63 was invariably low in these cells (Figure 3-16 A). However, there was a significant inverse correlation between the levels of intracellular basogranulin and CD63 expression upon stimulation with anti-IgE (Figure 3-16 B) (0.001-10%) (r_s= -0.820, **P= -0. 005, n=10). This association was seen also upon anti-FcεRI stimulation (Figure 3-16 C) (r= -0.829, P= 0. 0058, n=6) though not correlated when basophils were stimulated with grass pollen (r= -0.346, P= 0. 299, n=11) (Figure 3-16 D).

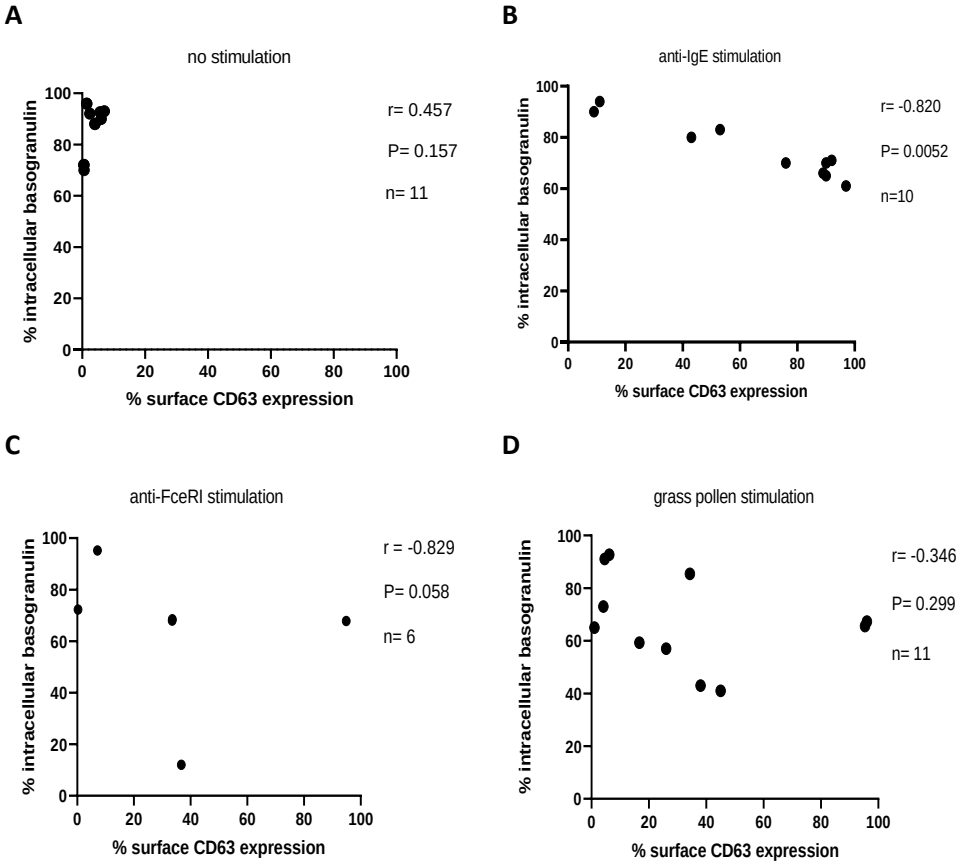


Figure 3-16. Association between levels of intracellular basogranulin and expression of surface CD63. Percentage of expression is shown for basophils with (A) no stimulation and for cells stimulated with (B) anti-IgE (0.0001- 10%), (C) anti-FcεRI (unknown concentration) or (D) grass pollen (0.0001-100 BSU). Analysis was by Spearman's test.

3.4 Measuring surface basogranulin expression

Storing basophils for 24 hours at 4°C was associated with decreased basophil reactivity/activation upon stimulation with *D. pteronyssinus* (10-5 to 100 SBU) (Figure 3 16). When the difference in basophil activation at 24 hours was compared with that within 4 hours, it was found that CD63 expression was significantly decreased (***P= .0006). These findings are preliminary and further work is needed for confirmation. It was decided to perform basophil activation testing at the same day of blood collection and preferably within the first 4 hours. Also, the effect of purification on basophil activation was determined by measuring CD63 expression whole blood and purified basophils using flow cytometry (data not shown). Although basophils were resuspended with stimulation buffer containing IL-3 for 30 minutes prior to the stimulation, the reactivity was lost almost completely with all stimuli (positive controls and allergen). However, these findings are preliminary, and it was thus to avoid basophil purification when basophil activation was intended.

We then measured alterations in expression of surface basogranulin in basophils stimulated within 4 hours of collection time. Basophils were identified by anti-CCR3 and low granularity of basophils on side scatter. Basogranulin positive cells were detected within the total gated basophil population (CCR3 positive cells) as described above (Figure 2-1 and Figure 3-11 A). Basogranulin was barely expressed on the surface of nonstimulated basophils. However, stimulating basophils with anti-IgE resulted in increases in basogranulin expression (Figure 3-18 A). The concentration response was semi-sigmoidal for surface expression of basogranulin with maximal expression at 1 and 10 µg/ml anti-IgE (Figure 3-18 B). Figure 3-19 showed expression of basogranulin and CD63 on the surface of basophils following stimulation. Nonetheless, there was a variation in expression of surface basogranulin though it was almost invariably higher than that of CD63. The overall expression of both markers was higher in response to stimulation with anti-FcεRI than with fmlp.

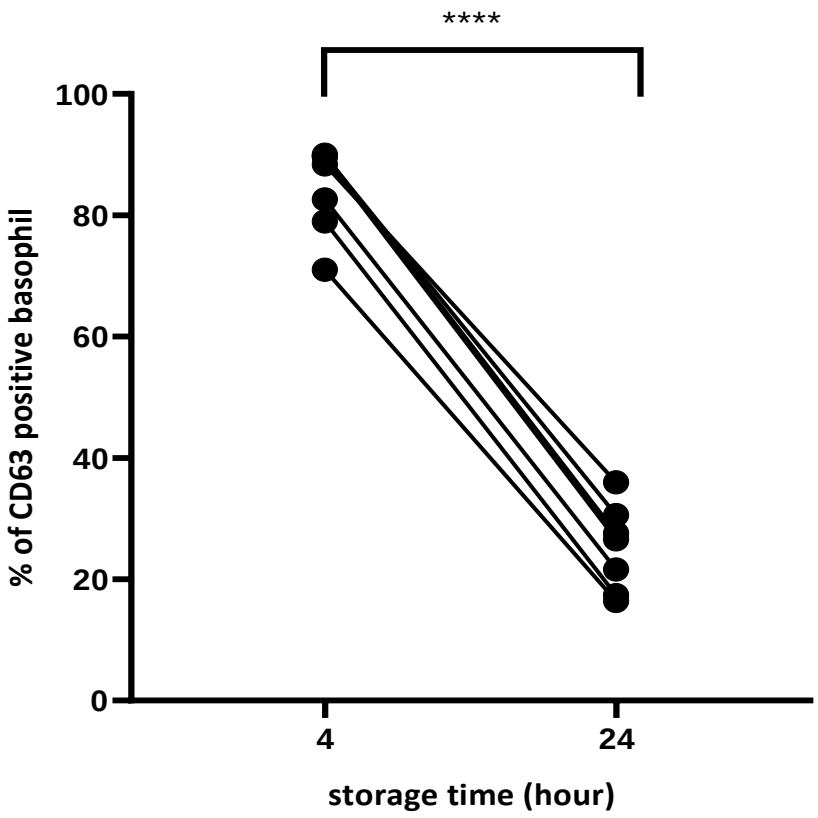


Figure 3-17. Storage time-dependent decrease in basophil activation. Basophil activation with *D. pteronyssinus* (10^{-4} -10^{2} SQ-U/ml) was performed within 4 hours after blood collection or after storage of 24 hours at 4°C (n = 7 experiments from one sample). Data was acquired by flow cytometry and statistical analysis was performed by t- test (P <0.0001).

Nonstimulated basophils exhibited surface basogranulin expression varied between 4.3 and 14.3%. Following stimulation with anti-FcεRI or fmlp, basogranulin surface levels increased with a range of 67 – 94% and 29-59% respectively. Likewise, CD63 expression increased in response to anti-FcεRI or fmlp by 47 to 94% and 25 to 47% respectively. However, basogranulin expression was more than that of CD63 in basophils stimulated with fmlp or various concentrations of anti-IgE (Figure 3-20). Further, as seen in Figure 3-21 basogranulin expression was upregulated and exhibited a bell shaped concentration response curve when stimulated with various concentrations of anti-IgE although the extent of expression was relatively low. In contrast, CD63 expression was not upregulated on these basophils. However, these findings were from one donor and more experiments are required for validation.

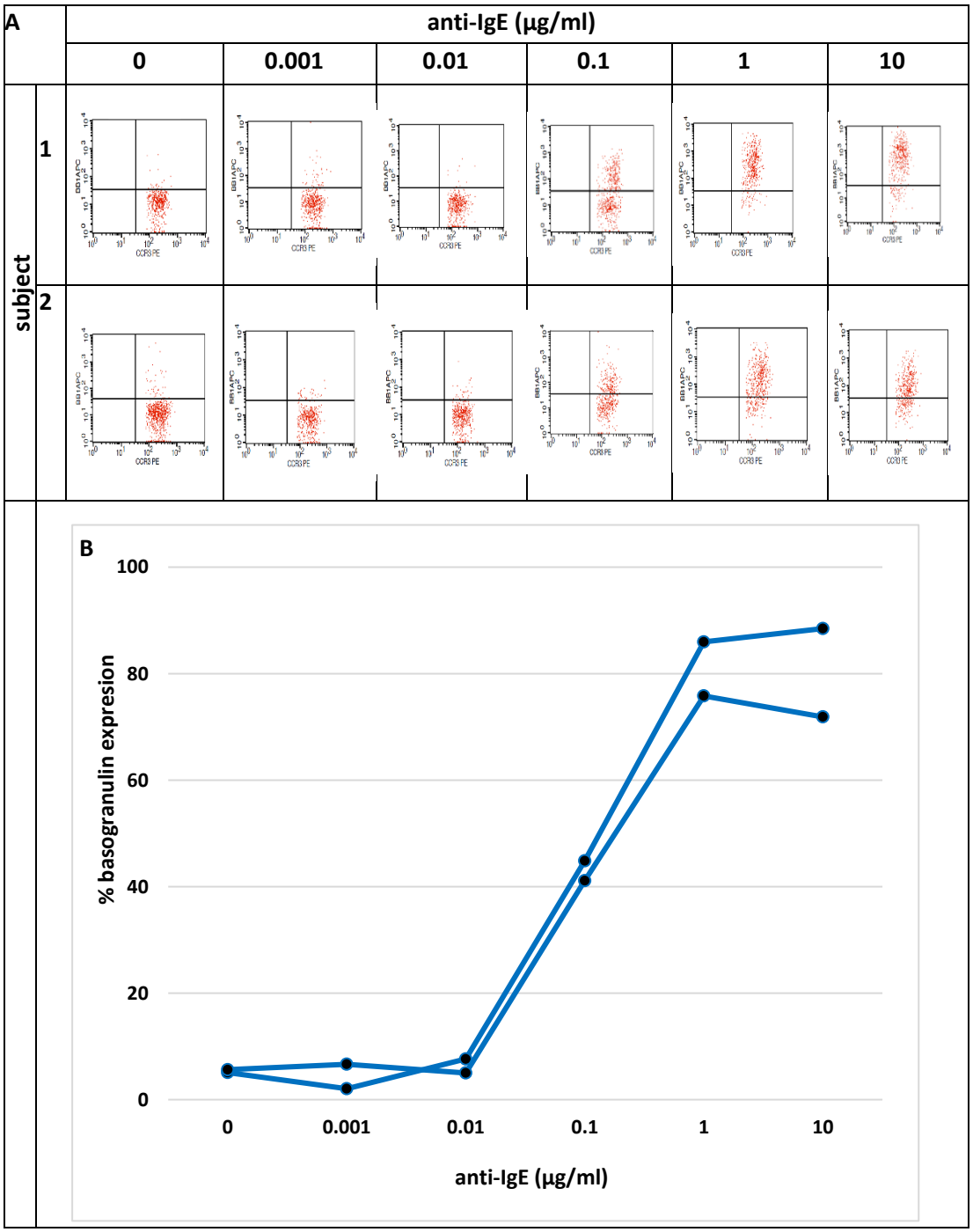


Figure 3-18. Dose-dependent increases in basogranulin surface expression on basophils in response to stimulation with anti-IgE. Basophils were incubated with increasing concentration of anti-IgE antibody (0.001 -10 µg/ml) or buffer alone (negative control). Representative flow cytometry plots showing (A) basogranulin surface expression in basophils from two subjects. (B) Basogranulin expression in response to increasing concentrations of anti-IgE. Data was analysed by flow cytometer using the CellQuest software.

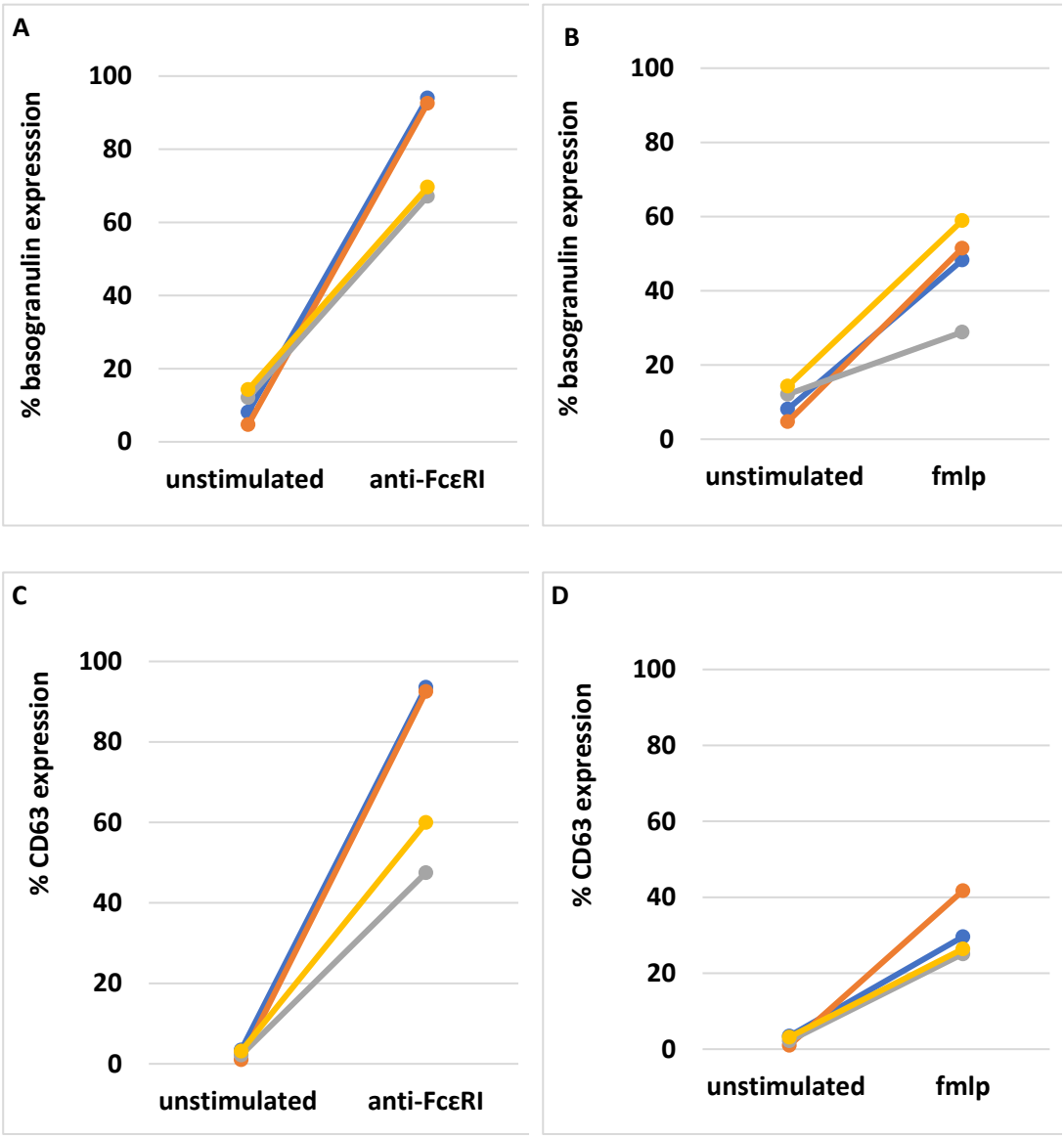


Figure 3-19. Basophil activation by anti-FcεRI or fmlp. Basogranulin and CD63 expression was determined by flow cytometry in unstimulated and basophils stimulated with anti-FcεRI or fmlp (n=4 subjects). Basophils were stimulated in whole blood and marker expression was calculated as the percentage of CD63 positive cells relative to the total amount of basophils gated (for which at least 1000 basophils were gated).

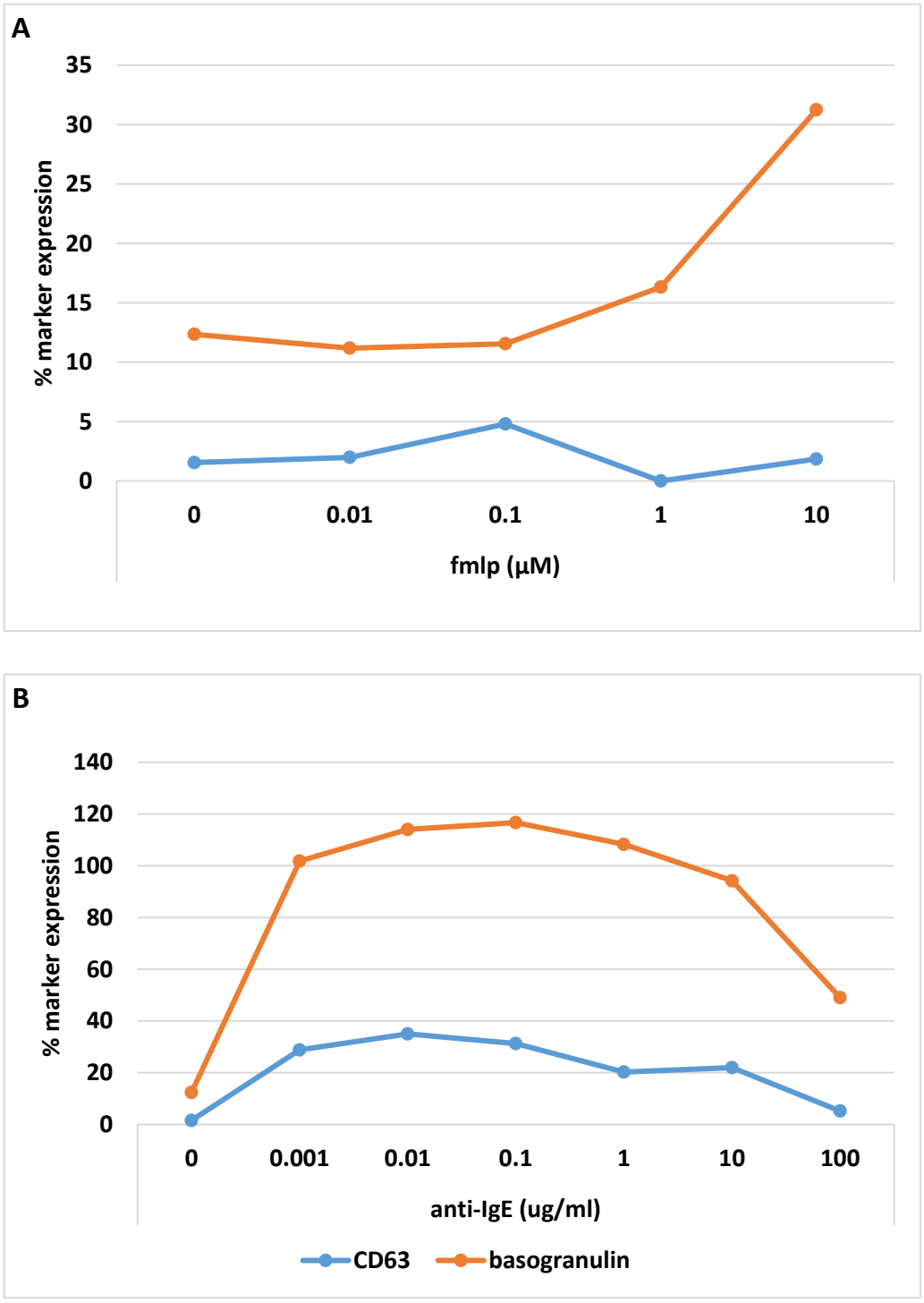


Figure 3-20. Increased expression of surface basogranulin in basophils stimulated with fmlp and anti-IgE. Basophils were stimulated with fmlp or anti-IgE or incubated with the stimulation buffer alone (negative control). Data was acquired by flow cytometry from one subject.

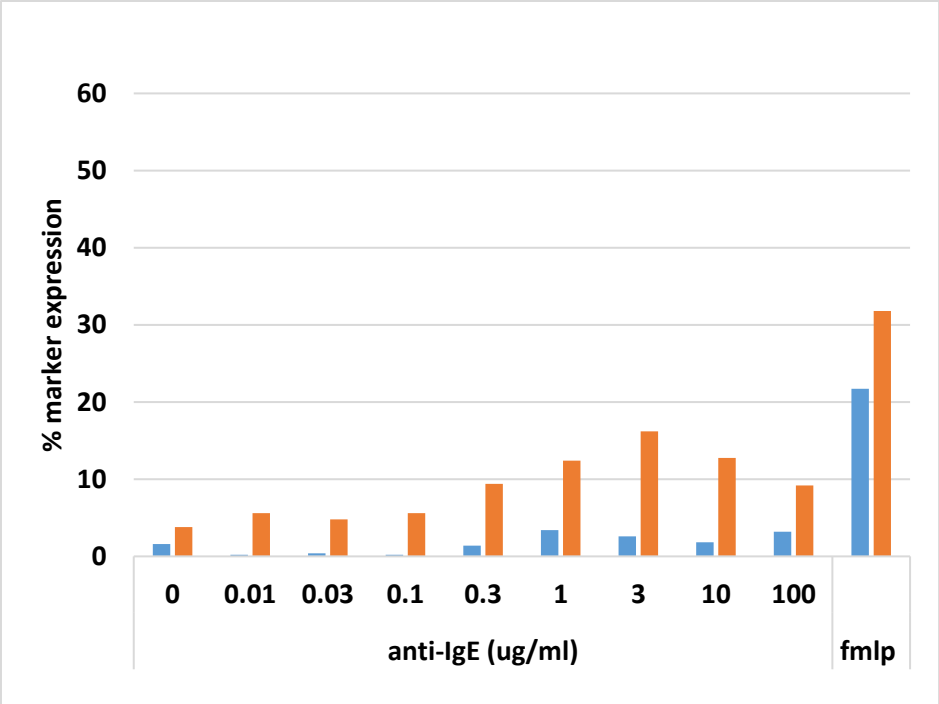


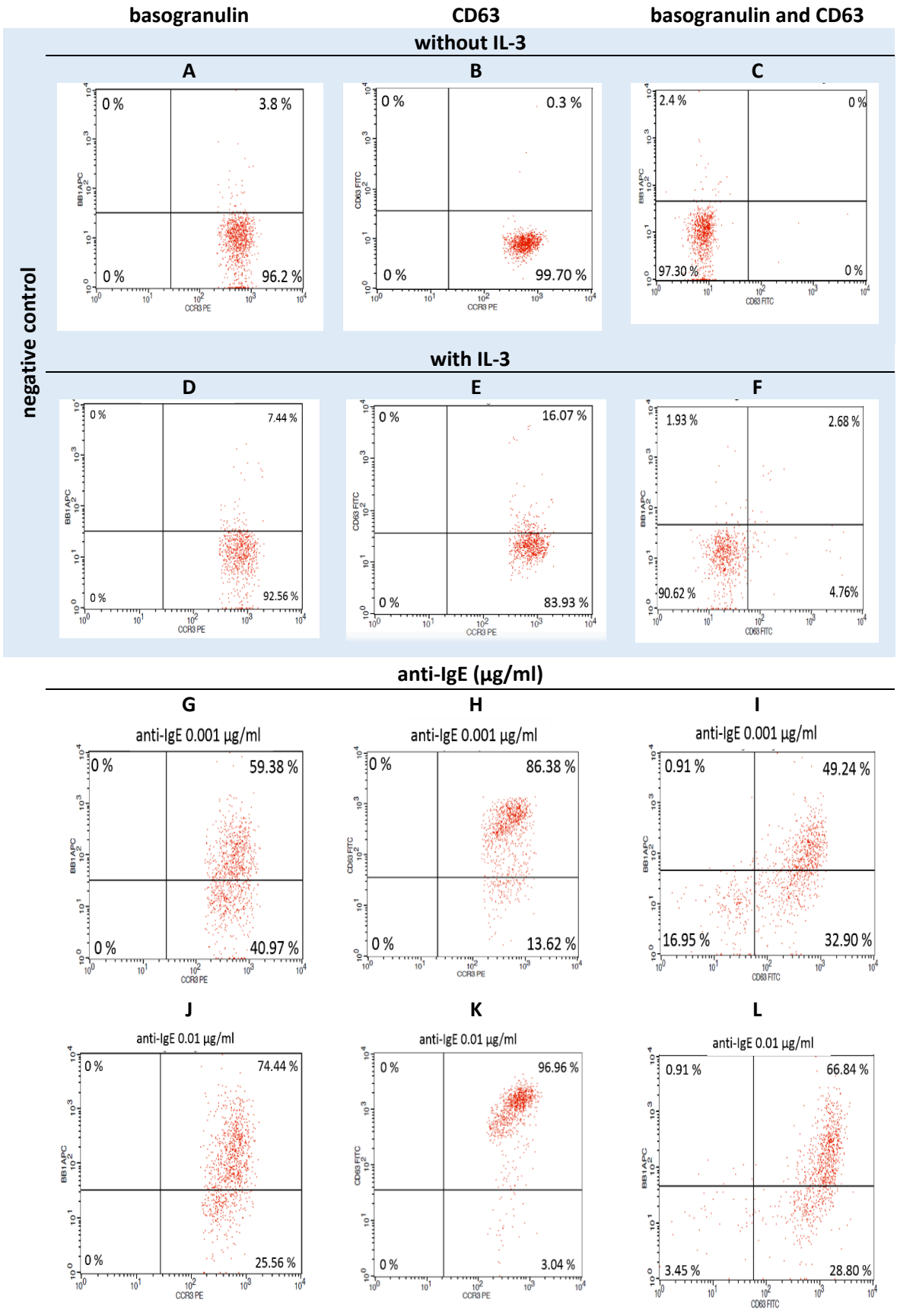
Figure 3-21. Surface basogranulin expression in basophils stimulated with anti-IgE and fmlp. Basophils in whole blood from one subject stimulated with fmlp and various concentrations of anti-IgE, and stained with antibodies specific to CD63 and basogranulin. Data was acquired by flow cytometry and analysed by CellQuest software.

Stimulating basophils induced changes in expression of basogranulin and CD63. However, there were four distinct basophil populations based on differential marker expression. Basophils could be classified as 1) double positive (expressing both basogranulin and CD63), 2) basogranulin positive and CD63 negative ('basogranulin alone'), 3) CD63 positive and basogranulin negative ('CD63 alone'), 4) double negative (expressing neither basogranulin nor CD63). In this section, of the nature of differential basogranulin and CD63 expression in basophils will be described in non-stimulated basophils primed with or without IL-3 and in basophils following stimulation with anti-IgE, fmlp or anti-FcεRI. Further findings about differential expression of basogranulin and CD63 are described later (Section 3.4.1.3).

There were differences in percentages of expression of all basophil subsets. Figure 3-22 presents dot plots for the expression of basogranulin and CD63 on the surface of basophils as determined by flow cytometry. Incubating basophils in buffer free of calcium and magnesium indicated that the vast majority of basophils were basogranulin negative cells (96.2%) and only 3.8% of basophils expressed basogranulin. However, CD63 positive basophils represented only 0.3% of the cells. There were no basophils that exhibited double

positive marker staining (i.e. no basophils were shown to express both basogranulin and CD63). Incubating basophils in stimulation buffer containing calcium and magnesium slightly increased expression of basogranulin to 7.4% whereas the rest of the cells did not. in contrast, CD63 expression was increased to 16%. Basophils expressing both markers were only 2.7 of total cells %. Stimulating basophils with anti-IgE (0.001 -100 µg/ml) induced apparent expression of basogranulin and CD63. The maximum expression of basogranulin 76% was with an anti-IgE concentration of 0.1 µg/ml which corresponded to expression of CD63 of 97%. Basogranulin expression was evenly distributed and slightly more scattered than that of CD63 for which expression was condensed (clustered), and occasionally split into two populations.

The proportions of basophils which expressed basogranulin alone and basophils that expressed both basogranulin and CD63 were consistently lower than for those that expressed CD63 alone. Concentration response patterns for all three types of marker expression were semi-sigmodal (Figure 3-23). However, the MFI of basogranulin and CD63 exhibited bell-shaped concentration-response curves with highest MFI with an anti-IgE concentration of 10^{-1} µg/ml for basogranulin and 10^{-2} µg/ml for CD63 (Figure 3-24). Detailed description about corresponding MFI for basogranulin and CD63 are described in the following section.


basogranulin
CD63
basogranulin and CD63
without IL-3
with IL-3
anti-IgE (µg/ml)
negative control
A
B
C
D
E
F
G
H
I
J
K
L
0 %
3.8 %
96.2 %
0.3 %
99.70 %
2.4 %
97.30 %
7.44 %
92.56 %
16.07 %
83.93 %
1.93 %
2.68 %
90.62 %
4.76%
anti-IgE 0.001 µg/ml
59.38 %
40.97 %
86.38 %
13.62 %
0.91 %
49.24 %
16.95 %
32.90 %
anti-IgE 0.01 µg/ml
74.44 %
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CD63 FITC

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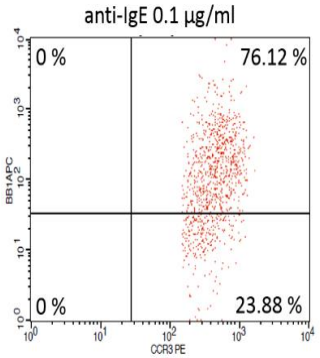

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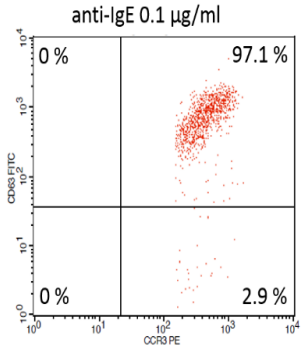

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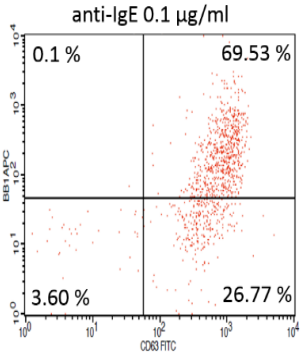

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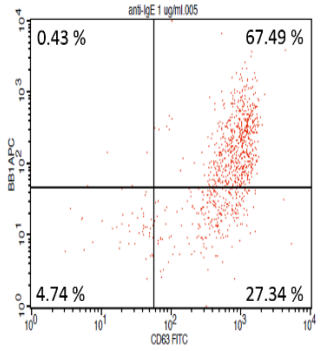

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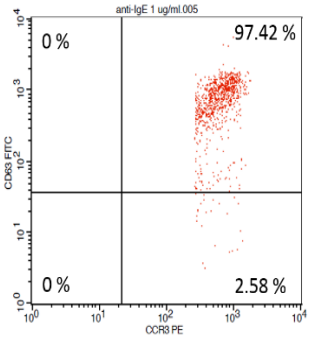

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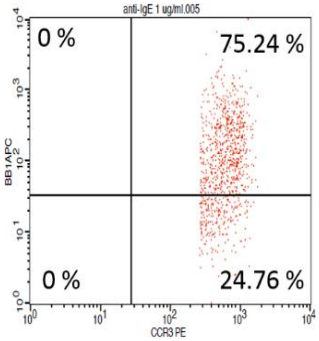

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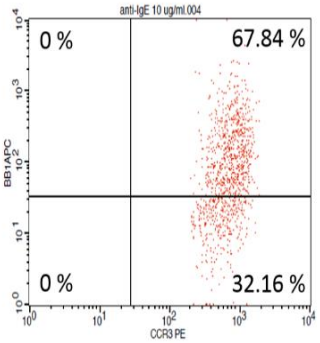

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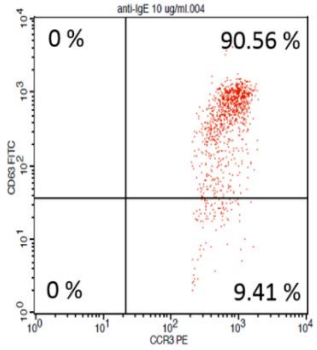
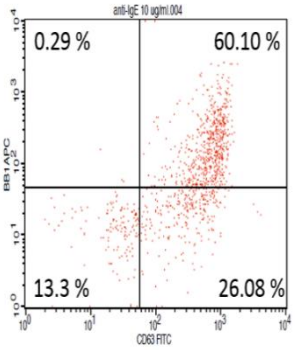
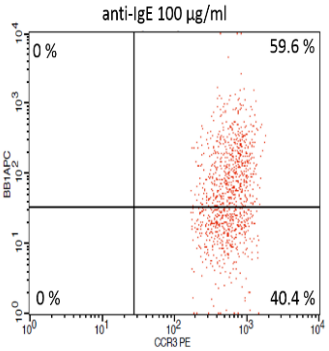


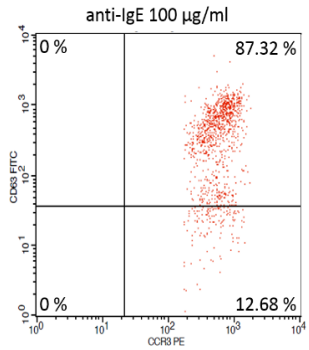
U



V



W



X

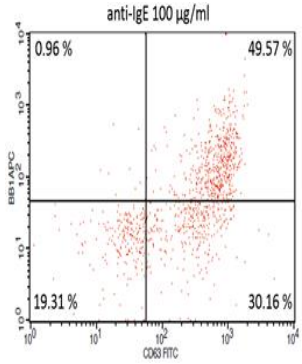


Figure 3-22. Dot plots of the expression of basogranulin, CD63 and both activation markers together on the surface of basophils in different conditions. The baseline of marker expression was determined in basophils incubated in stimulation buffer with or without IL-3. Basophils stimulated with anti-IgE at 0.001 to 100 µg/ml. Results are expressed as percentage positive basophils for basogranulin and CD63.

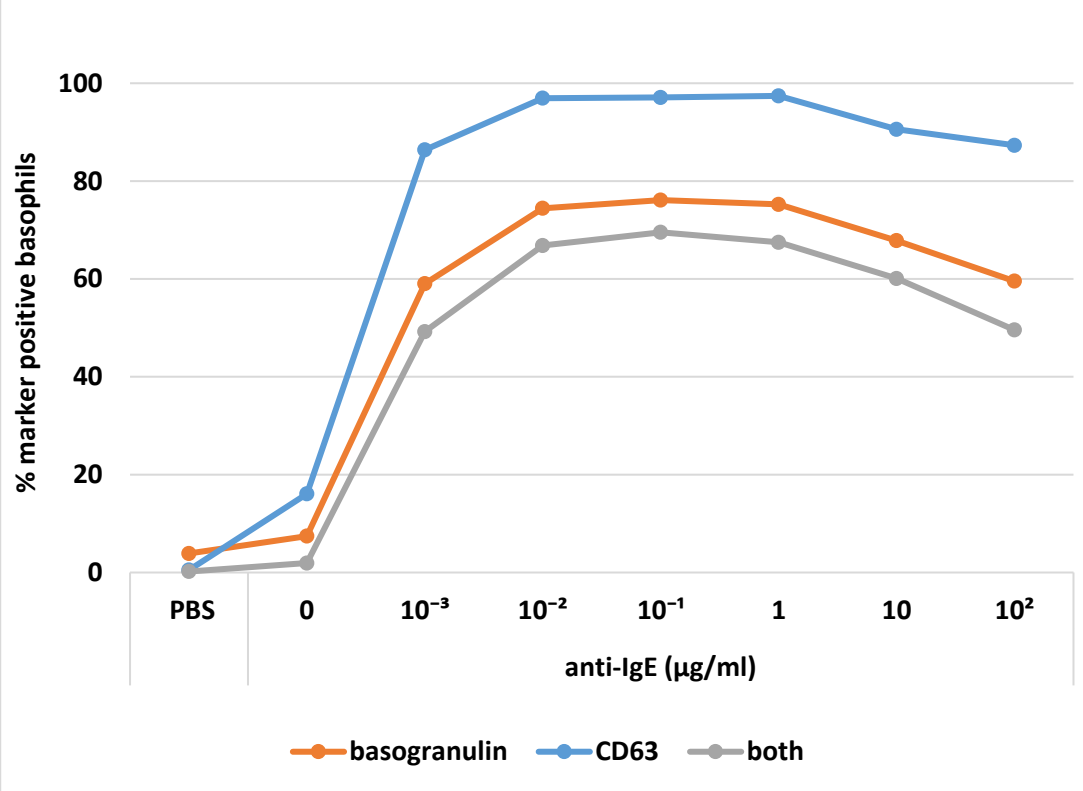


Figure 3-23. Percentage of basogranulin, CD63 and both (basogranulin and CD63) positive basophils. Basophils were stimulated with a range of concentrations of anti-IgE. Non-stimulated basophils were incubated with stimulation buffer, or PBS alone (without calcium and magnesium, IL-3) for basal expression. Expression of basogranulin on the surface of basophils upon stimulation by anti-IgE. Basophils were stimulated with anti-IgE (10^{-3}-10^{2} µg/ml) diluted in stimulation buffer or PBS alone (without Calcium and magnesium).

MFI of surface basogranulin and CD63 expression in basophils stimulated with anti-IgE

The changes in MFI of surface basogranulin and CD63 expression were monitored in basophils before and after stimulation with anti-IgE (Figure 3-24). Expression of basogranulin was relatively low in non-stimulated basophils which were incubated with buffer with or without calcium, magnesium and IL-3. However, stimulating basophils with anti-IgE resulted in a remarkable increase in MFI of surface basogranulin expression by

about 10-fold at an optimum anti-IgE concentration of 0.1 µg/ml. However, MFI decreased afterwards to about 50% though it did not completely return to the baseline.

The MFI of CD63 was low in non-stimulated basophils in absence of calcium, magnesium or IL-3 (MFI=8). However, incubating basophils in buffer that contained calcium, magnesium and IL-3 exhibited a little increase in MFI of CD63 to 21. Stimulation basophils with anti-IgE resulted in a strong apparent increase in MFI for CD63 reaching a maximum level with a concentration of 0.01 µg/ml. However, MFI of CD63 decreased upon higher concentrations of anti-IgE (0.1-100 µg/ml). MFI of basogranulin showed a bell-shape response curve with highest values at an anti-IgE concentration of 10^{-1} µg/ml whereas the maximum MFI of CD63 was with optimum concentration of anti-IgE of 10^{-2} µg/ml (Figure 3-24 B).

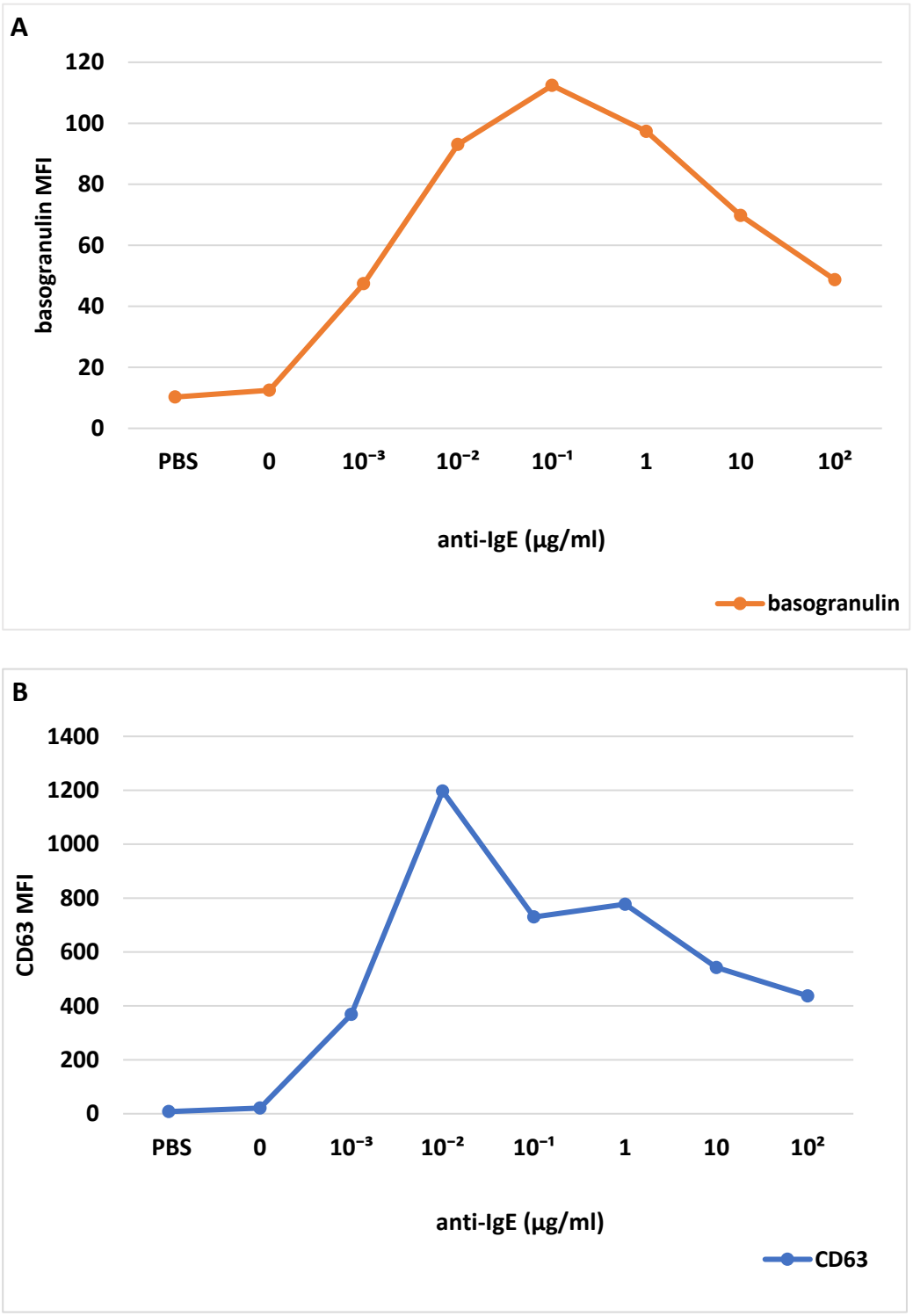


Figure 3-24. MFI of A) basogranulin and B) CD63 in basophils stimulated with anti-IgE. Basophils were incubated with various concentrations of anti-IgE diluted in stimulation buffer (containing calcium, magnesium and IL-3), or PBS only (without Ca++, Mg++). Data was acquired by flow cytometry from one donor.

3.4.1.1 Investigation of increased expression of surface basogranulin following basophil activation

Incubating non-stimulated basophils in buffer containing various concentrations of basogranulin which was prepared from highly purified basophil lysate for different time periods or incubating for various periods of time did not result in an increase in basogranulin bound to the surface of basophils (Figure 3-25 A and B)

Assessing specificity of BB1 antibody in measuring basogranulin by flow cytometry

The specificity of BB1 for basophils was confirmed by monitoring whether other cells such as eosinophils could express basogranulin upon stimulation by anti-FcεRI (Figure 3-26). Eosinophils were gated with high CCR3 expression and high side scatter ranging from 500 to 800 units compared to that in basophils with less than 200 units. Stimulation resulted in expression of basogranulin in more than 50% of activated basophils. In cells gated as eosinophils showed only 3% expressed basogranulin. CD63 expression on activated basophils was higher in this subject (76%), and10% of eosinophils expressed CD63. Figure 3-26 (G and H) presents data for expression of basogranulin and CD63 on stimulated and non-stimulated cells (basophils or eosinophils).

These findings provided confirmation that BB1 antibody does not cross react with eosinophils (Figure 3-26 B). BB1 did not react with any cells types other than basophils following activation with anti-FcεRI. However, there were substantial increases in expression of basogranulin and CD63 observed in basophils following activation by anti-FcεRI (Figure 3-26 C and E) whereas neither of these activation markers were increased in eosinophils (Figure 3-26 D and F). Basogranulin positive cells in those gated as basophils represented 56% of total leukocytes (and 76% positive for CD63), compared with just 3% of those gated as eosinophils (and 10% for CD63).

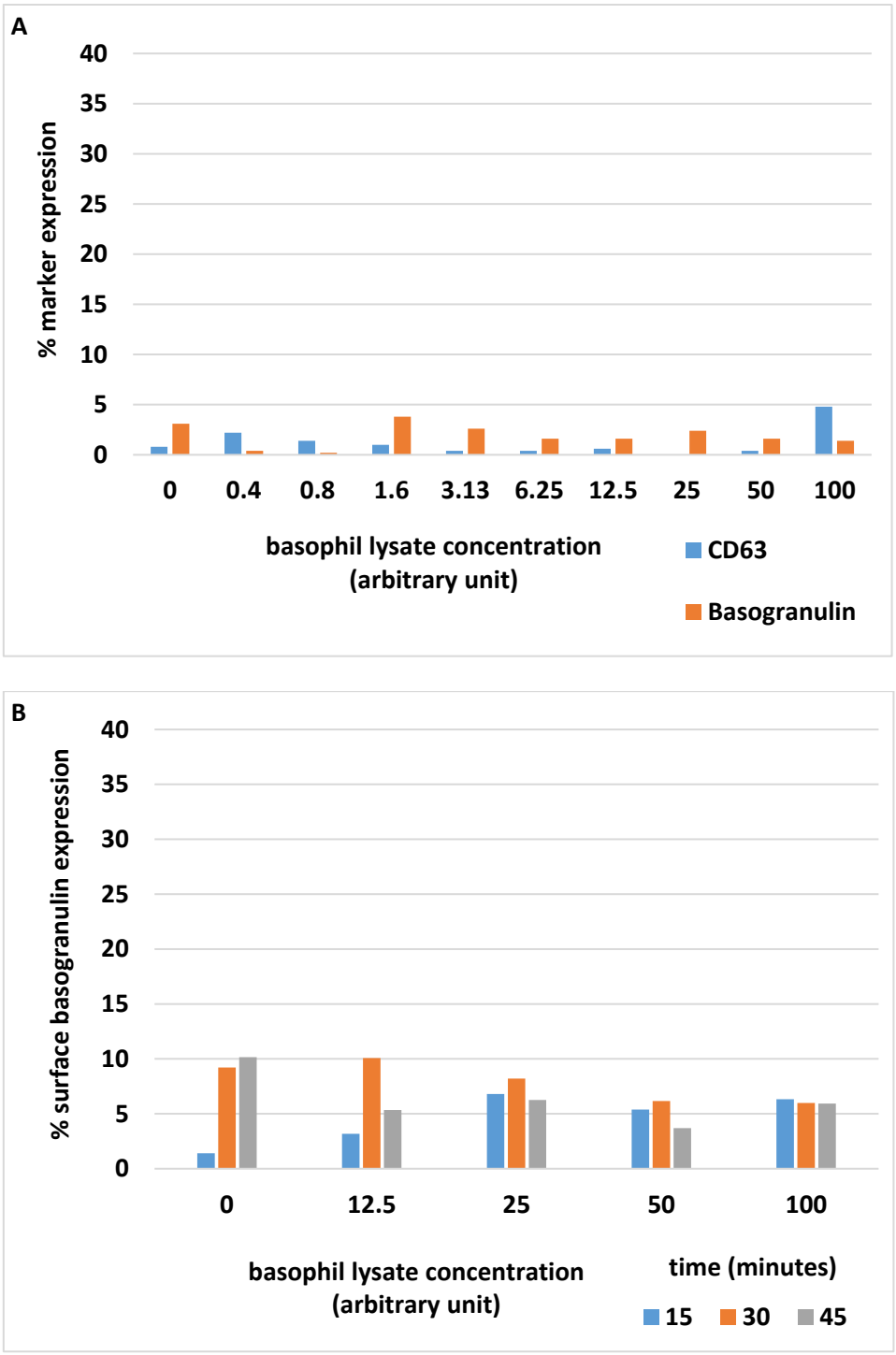
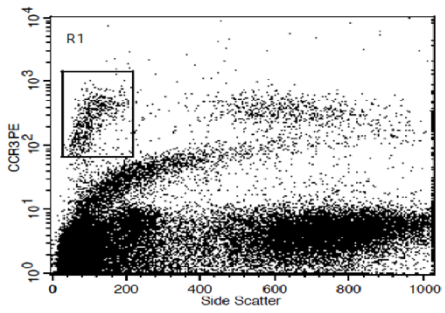
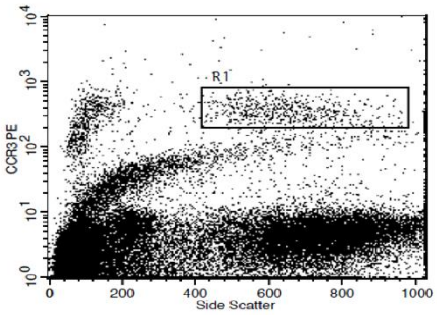


Figure 3-25. Binding of exogenous basogranulin to the surface of basophils. Basophils in whole blood were incubated with different dilutions of purified basophil lysate (10^6 basophils/ml) for (A)15 minutes and (B) at different time points. Basophils were identified by anti-CCR3, and side scatter characteristics, and the activation was monitored by BB1 and anti-CD63. The percentage of marker expression was calculated as numbers of positively stained basophils relative to the total of gated basophils (of which at least 1000 basophils were gated)

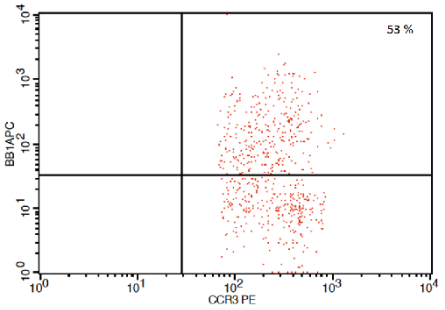
A


R1
CCR3 PE
Side Scatter

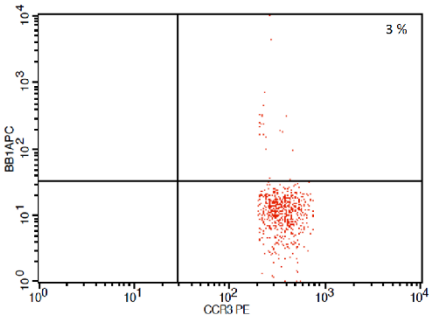
B


R1
CCR3 PE
Side Scatter

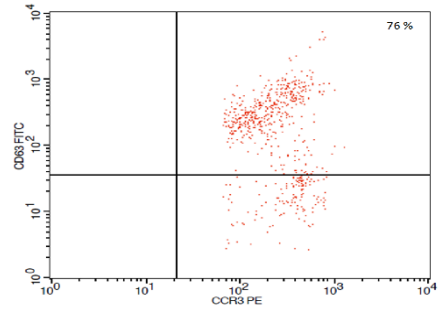
C


53 %
BB1APC
CCR3 PE

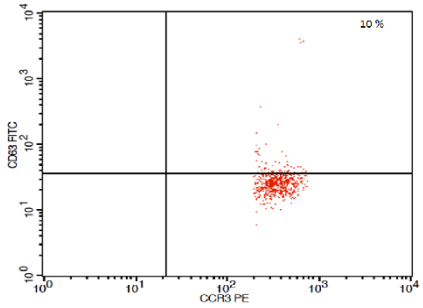
D


3 %
BB1APC
CCR3 PE

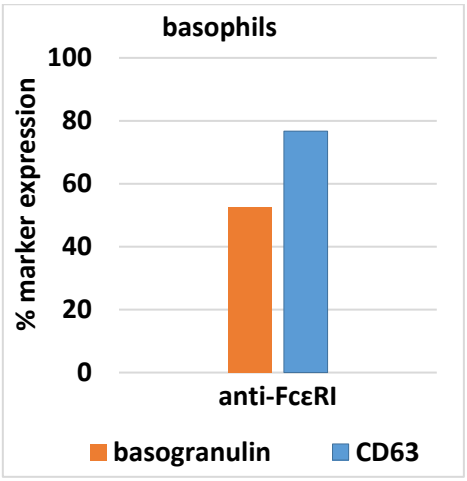
E


76 %
CD63 FITC
CCR3 PE

F


10 %
CD63 FITC
CCR3 PE

G


basophils
% marker expression
100
80
60
40
20
0
anti-FcεRI
basogranulin
CD63

H

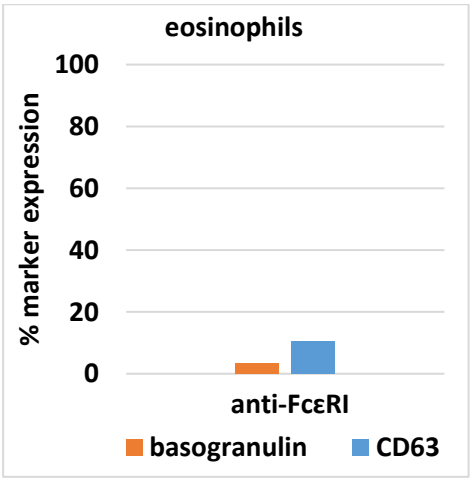

eosinophils
% marker expression
100
80
60
40
20
0
anti-FcεRI
basogranulin
CD63

Figure 3-26. Measuring of basogranulin expression in basophils and eosinophils in response to stimulation with anti-FcεRI *in vitro*. Dot plot of gated basophils and eosinophils are displayed for leukocytes with (A) high CCR3 and low side scatter (basophils), and (B) high CCR3 and high side scatter (eosinophils). Basogranulin expression in (C) basophils and (D) eosinophils, and CD63 expression in (E) basophils and (F) eosinophils were acquired by flow cytometry. (G,H) The expression of basogranulin and CD63 for both cell populations. At least 1000 cells were gated and basogranulin or CD63 positive cell percentages were identified as CCR3+ basophils expressing each of these markers.

3.4.1.2 Effect of IL-3 on basogranulin and CD63 expression

The potential effects of IL-3 on expression of basogranulin were assessed in basophils which were incubated with serial diluted IL-3 at 37 °C and simultaneously stimulated with anti-FcεRI and buffer as a control for 5 and 20 minutes. Incubating basophils with increasing concentrations of IL-3 seemed to slightly enhance the surface basogranulin expression, with a maximal effect of about 10% at concentrations of 10-20 of IL-3 ng/ml at both 5 and 20 minute-time points. In contrast, incubation of basophils with IL-3 for these periods s did not seem to alter expression of CD63 on the surface of basophils (data not shown).

Addition of anti-FcεRI in combination with IL-3 showed decreases in basogranulin expression at 5 ng/ml but basogranulin expression increased upon subsequent concentrations (20-100 ng/ml) (Figure 3-27). Incubating basophils with 200 ng/ml of IL-3 was associated with a slight decrease in basogranulin expression. Prolonging the incubation time of IL-3 with basophils alone to 20 minutes was associated with an increase in basogranulin expression to a maximum of 13.2% at 20 ng/ml. However, stimulating basophils in the presence of IL-3 for 20 minutes appeared to induce the opposite effect on basogranulin expression though the difference was only about 5%.

Incubating basophils with IL-3 (0-200 ng/ml) for 5 or 20 minutes had little effect on CD63 expression. Stimulation with anti-FcεRI for 5 minutes was associated with a slight decrease (8%) in basogranulin expression in basophils primed with IL-3 at 5 ng/ml. Stimulating basophils with anti-FcεRI in the presence of higher concentrations of IL-3 (10-200 ng/ml) increased basogranulin expression up to maximum of 11% at 100 ng/ml. Prolonging the incubation time to 20 minutes in the presence of IL-3 increased basogranulin expression almost by 97% at 10 ng/ml.

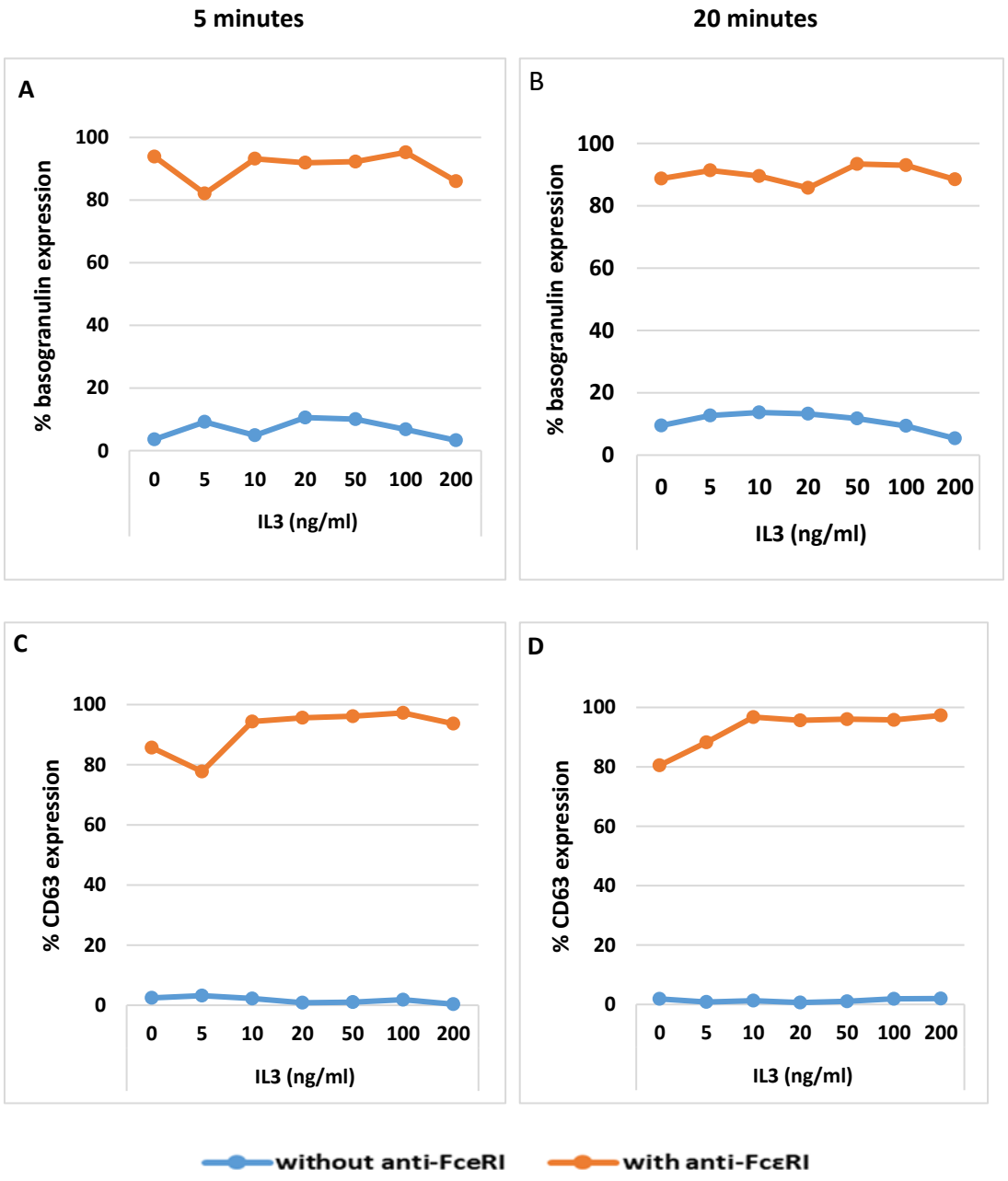


Figure 3-27. Effect on basogranulin and CD63 expression in of stimulating basophils stimulated in whole blood with anti-FcεRI in presence of various concentrations of IL-3 for (A, C) 5 or (B, D) 20 minutes as analysed by flow cytometry.

Optimisation of the basogranulin flow cytometric assay

In this section, are reported the results if different approaches applied to optimise the assay for measuring surface expression of basogranulin by flow cytometry. These included investigating whether the anticoagulants used had an effect on basogranulin expression. Also, whether addition of specific antibodies for staining basophils before or after

stimulation would affect measurements of basogranulin expression.(Figure 3-28). The MFI for basogranulin and CD63 in non-stimulated basophils did not differ between third from blood collected in EDTA or heparin. Basophils stimulated with fmlp in EDTA had lower expression of basogranulin and CD63 than those for which heparin was present (Figure 3-28 A). There was no apparent effect of EDTA or heparin on expression of basogranulin and CD63 in basophils induced by anti-FcεRI.

Immunostaining basophils before fmlp stimulation resulted in higher expression of basogranulin and CD63 than that immunostained after stimulation. Those basophils immunostained prior to stimulation with anti-FcεRI had higher expression of basogranulin and CD63 in than those from EDTA blood immunostained after stimulation. The differences in CD63 expression were greater than that observed for basogranulin. Nonetheless, heparinised blood showed almost no effect of immunostaining basophils before and after stimulation with anti-FcεRI on expression of basogranulin.

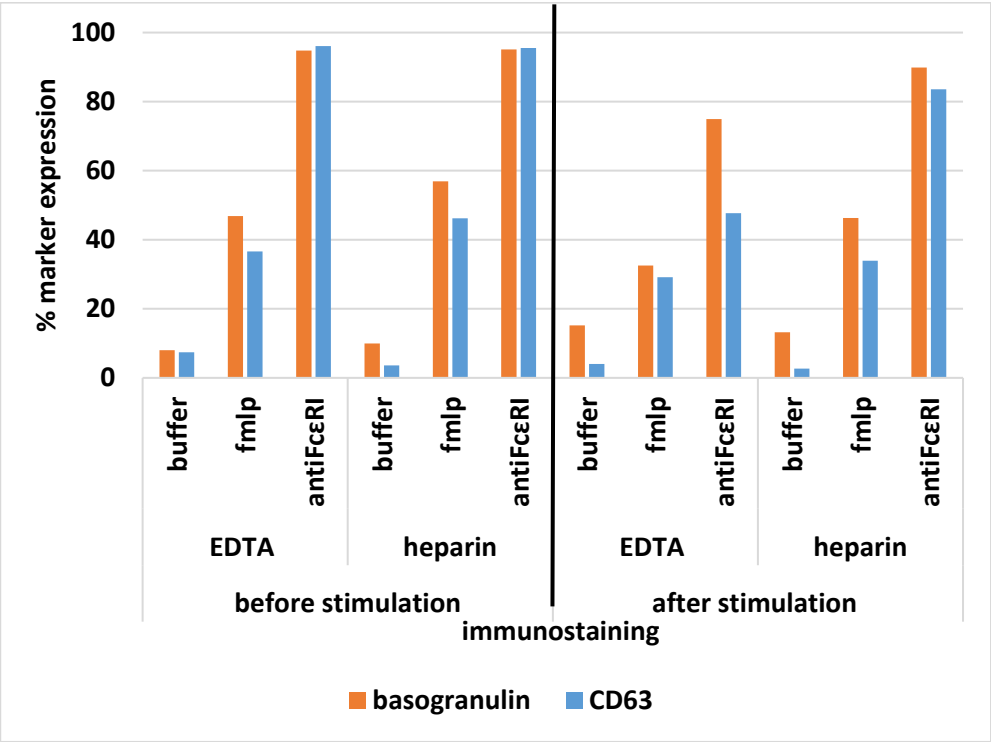


Figure 3-28. Anticoagulants and the effect of timing of immunostaining on basogranulin and CD63 expression. Basophils in blood which was anticoagulated with EDTA or heparin were stimulated with fmlp, anti-FcεRI, or buffer alone. Basophils were stained with specific labelled antibodies before or after the stimulation. The percentage of basogranulin, CD63, and both (basogranulin and CD63) positive basophils was analysed by flow cytometry.

3.4.1.3 Differential expression of basogranulin and CD63 in activated basophils

In this section, the nature of differential basogranulin and CD63 expression in basophils will be described in non-stimulated basophils and in basophils following stimulation with anti-IgE, fmlp or anti-FcεRI.

Anti-IgE stimulation

More than 90% of unstimulated basophils expressed neither basogranulin nor CD63 whereas only 3% of basophils expressed basogranulin alone, and 3 to 5% expressed CD63, while some 2 to 3% of basophils expressed both basogranulin and CD63 (Figure 3-29). However, stimulating basophils with anti-IgE (0.001 ug/ml)resulted in expression of basogranulin and CD63. Yet, about 49% basophils expressed both markers whereas 33% expressed CD63 alone and 1% expressed basogranulin alone. However, 17% of basophils expressed neither basogranulin or CD63. Increasing the concentration of anti-IgE 10-fold (0.01 µg/ml) increased the percentage of basophils that were positive for both markers to 67% whereas the percentage of CD63 positive basophils slightly decreased to 29%. Basogranulin positive basophils remained unchanged at 1%. However, basophils which were negative for both markers decreased to 3%. Further stimulation of basophils with higher concentration of anti-IgE (0.1 -1 µg/ml) resulted in nearly 70% of basophils expressed both basogranulin and CD63 and 27% expressing CD63 alone. However, no basophils positive for basogranulin were detected. Nonetheless, the percentage of double negative marker basophils were equal or less than 5%. Interestingly, stimulating basophils with anti-IgE 10 µg/ml had increased the percentage of double positive basophils to 91% whereas basophils stained with CD63 alone was 9%. No basophils stained for basogranulin alone. Also, double negative basophils were not detected. Nevertheless, increasing the concentration of anti-IgE to 100 µg/ml led to a decrease in the percentage of double positive basophils to 50%. The percentage of basophils which expressed CD63 was 30%. The percentage of double negative basophils increased to 19%. The percentage of basophils which expressed basogranulin was 1%. The percentages of differential marker expression at this dose is similar with anti-IgE at 0.001 µg/ml.

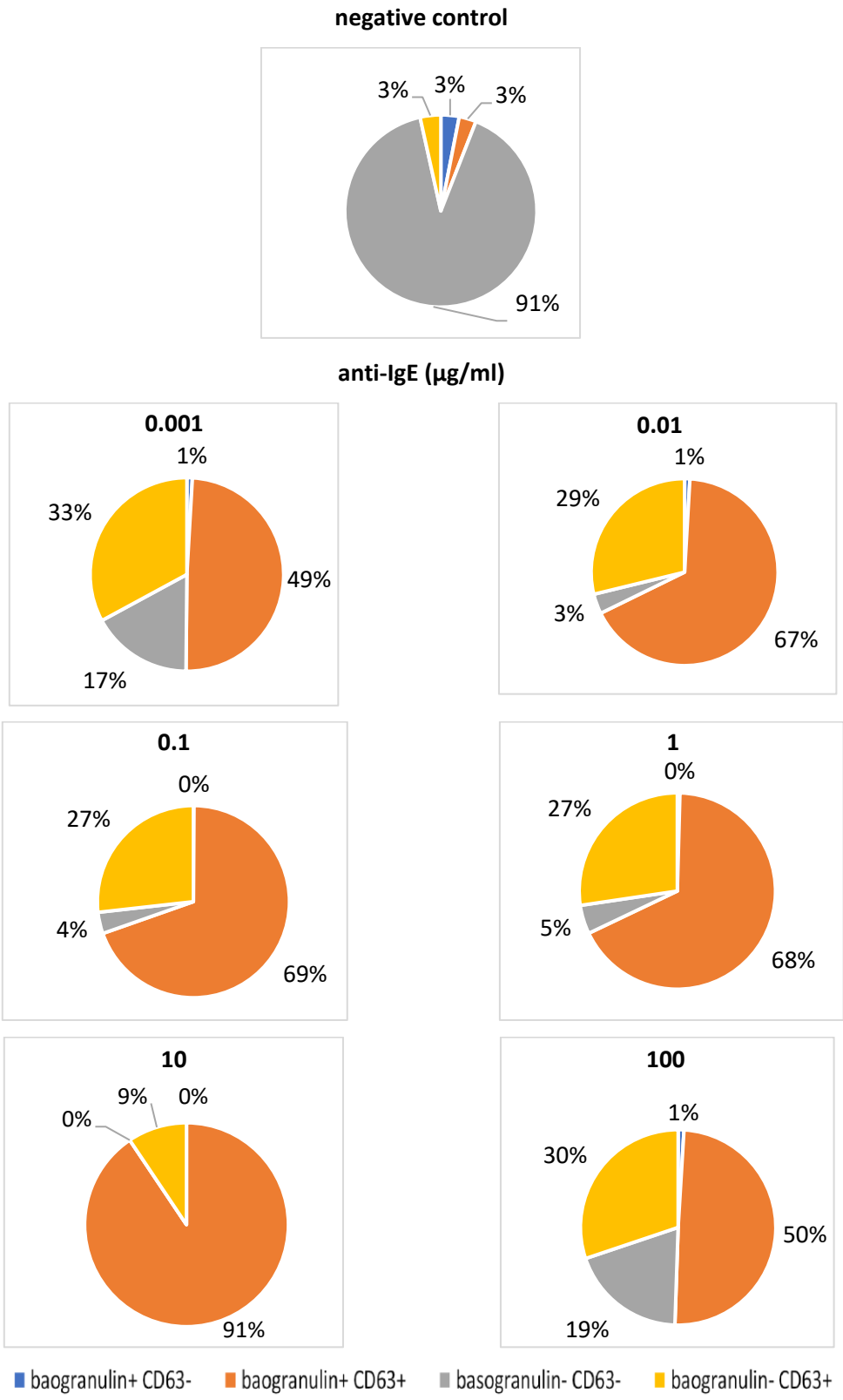

negative control
3%
3%
3%
91%
anti-IgE (µg/ml)
0.001
1%
33%
49%
17%
0.01
1%
29%
3%
67%
0.1
0%
27%
4%
69%
1
0%
27%
5%
68%
10
9%
0%
0%
91%
100
1%
30%
50%
19%
baogranulin+ CD63-
baogranulin+ CD63+
basogranulin- CD63-
baogranulin- CD63+

Figure 3-29. Differential expression of basogranulin and CD63 in unstimulated basophils and in basophils stimulated with various concentrations of anti-IgE. The percentage was counted for basophils that expressed either basogranulin (basogranulin + CD63-) or CD63 (basogranulin- CD63+), or neither (basogranulin- CD63-), or both (basogranulin+ CD63+), out of the population of basophils stimulated with anti-IgE or buffer alone.

Fmlp or anti-FcεRI stimulation

Differential expression of basogranulin and CD63 in basophils from five donors in response to stimulation with fmlp or anti-FcεRI is presented in Figure 3-30. As described for the donors reported above, incubating basophils in buffer alone was not associated with increased expression of basogranulin on the surface of basophils. The percentage of double negative basophils ranged from 86 to 98%. Double positive basophils were barely detected, representing 1% or less. Similarly, the percentage of CD63 positive basophils was 1%. However, percentage of basogranulin positive basophils varied from 1 to 13% in unstimulated basophils.

Stimulating basophils with fmlp resulted in different expression of basogranulin and CD63 on activated basophils (Figure 3-30, middle panel). About 50 to 70% of basophils were double negative. However, fmlp stimulation resulted in increase in double positive marker basophil population with maximum of 40% observed in subject 4. Coincidently, the maximum percentage of basogranulin positive basophils was observed at the same subject by 18%. Also, the percentage of CD63 positive basophils varied from a subject to another (3 to 18%) with maximum of 18% observed in subject 5.

Stimulation with anti-FcεRI resulted in an array of marker expression (Figure 3-30, right panel). The percentage of double positive basophils varied from 27 to 93%). Basogranulin positive basophils varied between 1 to 27%. Similarly, CD63 positive basophils varied between 2 to 14%. The percentage of double negative basophils varied between 3 and 50%.

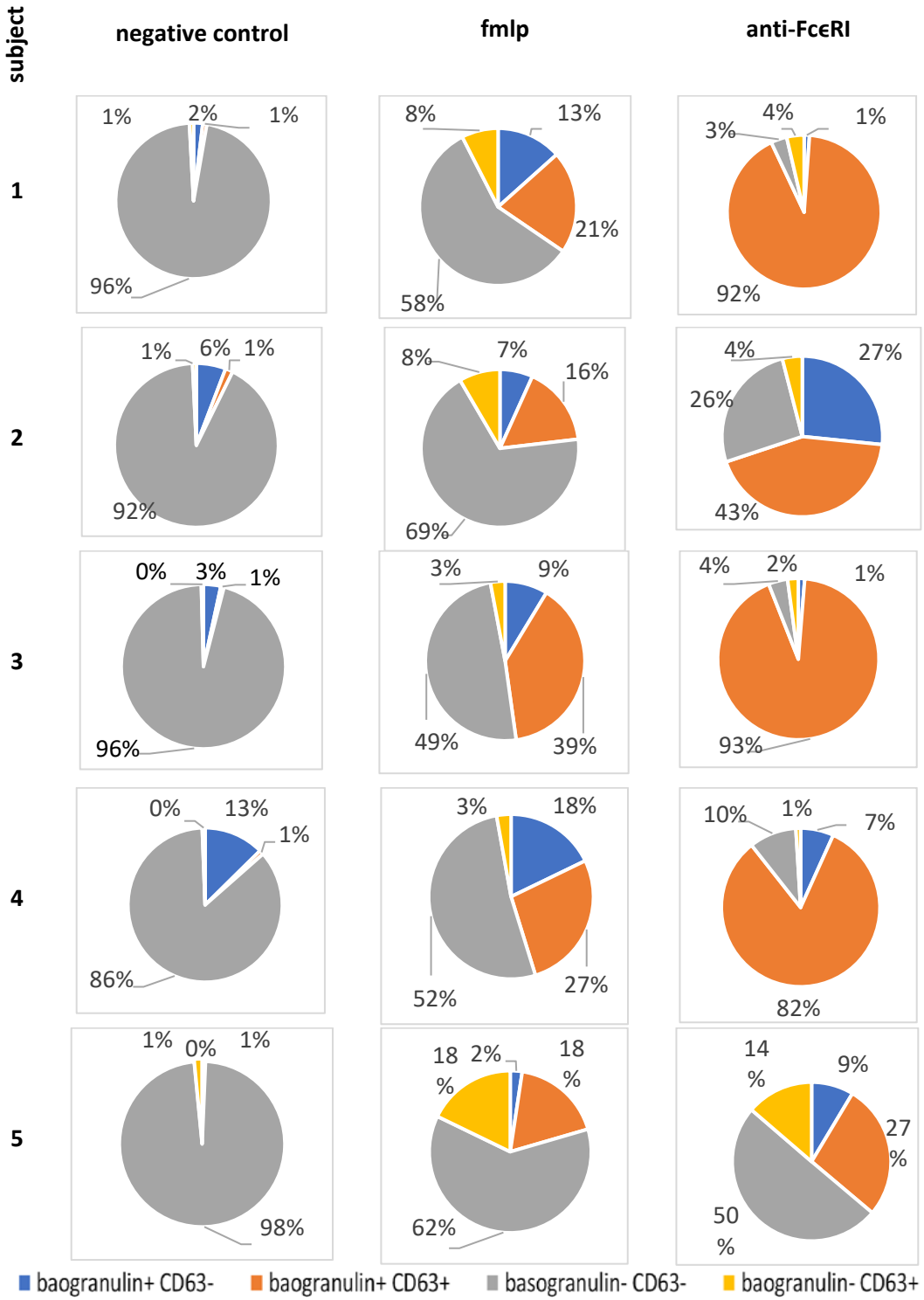


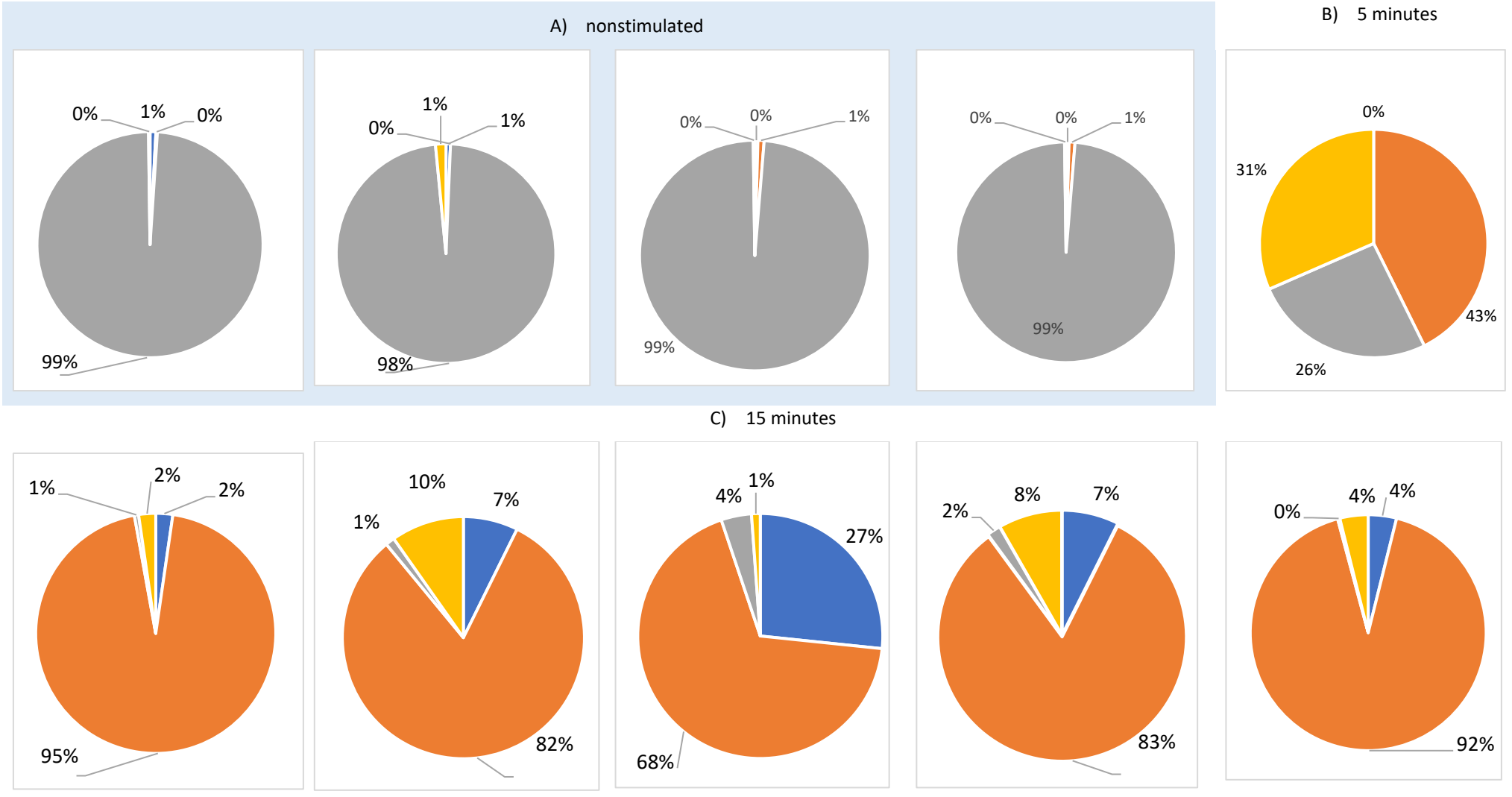
Figure 3-30. Differential expression of basogranulin and CD63 in basophils stimulated with fmlp or anti-FcεRI. The percentage was counted for basophils that expressed either basogranulin (basogranulin+ CD63-) or CD63 (basogranulin- CD63+), or neither (basogranulin- CD63), or both (basogranulin+ CD63+), out of the population of basophils stimulated with fmlp, anti-FcεRI or buffer alone (n=5 subjects).

Effect of activation time on expression of basogranulin and CD63

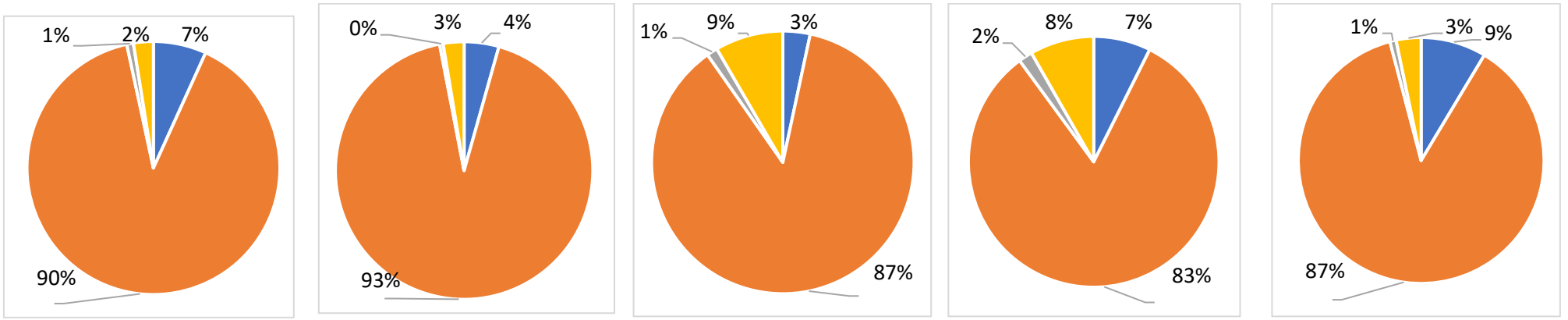
The time course for basogranulin and CD63 expression in basophils stimulated with anti-FcεRI as analysed by flow cytometry is indicated in Figure 3-31. Incubating basophils in buffer with or without calcium, magnesium and IL-3 for 20 minutes resulted in no increase in expression of basogranulin or CD63 where majority of basophils (98 to 99%) were double negative. However, stimulating basophils with anti-FcεRI for five minutes (n=1) induced expression of basogranulin and CD63 where 43% of basophils was double positive. A percentage of 31% of basophils were expressed CD63 alone and no basophils expressed basogranulin alone. However, 26% of basophils was double negative.

Extending the activation time by incubating basophils with anti-FcεRI for fifteen minutes led to increases in expression of basogranulin and CD63 (n=3) though variations were observed among subjects. The percentage of double positive basophils were 82 to 95%. Basophil which expressed basogranulin alone varied between 2 to 7% while 2 – 10% expressed CD63 alone. Only 1% of basophils did not express basogranulin and CD63 simultaneously (double negative).

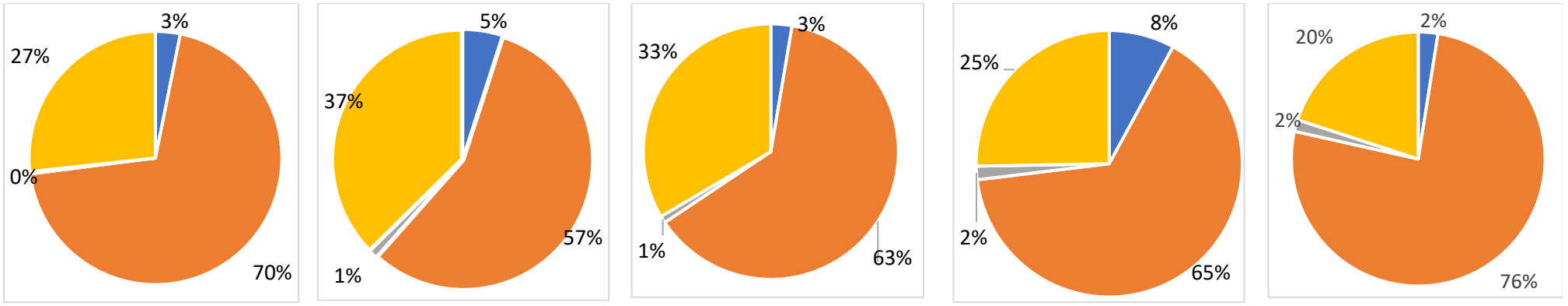
Doubling activation time to thirty minutes exhibited some alterations in expression of basogranulin and CD63. In general, the percentage of double positive basophils increased in some experiments which showed lower expression of these markers in a previous time point. However, other experiments showed a lower expression of basogranulin and CD63 than that of 15 activation minutes. The decrease in maker expression continued over time and shown to be much more in that of 60 minutes activation time though it did not return to the baseline expression. In the other hand, basophils expressing CD63 alone were increased but not those with basogranulin alone.


A) nonstimulated
0%
1%
0%
99%
1%
0%
1%
98%
0%
0%
1%
99%
0%
0%
1%
99%
B) 5 minutes
0%
31%
43%
26%
C) 15 minutes
1%
2%
2%
95%
10%
7%
1%
82%
4%
1%
27%
68%
2%
8%
7%
83%
0%
4%
4%
92%

D) 30 minutes



E) 60 minutes



baogranulin+ CD63- baogranulin+ CD63+ basogranulin- CD63- baogranulin- CD63+

Figure 3-31. Time-course expression of basogranulin, CD63 in basophils in response to stimulation with anti-FcεRI.
Basophils was stimulated with anti-FcεRI for 5, 15, 30 and 60 minutes. Nonstimulated cells (negative control) were incubated with buffer alone. The percentage was counted for basophils that expressed either basogranulin (basogranulin + CD63-) or CD63 (basogranulin- CD63+), or neither (basogranulin- CD63-), or both (basogranulin+ CD63+), out of the population of gated basophils. Data was analysed by flow cytometry with anti-CD63-FITC and BB1-APC antibodies in whole blood specimens from five anonymous donors.

Mean values for differential expression of basogranulin and CD63

Relative mean differential expression levels for basogranulin and CD63 in non-stimulated basophils and basophils stimulated with fmlp or anti-FcєRI is presented for five subjects in Figure 3-32. The mean of double positive basophils in nonstimulated basophils was less than 1% whereas 93% were double negative basophils (top panel). However, the mean of basogranulin positive basophils was only 5% whereas the mean of CD63 positive basophils represented 1%. A mean of 10% of basophils induced by fmlp was basogranulin positive basophils and 8% was CD63 positive (middle panel). Double positive basophils represented 24% of basophils. Nonetheless, 58% of basophils was double negative. A mean of 9% of basophils induced by anti-FcєRI was basogranulin positive basophils, and 5% of was CD63 positive (bottom panel). However, the mean of double positive basophils was 67% whereas 19% of basophils was double negative meaning that basophils did neither express basogranulin nor CD63.

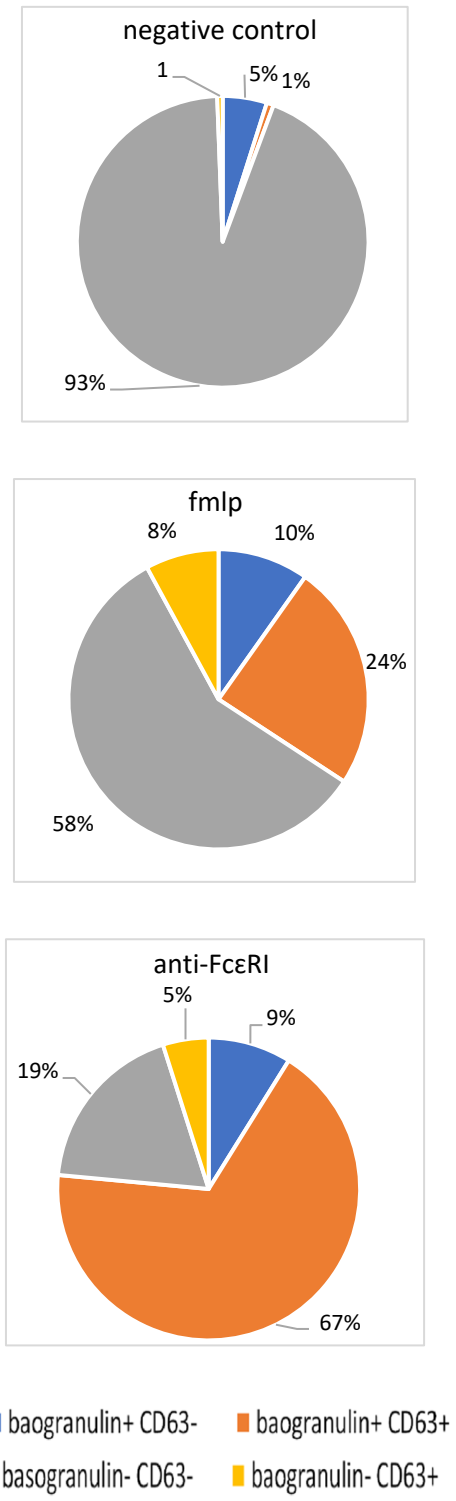


Figure 3-32. Relative numbers of basophils expressing both basogranulin and CD63, or just one of these markers alone or neither in non-stimulated basophils and basophils stimulated with fmlp or anti-FcεRI in whole blood. Data was analysed by flow cytometry from five anonymous donors.

3.5 Measuring CCR3 expression

As the expression of CCR3 was occasionally found to decrease or even disappear altogether following basophil activation. The effects of basophil activation on this marker was sought to be investigated under different conditions. Stimulating basophil with various concentrations of anti-IgE resulted in some changes in the MFI of CCR3 though those changes were inconsistent. But were affected by the addition of IL-3 as well as the stimulation with anti-FcεRI (Figure 3-34). Priming basophils with IL-3 (5-200 ng/ml) for 5 minutes resulted in a bell-shape like concentration dependent response of CCR3 MFI (Figure 3-34 A). Prolonging the period of priming for 20 minutes was associated with an almost doubling of the MFI with a response curve similar to that at 5-minutes response curve. Stimulating basophils with anti-FcεRI simultaneously with various concentrations of IL-3 for 5 minutes resulted in reduction in the CCR3 MFI with an inverse concentration response(Figure 3-34 B). However, further reduction of CCR3 MFI was associated with prolonging stimulation time for 20 minutes though the overall pattern of response curve remained almost the same of that with 5-minute response.

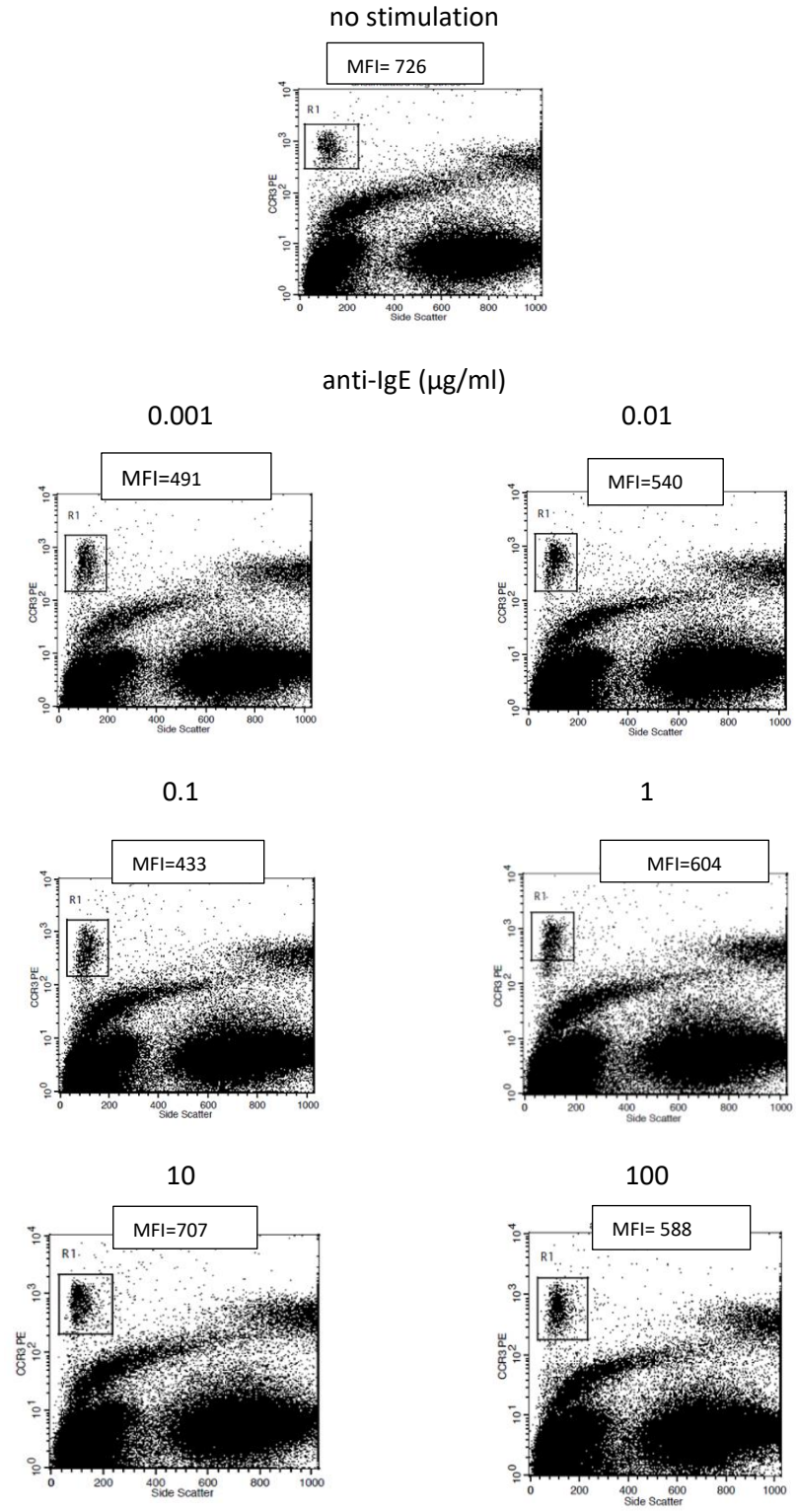


Figure 3-33. MFI of CCR3 in basophils stimulated with anti-IgE. Representative data of dot plots of MFI of CCR3 expression in basophils, at baseline (no stimulation), and with increasing concentration of anti-IgE (0.001- 100 µg/ml).

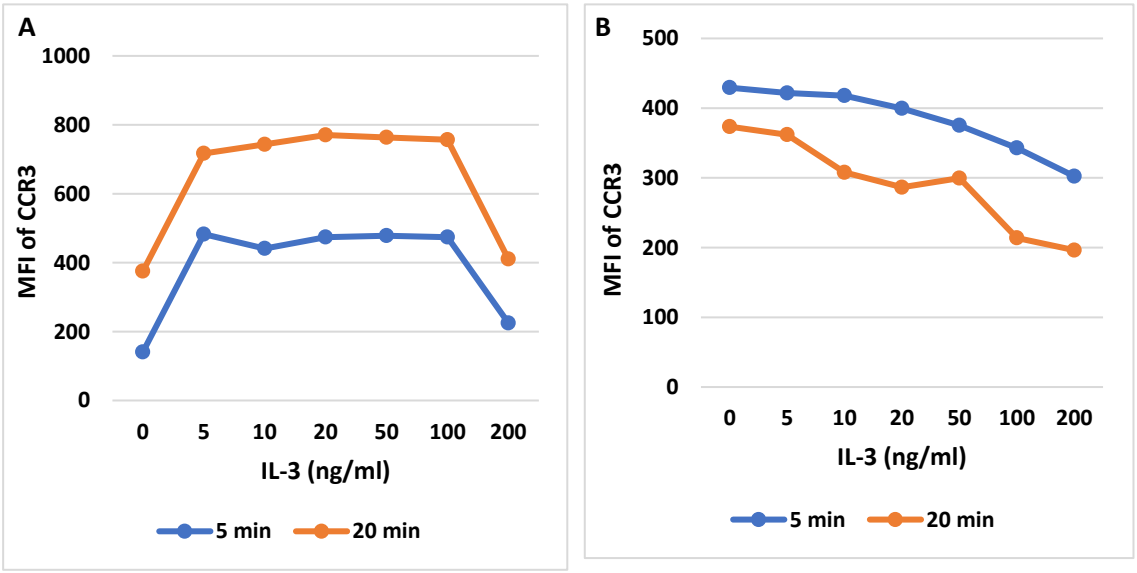


Figure 3-34. MFI of CCR3 in response to priming of basophils with IL-3 in the presence or absence of anti-FcεRI. Basophils in whole blood were incubated with increasing concentration of IL-3 (5-200 ng/ml) (A) without or (B) with anti-FcεRI at 37 ^{o}C for 5 or 20 minutes. Buffer without Il-3 was used for the basal line of basophil activation. Data was acquired by flow cytometry and analysed by CellQuest software.

3.6 Cellular localisation of basogranulin following basophil activation

Following the detection of basogranulin in LAD2 cell lysates, confocal microscopy studies were performed to investigate basogranulin expression in these cells. A small quantity of basogranulin was visualised but only on some cells (Figure 6-3 A).The subcellular distribution of basogranulin was analysed by confocal microscopy in response to simulation with anti-FcεRI or fmlp over various time points. Intracellular and surface expression were visualised in permeabilised and nonpermeabilised basophils respectively. In addition, CCR3 expression was monitored and colocalised with basogranulin. There was high expression of CCR3 in most basophils though variation between cells was observed. Staining of basophils with the irrelevant isotype matched control antibody was negative (Figure 6-3 B).

3.6.1 Intracellular basogranulin expression

When expression of intracellular basogranulin was analysed by confocal microscopy in permeabilised basophils from healthy subjects, it was found to decrease in response to stimulation in most of cases. Nevertheless, some basophils appeared to retain a large amount of basogranulin even after stimulation. We also noticed heterogeneity in the amount and the

distribution of basogranulin among stimulated basophils. Of the basophils stimulated with anti-FcεRI for 5 minutes, many had prominent staining of intracellular basogranulin within granules as well as some on the cell membrane (Figure 3-35). Some basophils appeared to exhibit a lower level of intracellular basogranulin expression suggesting depletion of intracellular basogranulin stores. Prolonging the time of stimulation to 10 minutes was associated with an apparent reduction in intracellular basogranulin expression (Figure 3-35 B). However, surface expression of basogranulin was also exhibited in some basophils. Further, stimulation basophils for 20 and 30 minutes showed a low expression of intracellular basogranulin whilst surface basogranulin was clearly visualised surrounding the cells.

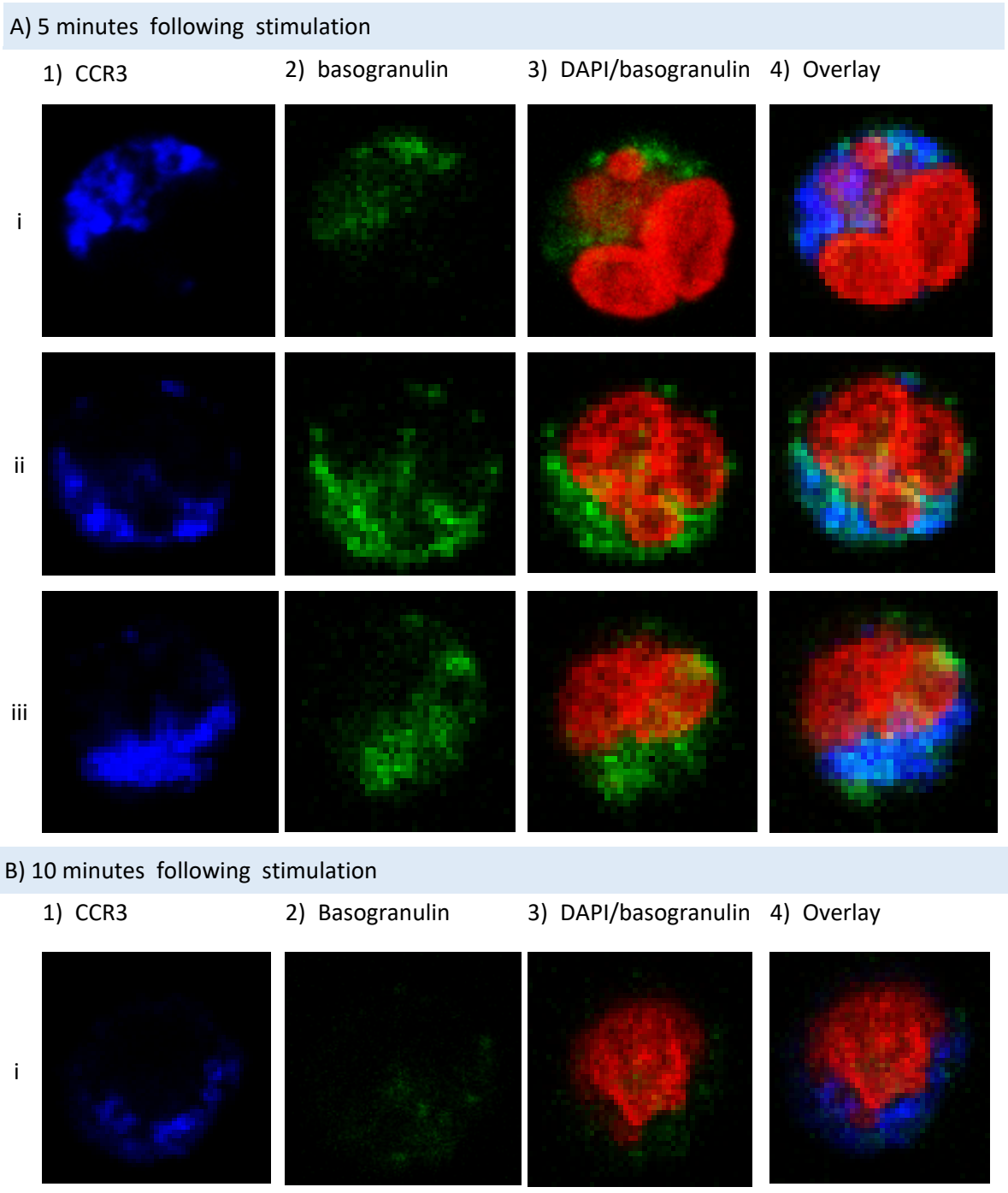
Fmlp stimulated basophils exhibited intracellular basogranulin and that expression was colocalised with CCR3 (Figure 3-36). Stimulating basophils with fmlp for 5 minutes initiated expression of basogranulin over all the cell. However, there has been disparity in expression of basogranulin in basophils stimulated for 20 minutes where some basophils seemed to retain high expression of intracellular as well as surface basogranulin whereas others exhibited low expression of basogranulin. Basogranulin colocalised with CCR3 and the best apparent example was shown in fmlp-stimulated basophils for 20 minutes (Figure 3-36 B).

3.6.2 Surface basogranulin expression

Findings of confocal microscopy confirmed our findings by confocal microscopy in that surface basogranulin is not expressed on unstimulated basophils, though it was upregulated upon stimulation. Stimulating basophils with anti-FcεRI for five minutes induced expression of basogranulin on the surface though there were quite different degrees of expression. Some basophils showed strong expression of basogranulin on over the whole surface area, though it appeared also within granules near the surface of activated basophils. However, some other cells showed weak expression of basogranulin, and others intermediate expression of surface basogranulin with substantial variation. CCR3 expression was high in all of these cells. After stimulation with anti-FcεRI, basogranulin was occasionally localised to a cap at one pole of the cell.

Expression of basogranulin was altered in basophils to which fmlp was added for 5 or 20 minutes (Figure 3-38). Stimulating basophils with fmlp for 5 minutes initiated expression of surface basogranulin (Figure 3-38 A). However, basogranulin expression in basophils induced by fmlp for 5 minutes was slightly lower than that for anti-FcεRI for the same period of

stimulation, consistent with expression patterns indicated by flow cytometry (*vide supra*). The degree of basogranulin expression exhibited by confocal microscopy was highly variable between basophils (i, ii, iii), though stimulating basophils with fmlp for 20 minutes led to strong expression of surface basogranulin on the surface (Figure 3-38 B).



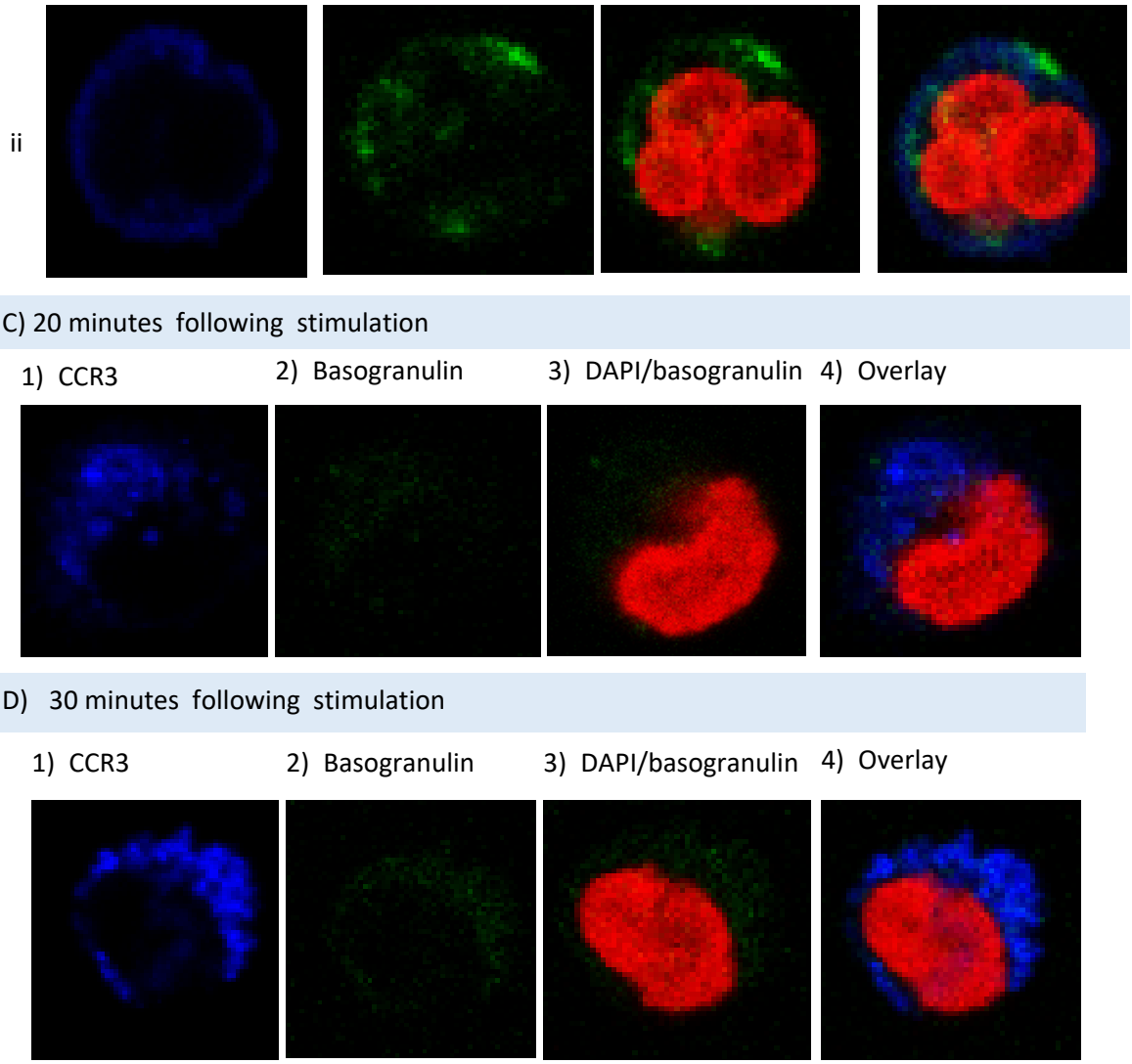


Figure 3-35. Intracellular basogranulin expression in basophils following activation with anti-FcεRI at 5, 10, 20 and 30 minutes . Following stimulation, basophils were permeabilised prior to immunostaining with the antibodies against basogranulin (green) and CCR3 (blue). Nucleus were stained with DAPI (red). Images were captured using Leica TCS-SP8 confocal microscope with x63 glycerol objective lens

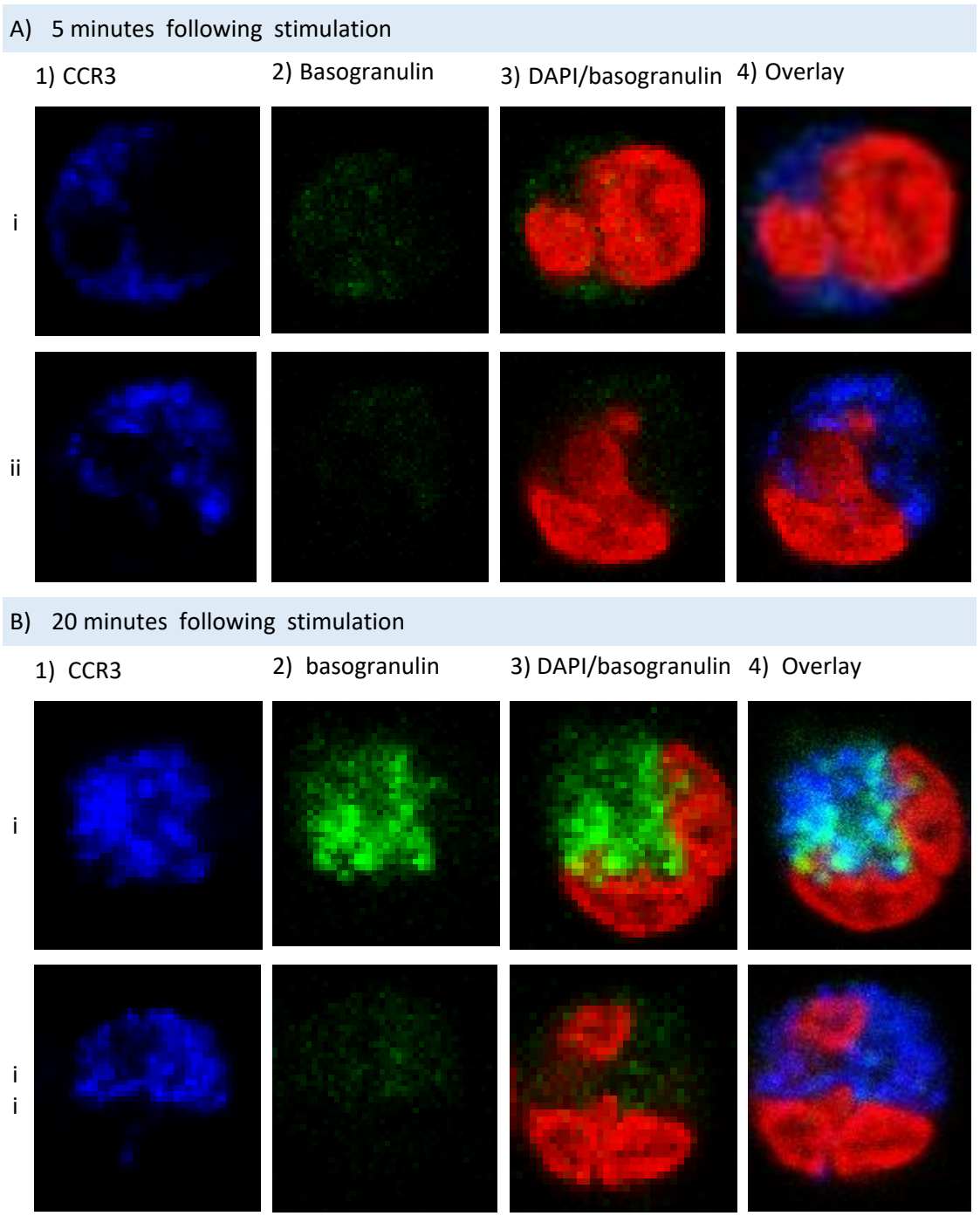
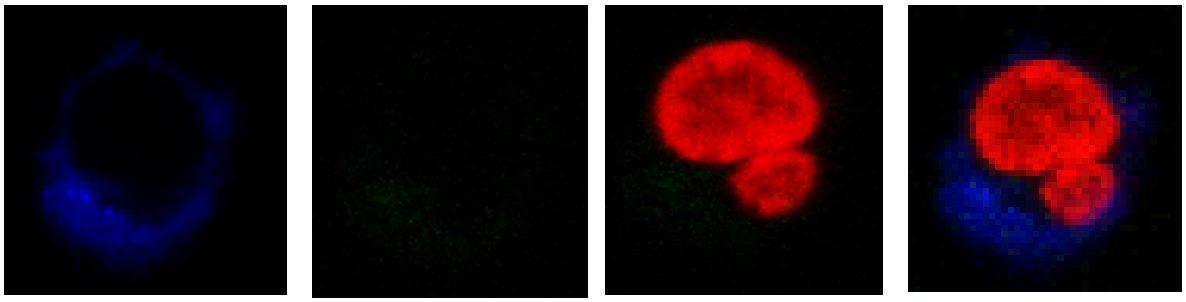


Figure 3-36. Confocal microscopy of intracellular basogranulin in basophils stimulated with fmlp at 5, 10, 20 and 30 minutes. Following stimulation, basophils were permeabilised prior to immunostaining with the antibodies against basogranulin (green) and CCR3 (blue). Nucleus was stained with DAPI (red).

A) no stimulation

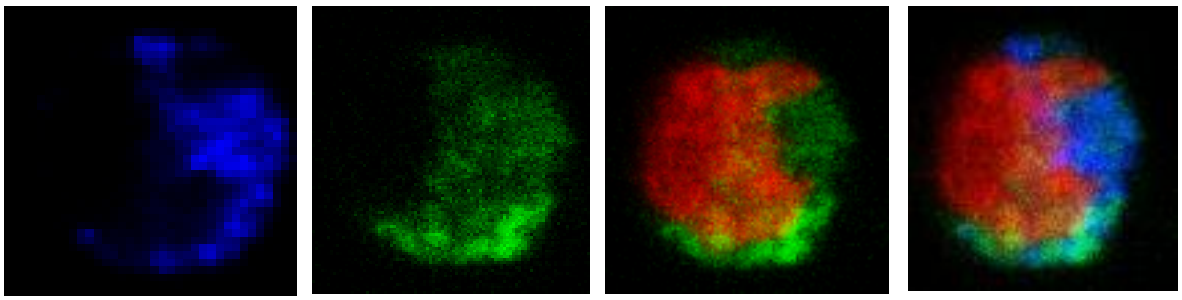
1) CCR3 2) Basogranulin 3) DAPI/basogranulin 4) Overlay



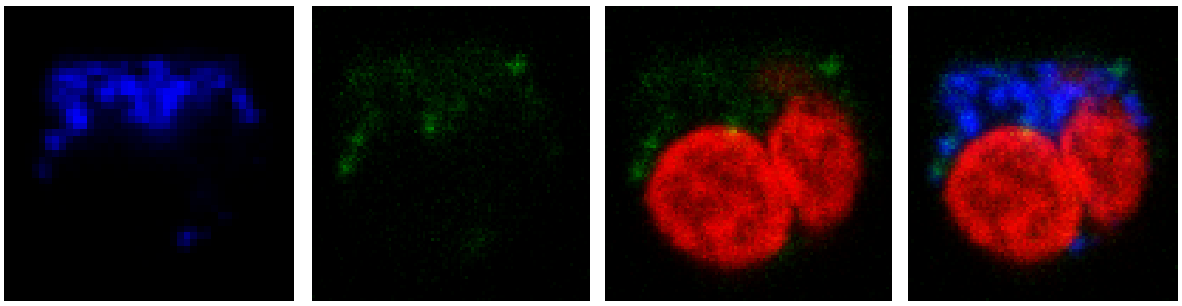
B) 5 minutes following stimulation

1) CCR3 2) Basogranulin 3) DAPI/basogranulin 4) Overlay

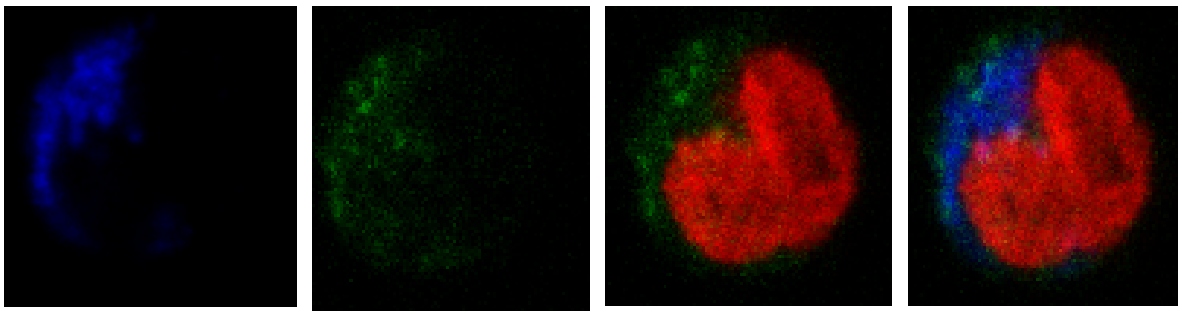
i



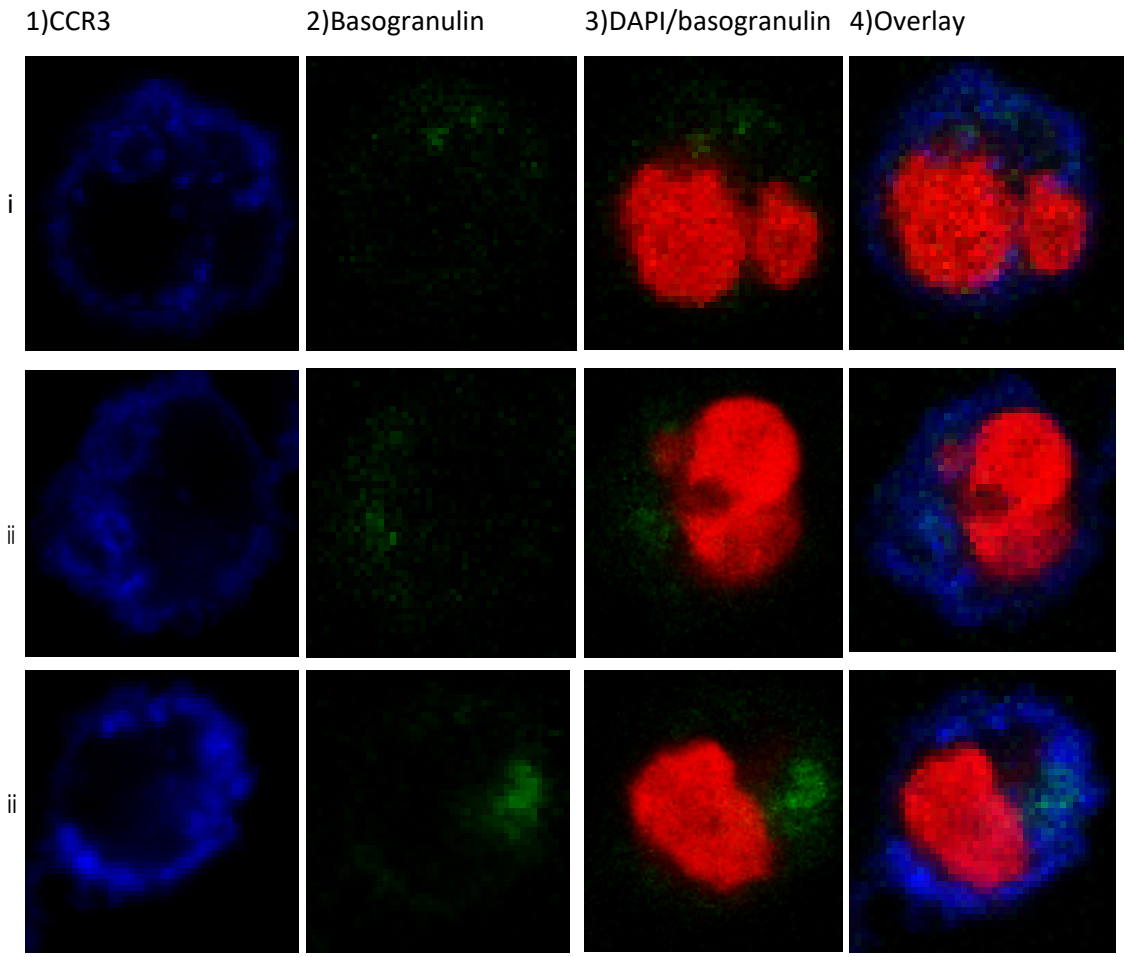
ii



iii



C) 20 minutes following stimulation



D) 30 minutes following stimulation

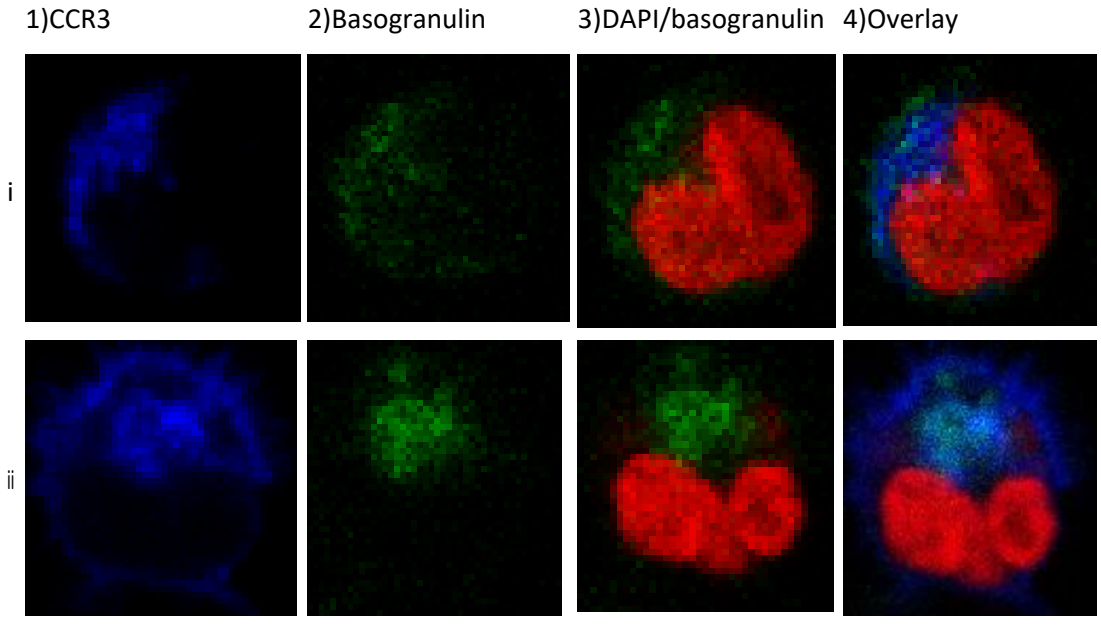


Figure 3-37. Surface expression of basogranulin in basophils stimulated with anti-FcεRI as determined by confocal microscopy. Representative confocal images of blood basophils stimulated with anti-FcεRI for (A) 5, (B) 10 min, (C) 20 minutes or (D) 30 minutes. Following stimulation, basophils were fixed and labelled with fluorescently conjugated BB1 (green), CCR3 (blue) and nuclei stained with DAPI (red). Images are shown for (1) CCR3 alone, (2) basogranulin alone, (3) DAPI and basogranulin, or (4) overlay. Images of several basophils (i, ii, iii …etc) are presented for every treatment where possible. Isotype controls for BB1, and anti-CCR3 antibodies are presented in the Appendix section.

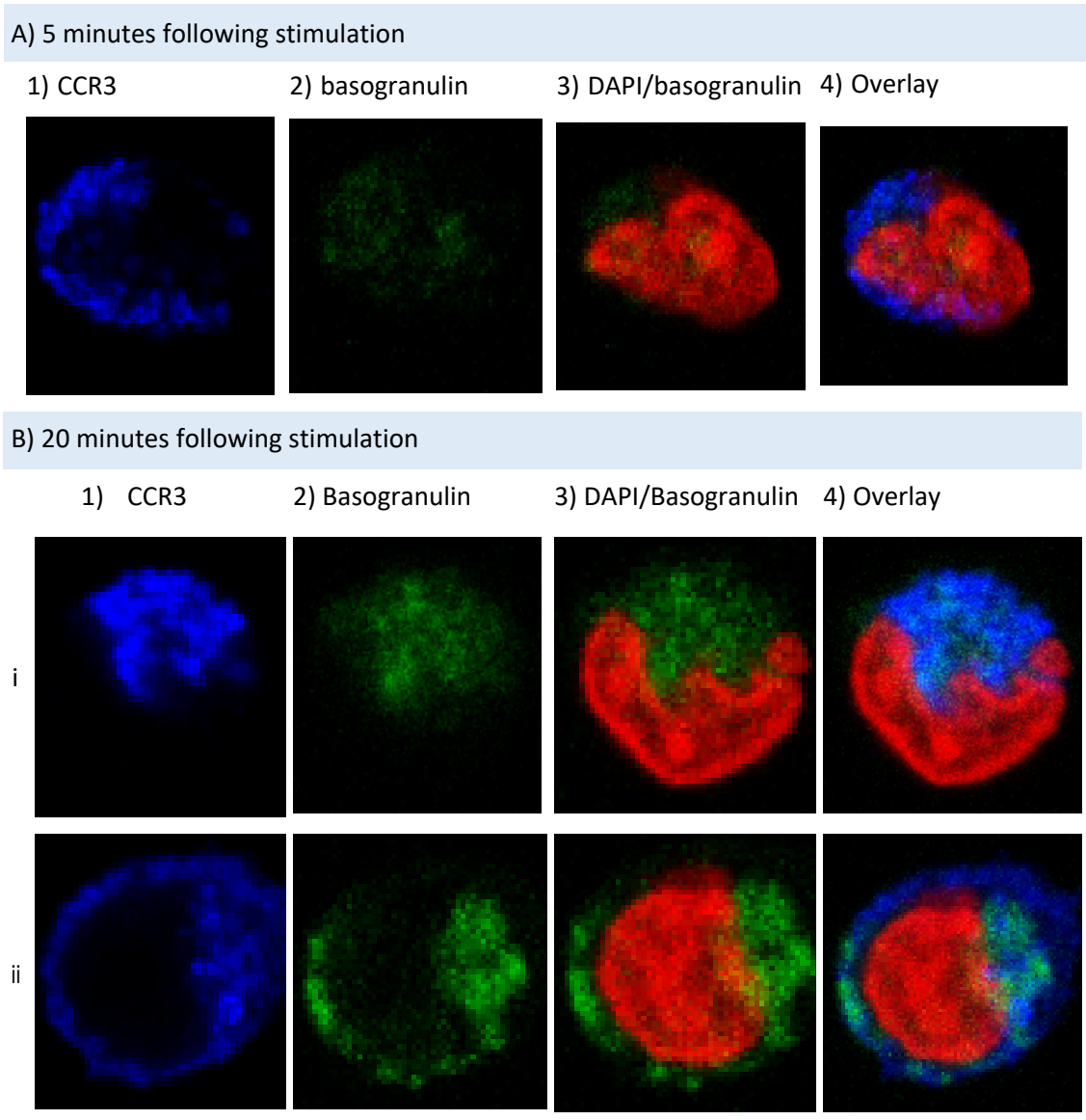


Figure 3-38. Surface expression of basogranulin in basophils stimulated with fmlp as determined by confocal microscopy. Representative confocal images of blood basophils stimulated with fmlp for (A) 5 or (B) 20 minutes. Following stimulation, basophils were fixed and labelled as described above. Basogranulin (green), CCR3 (blue) and nucleus stained with DAPI (red).

Chapter 4 Application of the newly developed basogranulin-based assays for assessing allergic sensitivity

In this chapter, are presented the results obtained when the newly developed basogranulin-based assays were applied to assess specific sensitivity to various allergens. This involved measurements of (a) basogranulin release from basophils using dot blotting, (b) alterations in intracellular basogranulin stores and (c) surface expression of basogranulin and CD63 in response to stimulation *in vitro* with various allergens.

4.1 Basogranulin release following basophil activation in response to stimulation with allergen

4.1.1 Measuring basogranulin release in supernatants from purified basophils

Basogranulin release was successfully measured by dot blotting in supernatants of purified basophils obtained from peripheral blood. Basogranulin release from four house-dust allergic subjects were measured by dot blotting (Figure 4-1). *D. pteronyssinus* stimulated basogranulin release from basophils of house dust sensitive subjects though concentrations determined differed between subjects. Challenging basophils with *D. pteronyssinus* resulted in increased release in the majority of subjects, and a typical bell-shaped curve was seen only in one subject over the concentrations of allergen investigated. The maximal basogranulin release was observed at a concentration of 0.1% of anti-IgE with about 35% of the total basogranulin content. The basogranulin response curve shifted to the right upon activation by *D. pteronyssinus*, with maximal release of about 32 % at 0.3 SQ-U/ml.

Basogranulin release from subject 2 showed a dose response at both anti-IgE and *D. pteronyssinus* treatments. The peak basogranulin release (28%) and (44%) was measured at 10% anti-IgE and 1 SQ-U/ml of *D. pteronyssinus*. Basogranulin release in subjects 3 displayed variable response upon anti-IgE stimulation. The maximum release was less than 12% at concentration of 10% of anti-IgE. A low release was observed upon stimulation with the first four concentrations of *D. pteronyssinus* (0.0001-1 SQ-U/ml). Although, release of basogranulin had increased upon subsequent higher concentration doses of *D. pteronyssinus*, maximal release was only about 16% of the total when stimulated with 1000 SQ-U/ml of *D.*

pteronyssinus. Unexpectedly, spontaneous release in this subject appeared higher than that stimulated with *D. pteronyssinus* (0.01 and 0.1 SQ-U/ml), and this may be due to variability in dot blotting.

In addition, basogranulin release was measured in basophil supernatants following activation with grass pollen (Figure 4-2). Spontaneous release was relatively low (7%). However, the release was greater after stimulation, with maximal net release (19%) at a grass pollen concentration of 1 SBU (Figure 4-2 A). In another donor, spontaneous release was high (17%) and the net release of basogranulin after stimulation was much higher when maximal release (55%) was at a grass pollen concentration of 0.1 SBU (Figure 4-2 B).

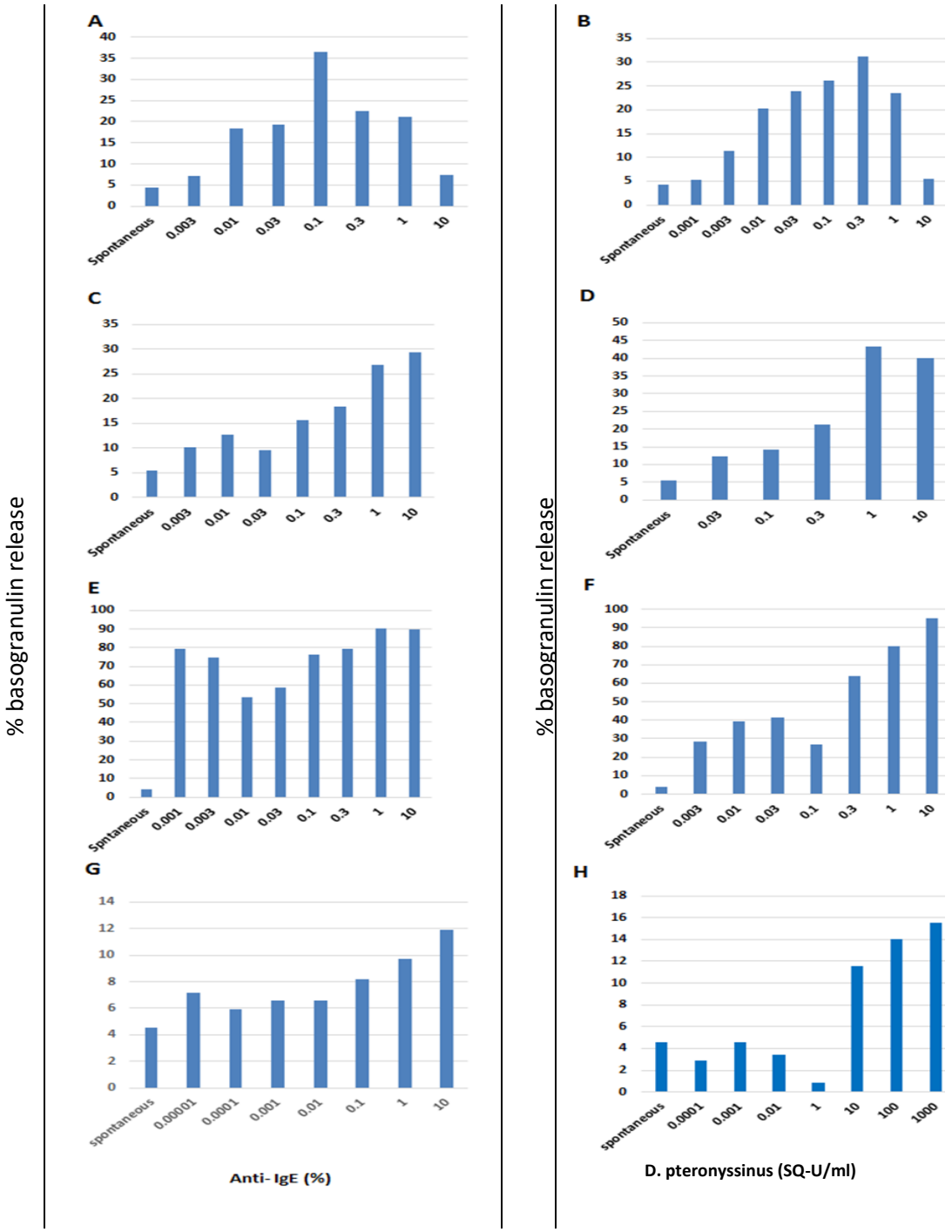


Figure 4-1. Basogranulin release from purified basophils. Basogranulin was measured in supernatant of purified basophils (n= 4) induced by anti-IgE (A, C, E and G) or *D. pteronyssinus* (B, D, F and H) using BB1. Subject 1 (A and B), 2 (C and D), 3 (E and F) and 4 (G and H). Spontaneous release was that from unstimulated basophils release. Vertical axis indicates the percentage of basogranulin release of total cell content. Mean of triplicate determinations are shown.

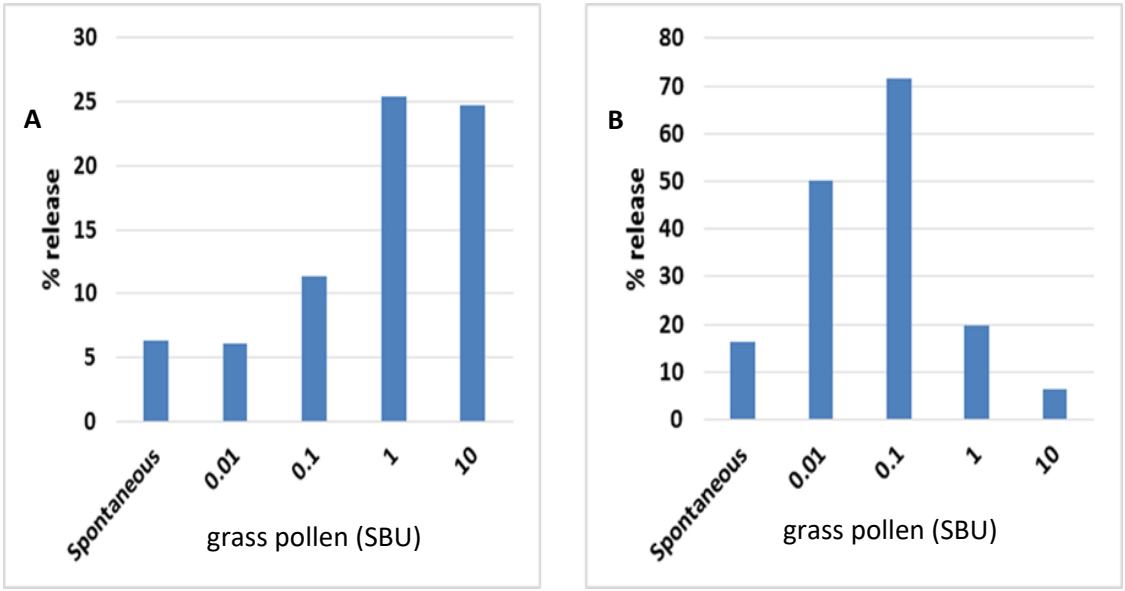


Figure 4-2. Basogranulin release from purified basophils induced by grass pollen allergen using dot blotting. Purified basophils from two subjects were simulated by grass pollen at concentration of 0.01-10 SBU. Spontaneous release indicates that from nonstimulated basophils. Data was calculated against the percentage of total content basogranulin in a lysate of the cells. Results of triplicate determinations are shown.

4.2 Measuring intracellular basogranulin by flow cytometric assay

Flow Cytometry was applied to measure the depletion of intracellular basogranulin stores in activated basophils. Stimulating basophils with grass pollen resulted in a concentration dependent depletion of intracellular basogranulin expression. The maximal intracellular basogranulin depletion was about 24% with a grass pollen concentration of 10 SBU (Figure 4-3 A). In contrast, surface CD63 expression increased in a concentration dependent manner of up to 95% at 10 SBU. Non-stimulated basophils exhibited high expression of intracellular basogranulin (95%) and low CD63 expression (0.5%).

Subject 2 showed maximal depletion of intracellular basogranulin at 10 SBU and the depletion continued afterward at 100 and 1000 SBU (Figure 4-3 B). However, intracellular basogranulin appeared to gradually increase though it did not return to the those basogranulin levels seen in non-stimulated basophils. On the other hand, surface CD63 expression increased in concentration dependent manner and exhibited a bell-shaped curve with a maximum expression of 45% at 100 SBU. Overall, the depletion of intracellular basogranulin was inversely associated with the increase in surface CD63 expression.

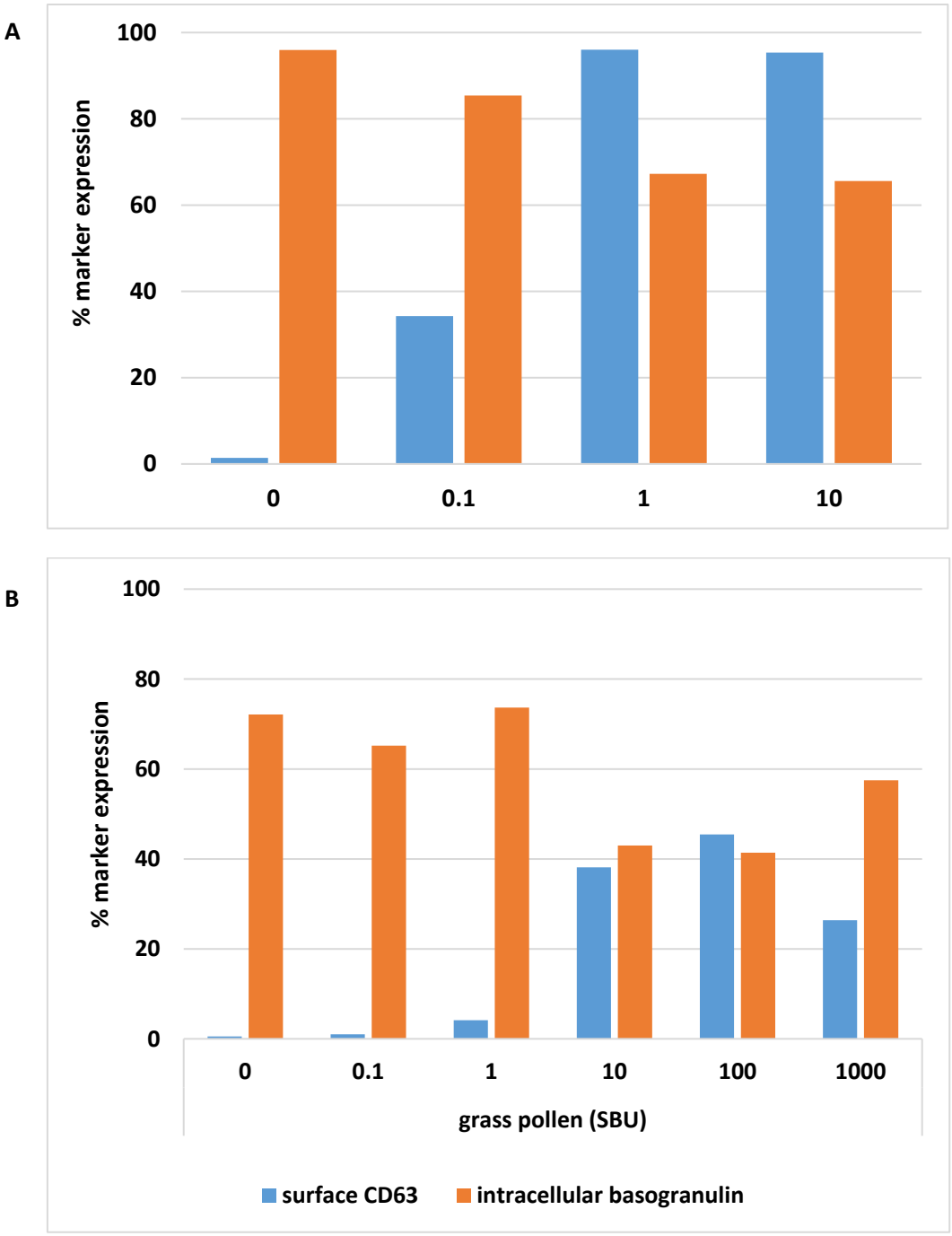


Figure 4-3. The depletion of intracellular basogranulin expression in basophils stimulated with grass pollen. Basophils in whole blood from two subjects were stimulated with grass pollen 0.1-1000 (SBU), and stained with CD63-specific antibody and BB1 coupled to fluorochromes. Data was acquired by flow cytometer and analysed by CellQuest software.

4.3 Measuring expression of surface basogranulin in response to stimulation with specific allergen

The newly developed flow cytometric assay was applied for measuring surface basogranulin in basophils of allergic patients in response to stimulation with various specific allergens. This involved grass pollen, dust mite and numerous food allergens. Overall, basogranulin and CD63 were barely expressed on nonstimulated basophils and increased following activation. However, after stimulation, basogranulin and CD63 were increased though the variable (Figure 4-4, and Figure 4-5). Basogranulin expression appeared to be one to two fold more than that for CD63 upon stimulation with fmlp, anti-FcεRI and various concentrations of grass pollen. A low expression of both CD63 (1.1%) and BB1 (2.3%) was observed in nonstimulated basophils. The expression of CD63 was shown to be dose dependent with an optimal expression (16%) at fmlp µM. Surface basogranulin was expressed in a concentration-dependent manner with maximal expression (22%) observed at fmlp concentration of 10 µM (Figure 4-5 1A).

Stimulating the basophils with anti-FcεRI induced low expression of CD63 (4%) and basogranulin (7%) at a low level. Furthermore, both CD63 (mean 1.5%) and BB1 (mean 2%) were relatively low after stimulation by grass pollen suggesting that these basophils may have been of the non-responder phenotype (Figure 4-5 B). Unstimulated basophils (subject 2) showed slightly higher expression of CD63 compared to poor expression of basogranulin (Figure 4-5 D). Basophils stimulated with stimulated with fmlp induced dose dependent expression of both CD63 and BB1 with maximal expression of CD63 (18.8%) at 1 µM and BB1 (27.4%) at fmlp 10 µM by grass pollen, both basogranulin and CD63 showed a typical bell-shaped dose response with maximal expression of CD63 (26.6%) and basogranulin (24%) at grass pollen concentrations of 1 and 0.1 SBU respectively (Figure 4-5). Further experiments were performed to confirm the validity of basogranulin as am marker for basophil activation in response to stimulation with grass pollen (Figure 4-6). It showed that basogranulin is competent maker for measuring basophil activation in response to stimulation with grass pollen.

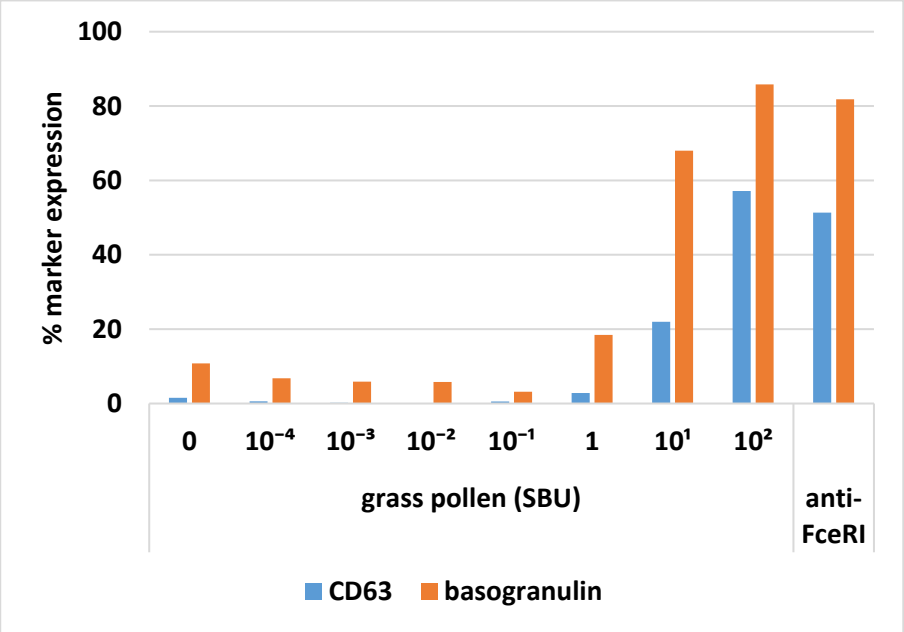


Figure 4-4. Surface basogranulin expression in basophils stimulated with grass pollen. Basophils in whole blood from one subject stimulated with various concentrations of grass pollen, and stained with antibodies specific to CD63 and basogranulin. Anti-FcεRI stimulation served as a positive control. Data was acquired by flow cytometry and analysed by CellQuest software.

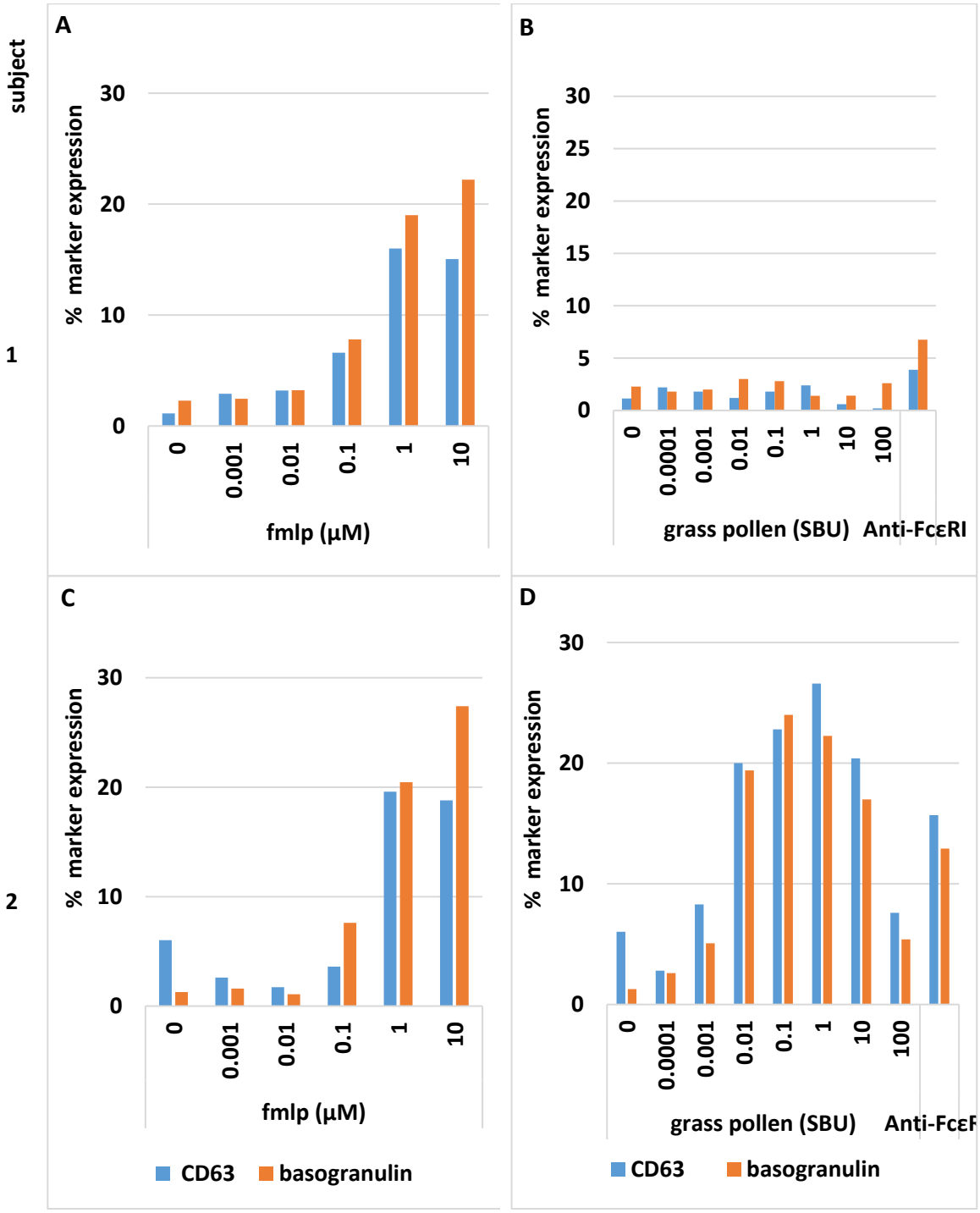


Figure 4-5. Upregulation of surface basogranulin and CD63 in basophils stimulated with fmlp, grass pollen or the stimulation buffer alone. Basogranulin was measured in basophils from two subjects. Data acquired by flow cytometer and analysed by CellQuest software.

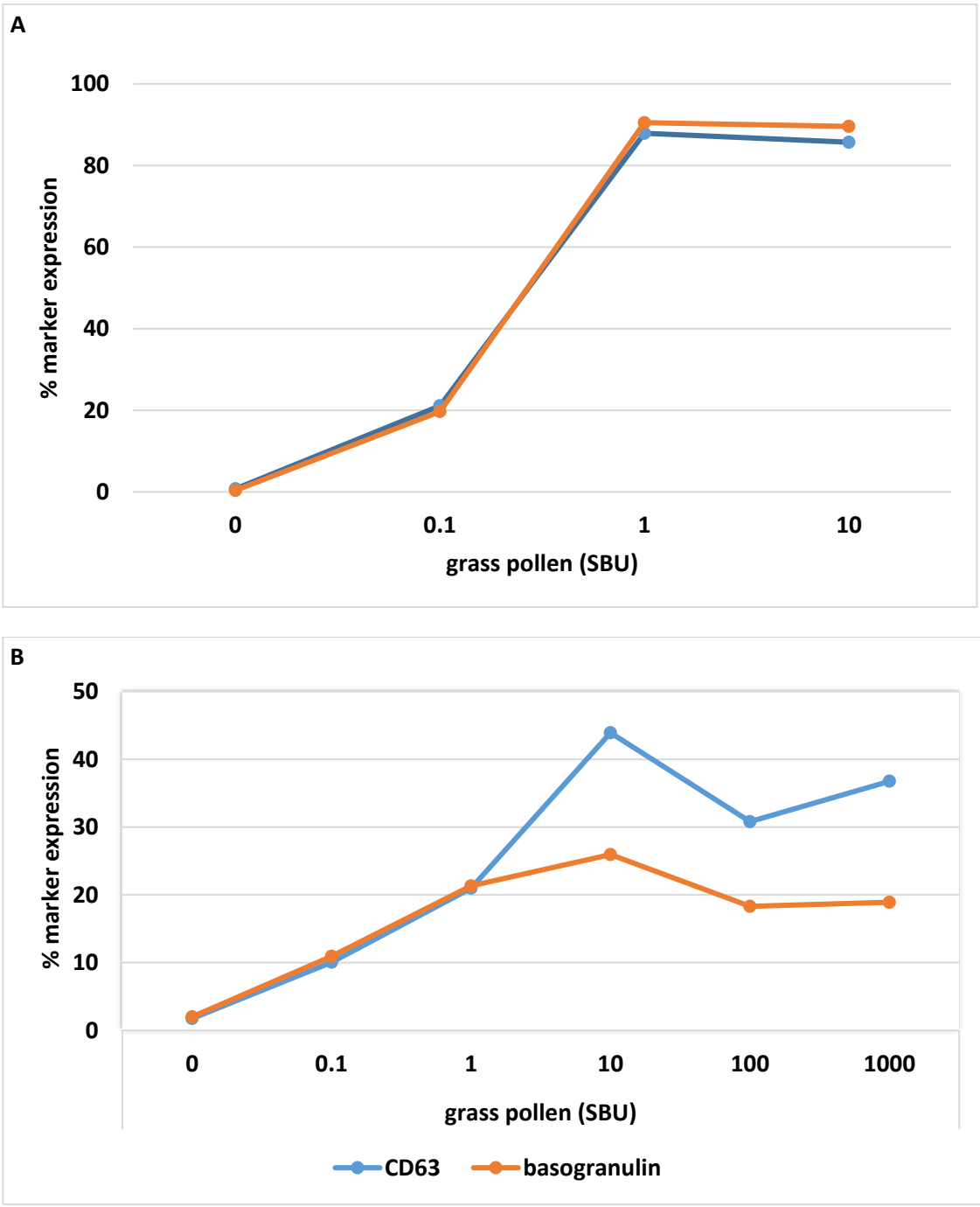
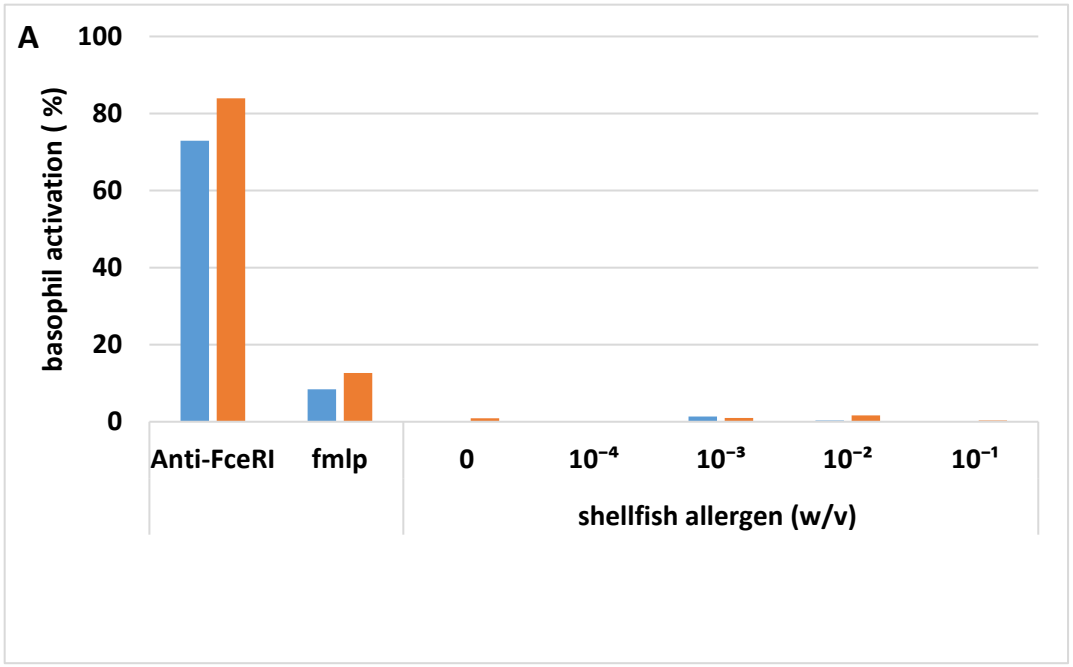


Figure 4-6. Increased expression of basogranulin on basophil membrane upon activation by grass pollen in vitro. Basophils were stimulated with grass pollen, or stimulation buffer alone as a negative control. Basophils in whole blood were identified by high expression of CCR3 and side scatter characteristics. Anti-CD63 was also used as a positive control for basophil activation. Data acquired by flow cytometer and analysed by CellQuest software.

Further, basogranulin and CD63 expression were consistant with the skin prick tests. Figure 4-7 showed that basogranulin and CD63 did not increase in basophils from subjects whom skin prick tests were negative for the same allergens. On the other hand, basogranulin and CD63 expression was meaured in basophils obtained from a subject with postive SPTs for *D. pteronyssinus*, *D. farinae*, crab, shrimp and oyster (Figure 4-8). In contrast, CD63 was only upregulated upon stimulation at the highiest concentrations of allergens. Further, basogranulin expression was measured in basophils from a person whose skin prick test were posive for peanut, almond, hazelnut (Figure 4-9). Although both basogranulin and CD63 increased following stimulating basophils with various cenocentrations of peanut, almond and hazelnut, basogranulin expression was higher than that for CD63.



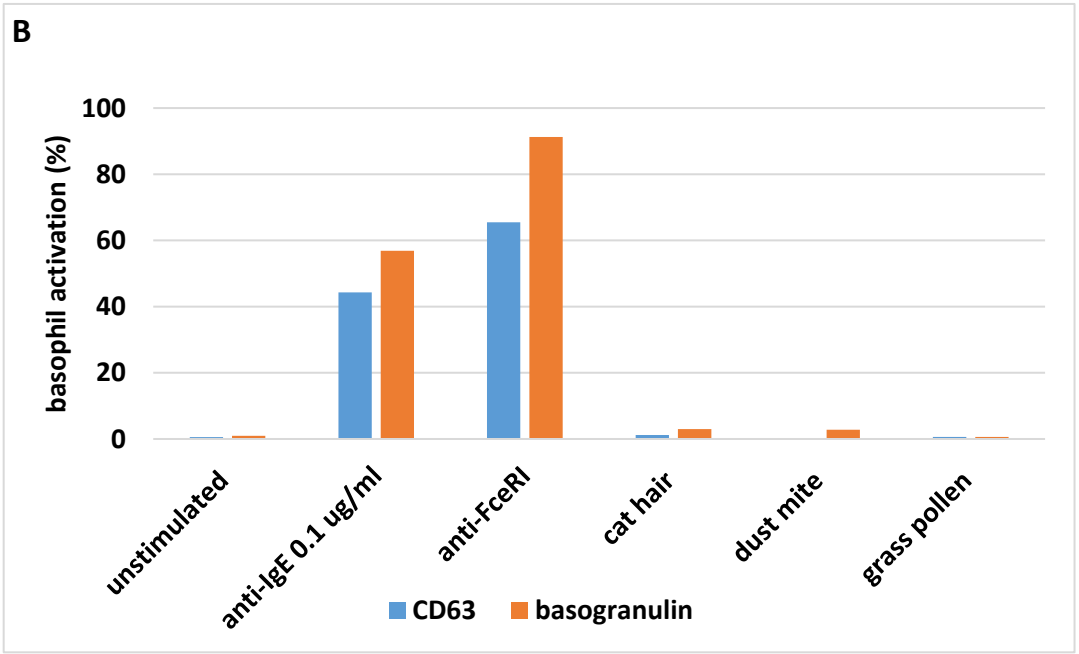
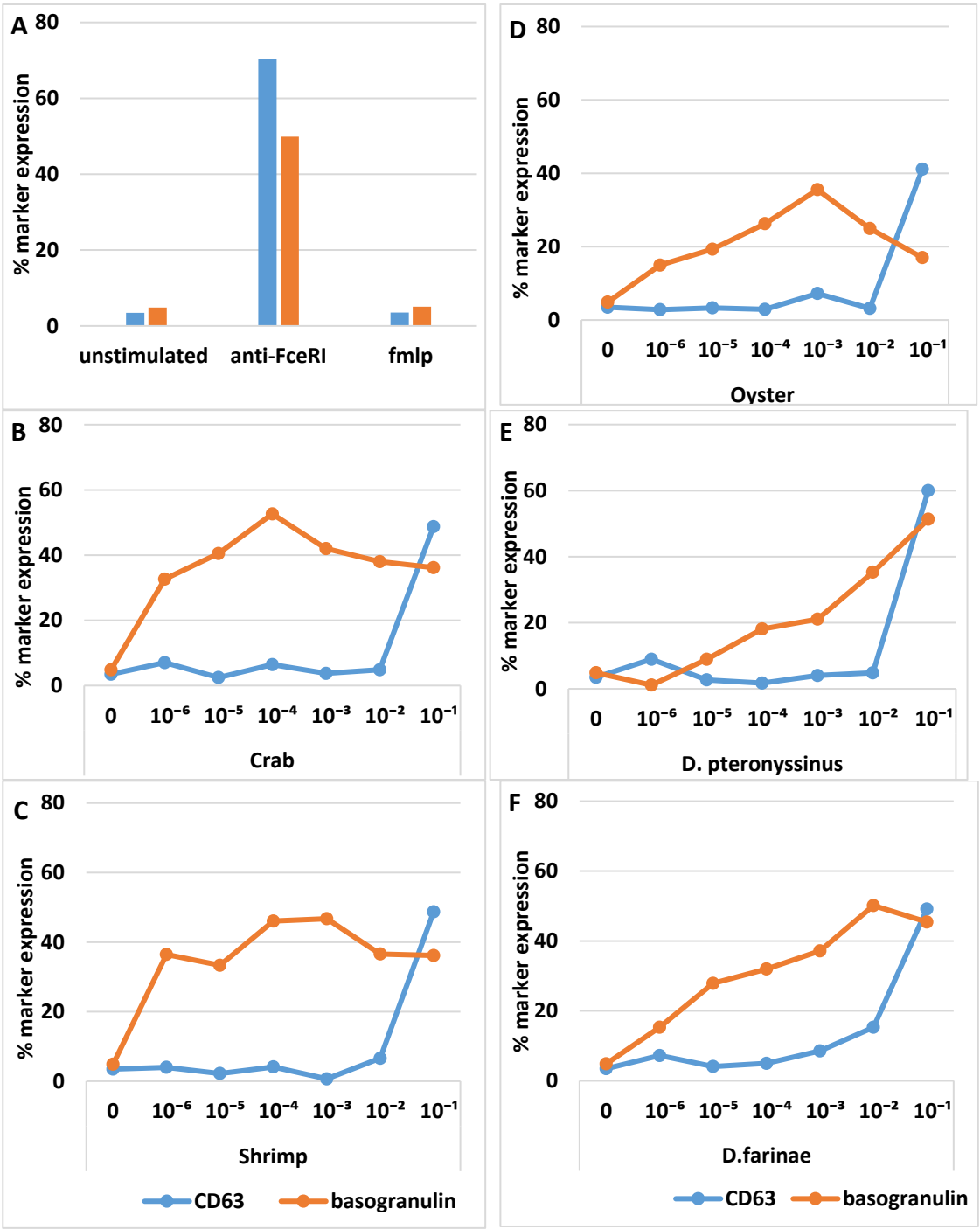


Figure 4-7. Relative expression of surface basogranulin in patients with negative skin prick test reaction to shellfish, cat hair, dust mite and grass pollen allergens. Basophils from one subject in whole blood stimulated with (A) anti-FcεRI, fmlp, or buffer alone, or serially diluted shellfish allergen (10^{-4}-10^{-1} %) and (B) cat hair, dust mite and grass pollen and stained with antibodies specific for basogranulin and CD63. Data acquired by flow cytometry and analysed by CellQuest software.



Test	positive control (histamine)	negative control (normal saline)	*D. pteronyssinus*	*D. farinae*	crab	shrimp	oyster
Wheal size (mm)	8×8	1×1	9×6	4×4	14×10	12×11	7×6

Figure 4-8. Surface basogranulin expression in response to various allergens. Basophils were obtained from a subject with positive skin prick test for oyster, shrimp, crab, *D. pteronyssinus*, *D. farinae*. Basophils were stimulated with different allergens diluted in stimulation buffer or stimulation buffer alone for negative control. Data was acquired and analysed by flow cytometry. Skin prick tests were performed with the same allergen extract.

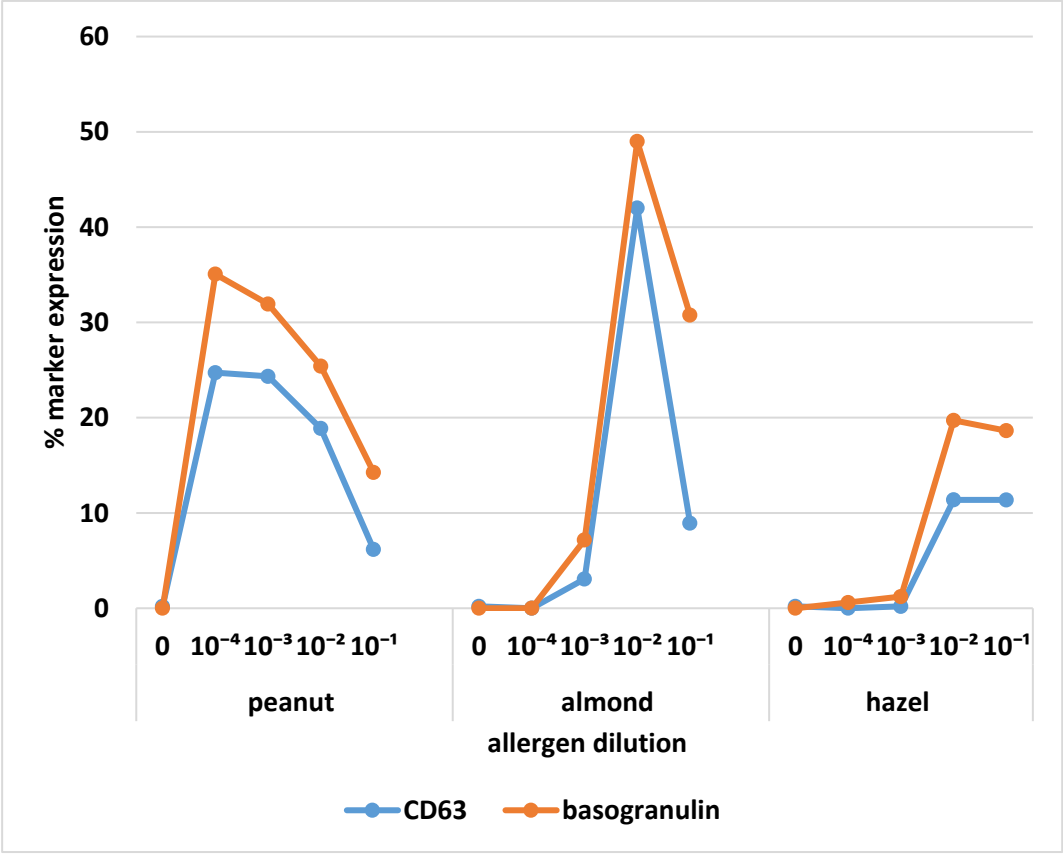


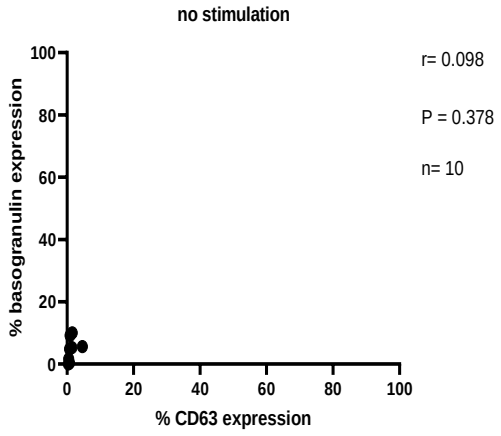
Figure 4-9. Surface basogranulin expression in basophils in whole blood in response to in vitro stimulation with various concentrations of peanut, almond and hazelnut. The sample was collected from a patient skin prick test positive for the same allergen extracts. Data was acquired by flow cytometry and analysed by CellQuest software.

4.3.1 Association of basogranulin and CD63 expression

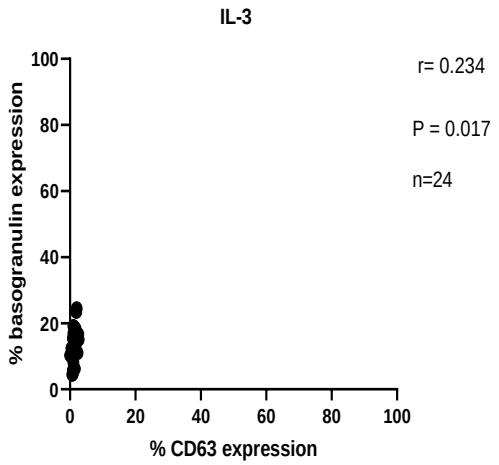
There was correlation between the basogranulin and CD63 expression in non-stimulated basophils (0.829, $P < 0.005$, n=10) (Figure 4-10). Addition of IL-3 to basophils slightly induced basogranulin expression (though by more than 20% in some cases), whereas surface CD63 remained unchanged. The levels of basogranulin and CD63 expression were strongly correlated upon stimulation with fmlp r=0.914, $P< 0.0001$, n=9), anti-IgE (r=0.877, $P< 0.001$, n=51) or anti-FcεRI (r=0.680, $P< 0.0001$, n=20). However, stimulation of basophils with different allergens

including *D. pteronyssinus*, *D. farinae*, crab, shrimp, and oyster) resulted in different patterns of expression for both markers and the correlation between the two were variable. *D.pteronyssinus* (r=0.549, P= 0.092, n=6), *D. farinae* (r=0.314, P= 0.247, n=6), crab (r=0.087, P= 0.571, n=6), shrimp (r=0.089, P= 0.566, n=6), and oyster (r=0.092, P= 0.559, n=6). Surface basogranulin and CD63 expression induced by all stimuli and the levels of spontaneous expression were correlated (r =. 0.95, P < .0001, n = 175), and the percentage expression of basogranulin was almost always more than that of CD63 (Figure 4-11).

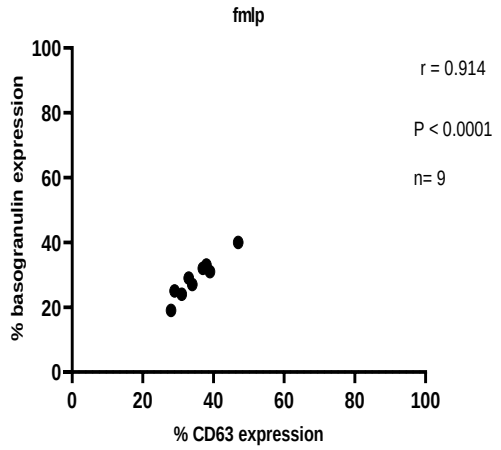
A


no stimulation
% basogranulin expression
% CD63 expression
r= 0.098
P = 0.378
n= 10

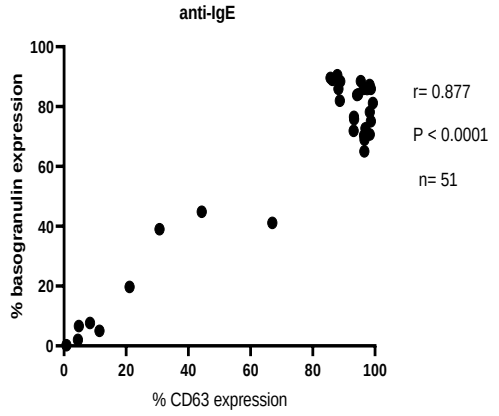
B


IL-3
% basogranulin expression
% CD63 expression
r= 0.234
P = 0.017
n=24

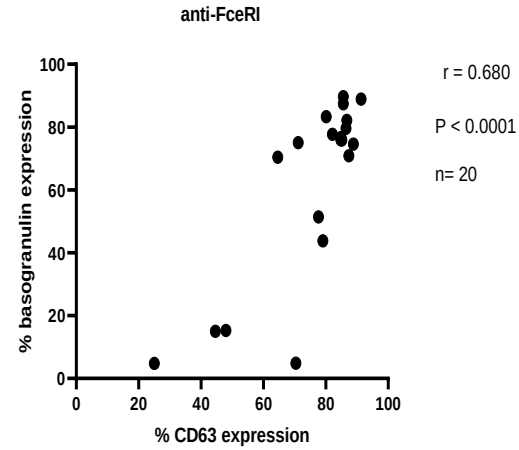
C


fmlp
% basogranulin expression
% CD63 expression
r = 0.914
P < 0.0001
n= 9

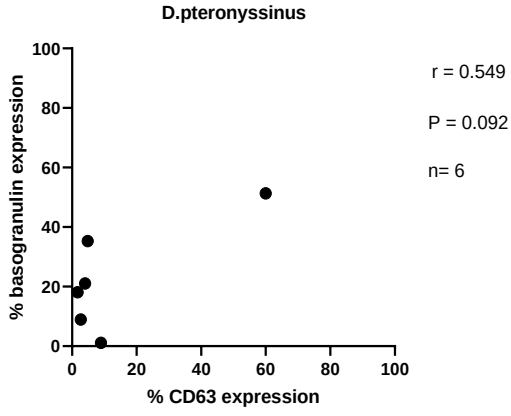
D


anti-IgE
% basogranulin expression
% CD63 expression
r= 0.877
P < 0.0001
n= 51

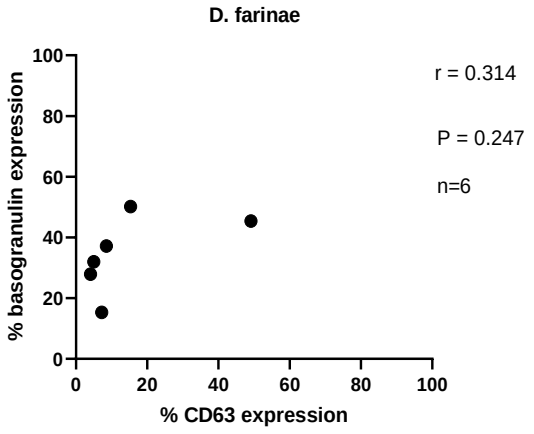
E


anti-FceRI
% basogranulin expression
% CD63 expression
r = 0.680
P < 0.0001
n= 20

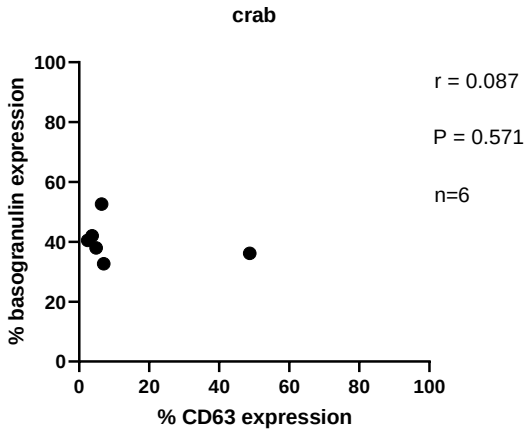
F


D.pteronyssinus
% basogranulin expression
% CD63 expression
r = 0.549
P = 0.092
n= 6

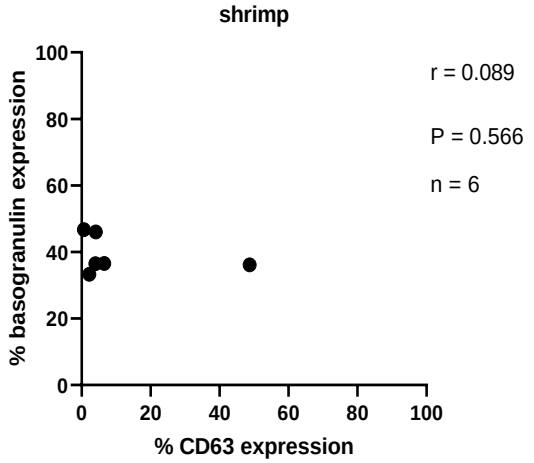
G



H



I



J

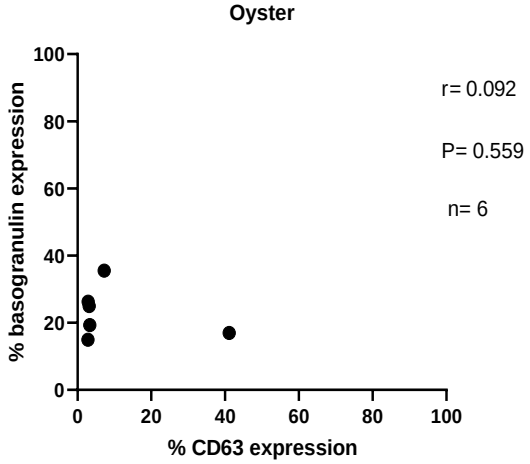


Figure 4-10. Associations between surface basogranulin and CD63 expression as determinedby flow cytometry. Percentage marker expression is shown for non-stimulated controls, and cells stimulated with IL-3, fmlp, anti-IgE antibody, anti-FcεRI, D. *pteronyssinus*, *D. farinae*, crab, shrimp, and oyster (at concentration of 10^{-6} -10^{-1} of the original material). Analysis was by Pearson's test.

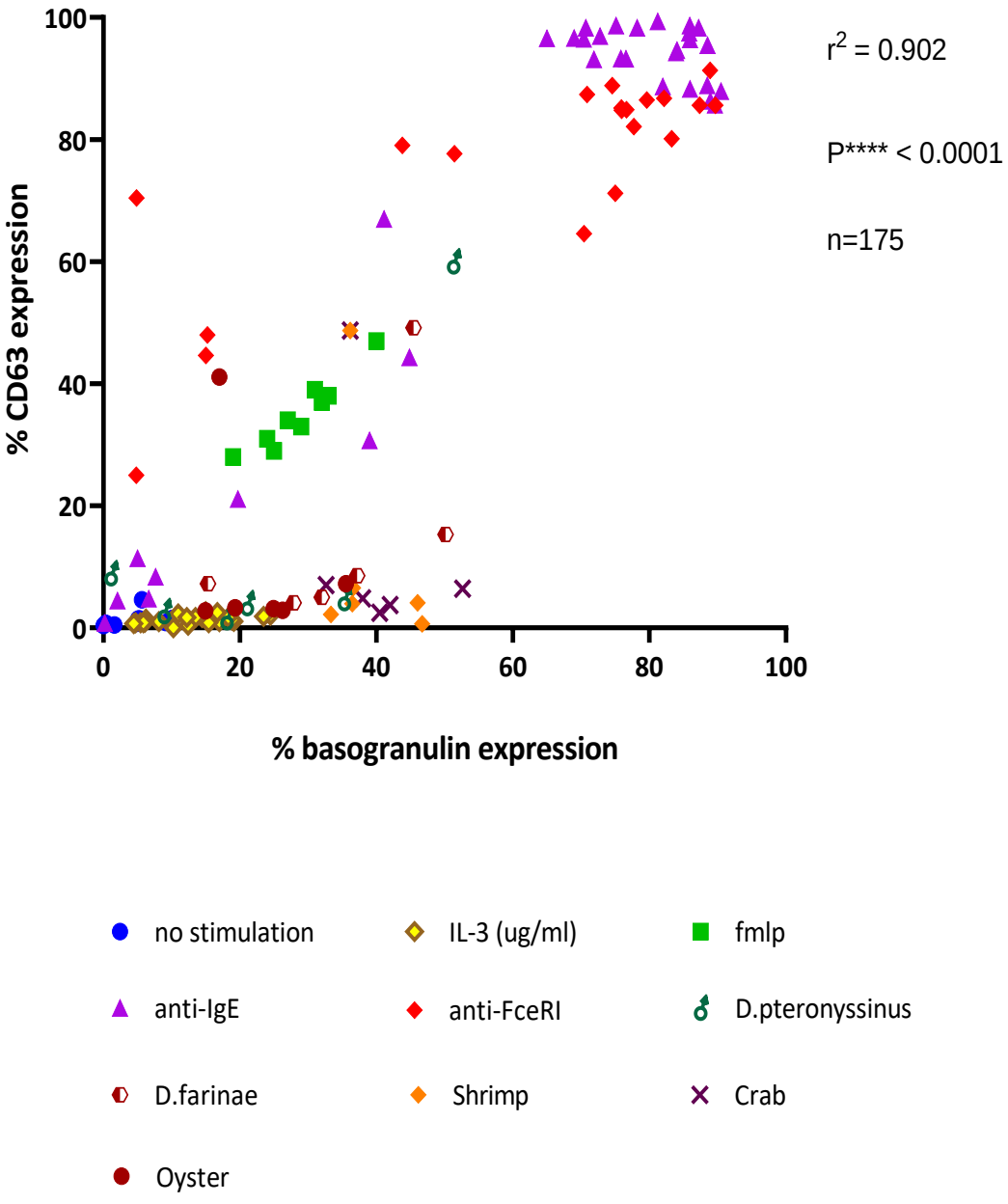


Figure 4-11.Association between basogranulin and CD63 expression.
Percentage of expression is shown for nonstimulated controls ( ), cells stimulated primed with IL-3 (5-200 µg/ml) ( ), and cells stimulated with anti-IgE antibody (0.0001% to 10%) ( ), fmlp (unknown concentration) ( ), anti-FcεRI ( ), and allergens: *D. pteronyssinus* ( ), *D. farinae* ( ), crab ( ), shrimp ( ), oyster ( ) (with concentration of 10^{-6}-10^{-1} of the original material). Analysis was by Pearson's test.

Chapter 5 Assessing basogranulin as a marker for basophil activation in clinical disease

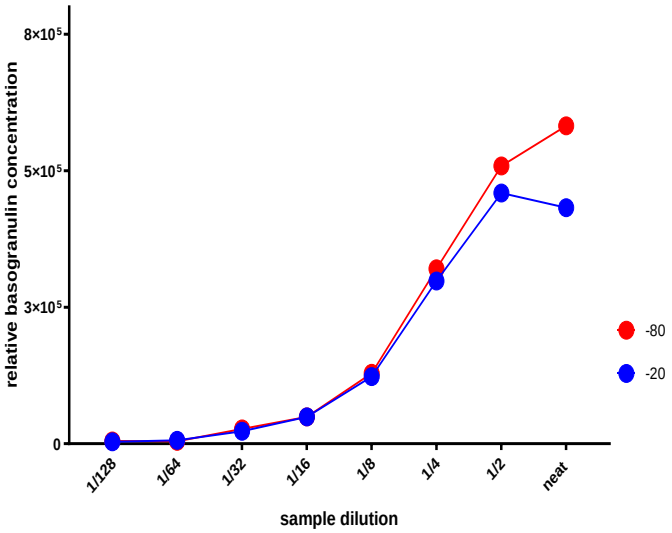
In Chapter 5, are presented the findings when basogranulin levels were investigated in body fluids as a means for assessing the extent of basophil activation in clinical diseases. This involved (a) optimising and applying dot blotting to measure basogranulin in biological fluids including saliva and BAL fluid, (b) the measurement of basogranulin in saliva samples from peanut allergic patients before and after allergen oral allergen challenge and (c) determining f basogranulin levels in BAL fluid from patients with asthma and healthy subjects by dot blotting assay.

5.1 Optimisation basogranulin dot blotting assay for saliva samples

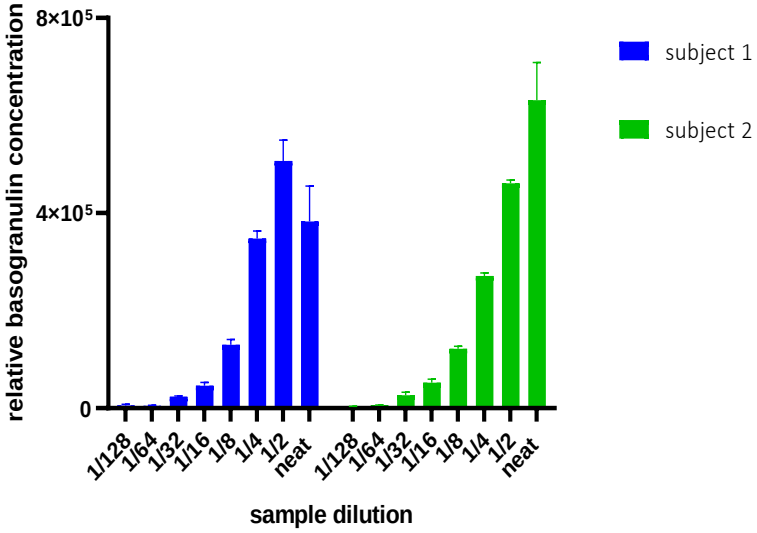
Attempts were made to optimise basogranulin dot blotting assay (Figure 5-1). When basogranulin was measured by dot blotting in saliva stored in -20 or -80 °C, the difference was not statistically significant as determined by t-test (Figure 5-1A). Diluting samples seemed to align the signal of the different stored samples at the same point of dilution. Diluting saliva samples 1 in 2 with normal saline in both cases, dilution resulted in lower measurements (negative correlation), and no detection below 1:32 (Figure 5-1B).

Diluting saliva in PBS-DTT resulted in some changes in the measurements of basogranulin by dot blotting (Figure 5-1 C). However, the changes in basogranulin measurement were not always associated with the dilution factor. The level of basogranulin did not exponentially correlate with dilution factor. Further, in some cases the basogranulin was higher in the second (1:2) dilution than the first one (1:1). Also, in some samples, basogranulin levels were not affected by diluting in PBS-DTT.

A


relative basogranulin concentration
8×10⁵
5×10⁵
3×10⁵
0
1/128
1/64
1/32
1/16
1/8
1/4
1/2
neat
sample dilution
-80
-20

B


relative basogranulin concentration
8×10⁵
4×10⁵
0
1/128
1/64
1/32
1/16
1/8
1/4
1/2
neat
sample dilution
subject 1
subject 2

C

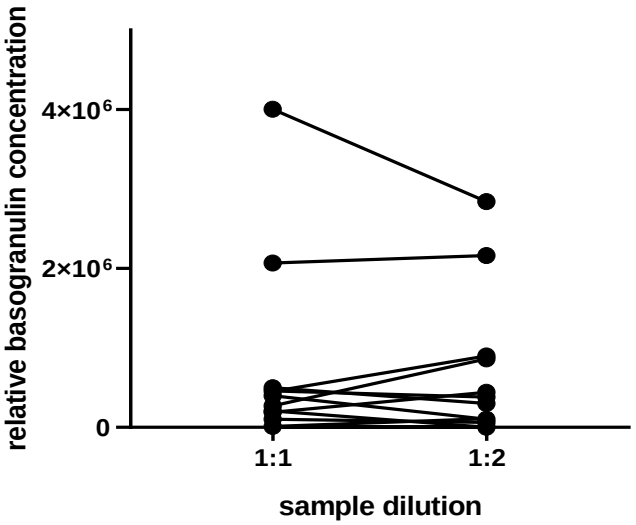
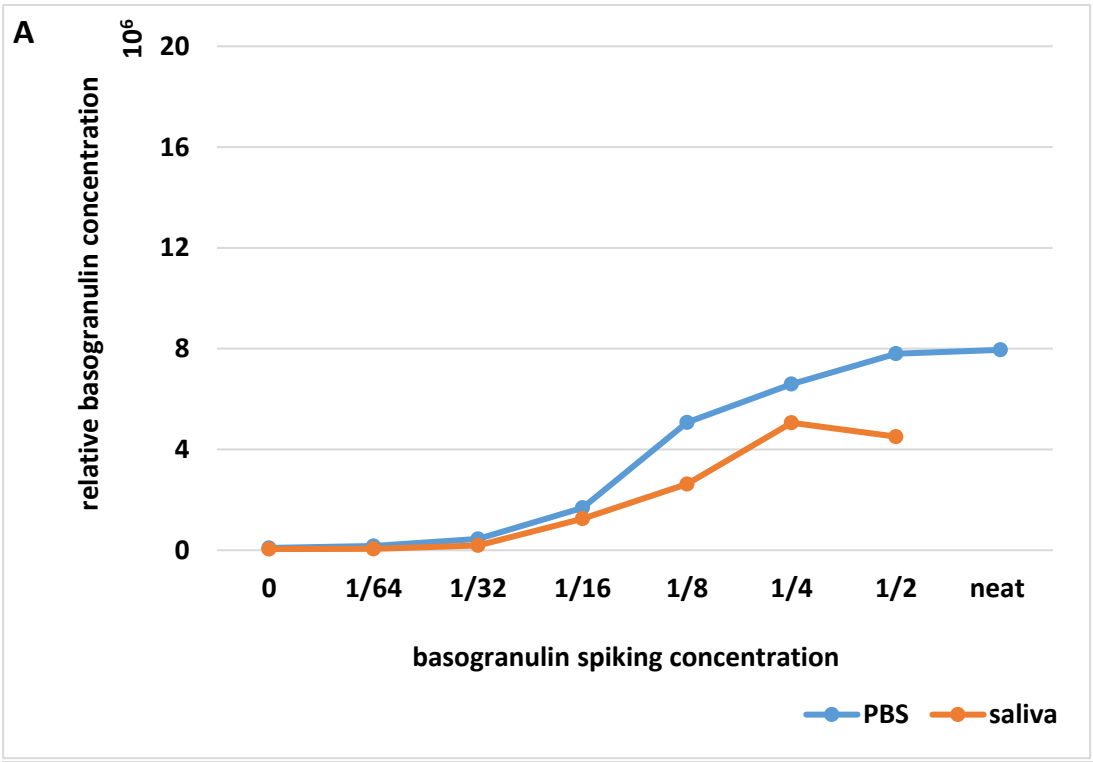

relative basogranulin concentration
4×10⁶
2×10⁶
0
1:1
1:2
sample dilution

Figure 5-1. Optimisation of basogranulin dot blotting assay. (A)The influence of storage temperature on the detection of basogranulin in saliva samples. Basogranulin were measured in saliva stored at -20 °C or -80 °C from one sample before or after serial dilution using dot blotting assay. (B) The effect of dilution on basogranulin measurement in saliva. The basogranulin concentration in saliva samples from saliva samples from two subjects was measured (before and after dilution) by dot blotting. The median and range of quadruplicates are shown. (C) Effect of sample dilution on the measurements of basogranulin in saliva by dot blotting. (Saliva were mixed with PBS containing DTT (50 µM) 1:1 and 1:2 v/v before blotting (n=13).

Spiking saliva with a range of different concentration of basogranulin indicated interference in the measurement in basogranulin by dot blotting (Figure 5-2). Levels of basogranulin in saliva were lower than that in buffer when upon developing the image after 30 seconds though both curves did not intersect (Figure 5-2 A). However, prolonging the period for developing the image to 60 seconds resulted in some changes in basogranulin levels detected as the basogranulin curves overlapped at a dilution 1/16 (Figure 5-2 B). In addition, basogranulin levels at higher concentrations(1/8, 1/4, 1/2) were higher in saliva that those in buffer. The highest level of basogranulin detected was at 1/4 and it was higher than that in saliva in all dilutions (1/4, 1/2 and neat).



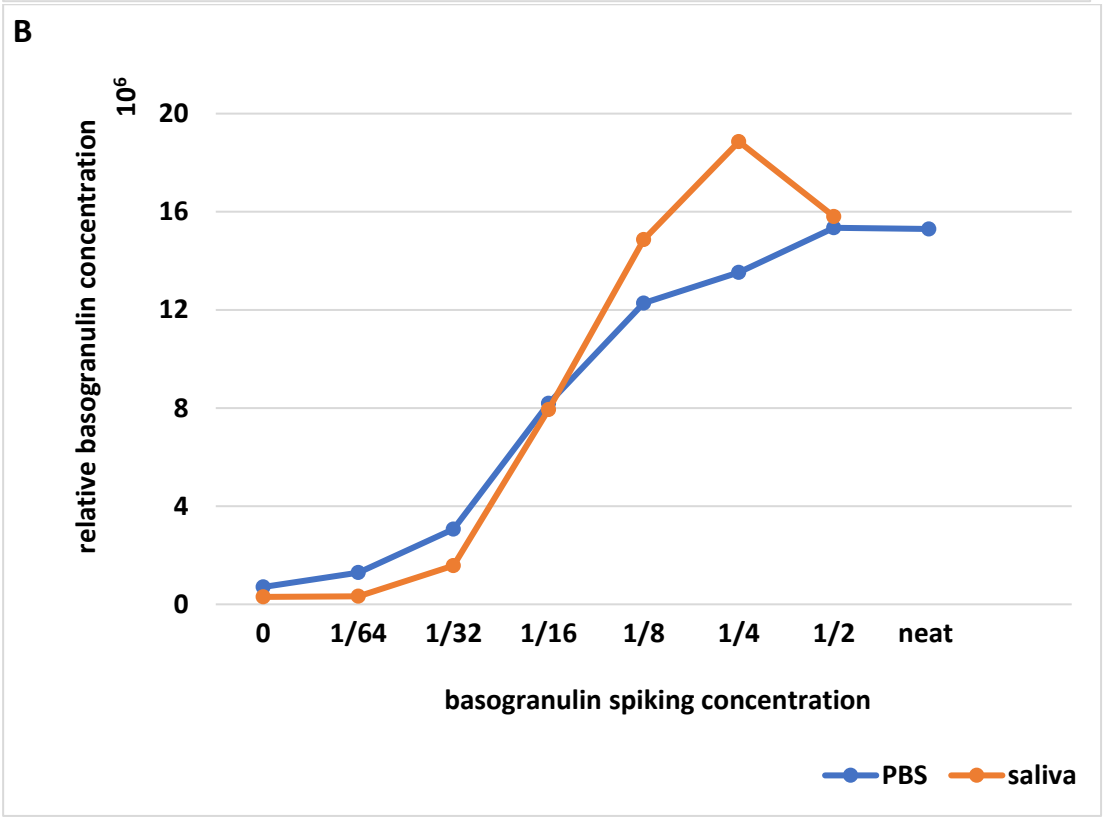


Figure 5-2 . Spiking saliva and PBS (as a diluent or matrix) for the basogranulin assay by dot blotting. Pretested saliva samples with undetectable basogranulin was spiked with various dilutions of a basophil lysate (10^4/ ml) in PBS used as a source of basogranulin. PBS alone was employed as a negative control. The levels of basogranulin was measured by dot blotting and the images were developed for (A) 30 or (B) 60 seconds.

5.2 Measuring basogranulin in saliva form peanut allergic subjects

When basogranulin was measured by dot blotting in saliva samples collected from 83 subjects or one or two hours after allergen challenge. Levels of basogranulin at baseline (i.e. before challenge) tended to be higher than those after challenge at these time point. However, a few cases had slight increases in basogranulin levels after the challenge (Figure 5-3). Samples were collected by Dr Paul Turner and colleagues at Kings College London as part of an ongoing study for which the code has not yet been broken.

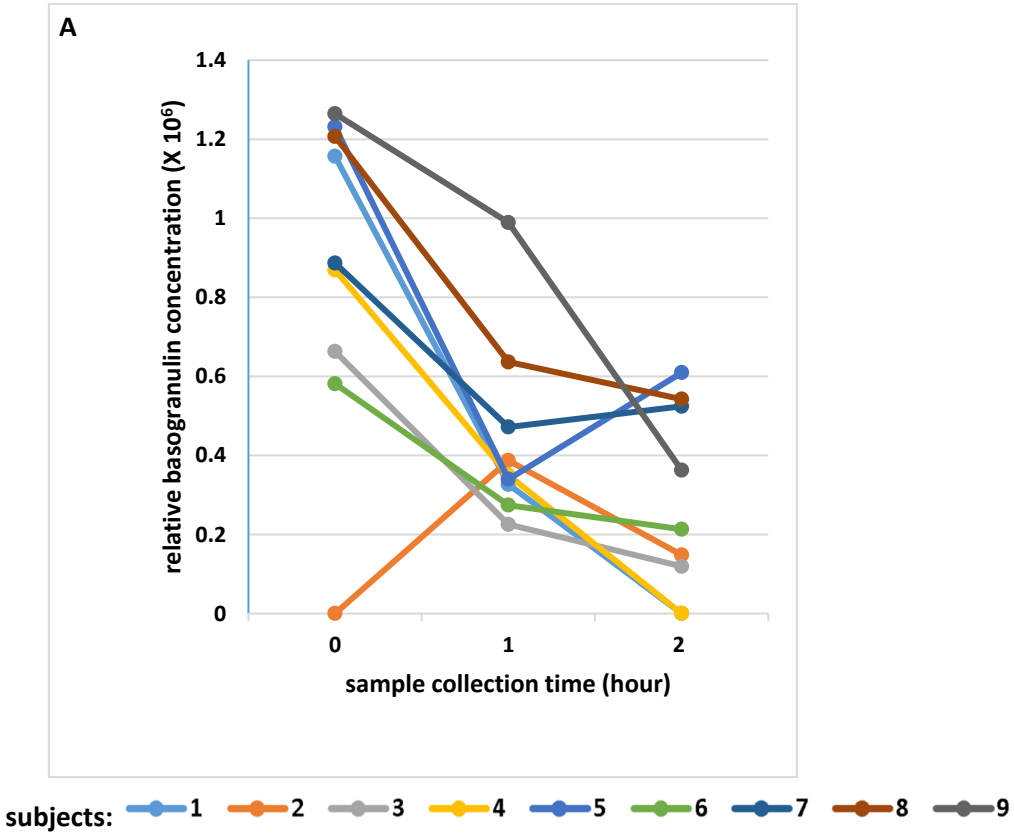


Figure 5-3. Representative of basogranulin levels in saliva from patients before and after oral challenge. Basogranulin in saliva from83 patients allergic to peanut was measured in three different time points: before (0), one hour (1) and two hours (2) after oral food challenge.

5.3 BAL fluid from subjects with asthma

Basogranulin was detected in BAL fluid samples collected from normal healthy subjects and from patients with atopic asthma and severe asthma using dot blotting (Figure 5-4). Basogranulin concentrations in BAL fluid samples from patients with atopic asthma were

higher than those of normal healthy subjects. However, basogranulin concentrations in patients with severe asthma were lower than those with atopic asthma and nearly equal to that in normal samples as analysed by t-test. There was no significant correlation between the levels of basogranulin levels between the three groups. Complete information about the subjects and/or the samples were not available. However, when basogranulin concentration was determined in five BAL fluids against basogranulin standard, the levels of basogranulin of all the samples were below the lower limit of quantification as determined by the guidelines of FDA bioanalytical method validation [284] (Figure 6-6).

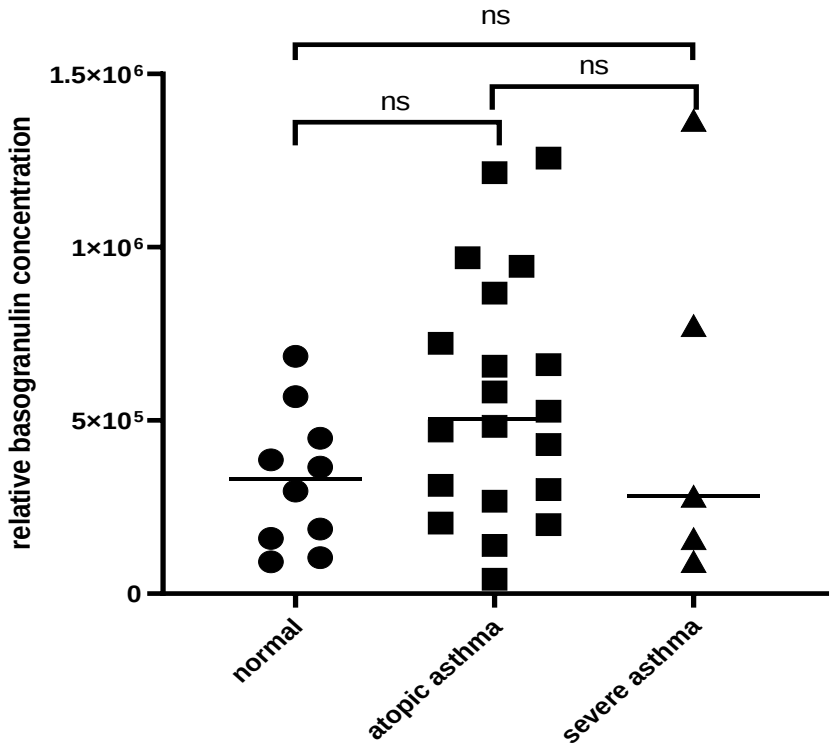


Figure 5-4. Measurement of basogranulin by dot blotting in BAL samples.
(A) Representative dot blotting image for detection of basogranulin in BAL (n=18). (B) Basogranulin concentration in BAL samples from normal healthy subjects (n=10), subjects atopic and asthmatic (n= 20) and severe asthmatic (n=5) subjects were measured by dot blotting. Data are expressed as the mean. Data was analysed by t-test. Mean of duplicates for each sample was determined.

Chapter 6 Discussion

We have successfully developed new ways for measuring basogranulin release and for monitoring alterations in intracellular and surface expression of this marker in response to basophil activation in vitro and in vivo. The finding that basogranulin is released in response to stimulation suggests that measurement of basogranulin release may be of diagnostic value in assessing allergic sensitivity. We have evidence that intracellular basogranulin stores are depleted following basophil activation, and that surface expression of basogranulin may be strikingly upregulated, suggesting that these could provide a reliable means for measuring basophil activation. The alterations in basogranulin expression paralleled those for expression of the basophil activation marker CD63, and in some cases provided a more sensitive method of determining allergic sensitivity. The newly developed assays were validated by applying them to assess basophil sensitivity to various allergens including grass pollen, dust mites and several food allergens. Our finding that basogranulin can be detected in saliva of subjects with food allergy and in BAL fluid from asthmatic subjects, opens the way for the first time to obtain discriminating evidence for basophil activation in clinical disease.

6.1 Development and optimisation of assays for assessing basophil activation

Separation of nucleated cells from erythrocytes using an erythrocyte aggregation and sedimentation method has been shown to be essential for achieving the highest possible purity with only one round of immunomagnetic selection. However, it has a little effect on basophil purity, but was successful in enriching basophils, amongst other contaminating nucleated cells sufficiently enough for further purification by negative immunoselection. Negative selection with magnetic beads was a method that resulted in higher purities of basophils. Although the antibody cocktail contains an anti-glycophorin A antibody, which efficiently removed residual erythrocytes, residual red blood cells seemed to compromise the final purity. Almost half of basophils were lost during the purification procedures, and the final yields were variable. The viability of basophils was relatively high (95%) as determined by the trypan blue exclusion test, but purity was relatively low in comparison to what it has been reported previously [285].

Achieving efficient basophil purification can be challenging and it can impact basophil responsiveness to stimuli. Thus, for example a marked reduction in expression of the basophil activation marker CD63 was noted in purified basophils following stimulation compared with that of basophils in whole blood. This could be the result of cell manipulation and possibly depletion of energy stores and essential physiological elements such as calcium and magnesium. The possibility of performing basophil activation studies in whole blood was considered as a means for overcoming purification-related loss of function, but this was not successful.

Extraction of basogranulin from lysates of purified basophils represented a crucial step for developing assays for measuring basogranulin. Purified basophil lysates were used as a source of basogranulin as there is not an established method for basogranulin purification. Optimal extraction of basogranulin was obtained in the present studies by lysis of purified basophils with SDS and deoxycholic acid. Addition of SDS led to a glutinous lysate product which diffused over the immunoblotting membrane. Extraction of basogranulin using 1% SDS seemed to be more effective than with other detergents. This was possibly because SDS induces disruption of the cell membrane and also solubilises basogranulin and results in dissociation from other proteins. However, deoxycholic acid was employed in further experiments for lysing basophils on account of the extensive diffusion on dot blotting observed with the SDS preparations.

Somewhat surprisingly basogranulin was detected in lysates of LAD2 cells, a cell line which has been characterised being as of the mast cell lineage. LAD2 cells therefore may serve as an alternative source of basogranulin in some experiments, though by confocal microscopy expression of basogranulin was quite low in these cells. LAD2 cells may possess some features of basophils as well as of mast cells. However, when passively sensitised with patients' IgE, they have been reported to respond on activation differently from donor basophils as analysed by CD63 expression [286]. Activation of passively sensitised LAD2 cells have been employed for assessing of allergic sensitivity through measurement of β-hexosaminidase release [144]. It might be useful to investigate whether basogranulin could also be employed as an activation marker for these cells.

The present studies indicate that IgE-dependent and non-IgE-dependent stimulation induced basogranulin release, depletion of intracellular basogranulin stores and increased of surface expression. These findings are consistent with idea that basogranulin may represent a reliable

marker for measuring basophil activation over a range of conditions and could form the basis for novel strategies in allergy diagnosis. Basogranulin was released in response to the various stimuli into cell supernatants with different basophil purities, and generally with bell-shaped response curves. Such patterns of release from activated basophils have been noted previously for basogranulin [275], as well as for histamine and other mediators [275, 287]. Possible explanations for the bell-shaped responses have included the possibility that higher concentrations of stimulus can cause cytotoxicity that diminishes basophil activation at those "supraoptimal concentrations". Thus, for example fmlp may bind with high affinity to a specific receptor, and also bind to another lower affinity receptor that may induce metabolic blocking effects [288]. As concentrations of the stimulus increase and specific receptors will approach saturation, the inhibitory effects become more powerful and the net polarising effect is reduced.

The bell-shaped dose-response curve represents a feature of FcεRI activation, that has been seen also with FcγRIII and B cell antigen receptors [289]. A bell-shaped curve was observed in basophil activation studies whether involving basogranulin release into cell supernatants or flow cytometric determination of basogranulin expression. There were, however, cases for which there was a progressive increase or decrease without any apparent bell shape being observed. This could be because the data represents either the ascending or descending arms of a bell-shaped curve, and obtaining a bell-shaped curve may have required a wider range of concentrations of stimulus than was employed.

A possible explanation that has been advanced for bell-shaped IgE-dependent responses is that co-aggregation of FcεRI may result in an overall inhibitory effect [290]. In addition, a bell-shaped response curve has been observed conditions of supraoptimal stimulation with by Src-homology 2–containing 59-inositol phosphatase (SHIP)–1 [291], where increasing the antigen dose may result in the formation of monovalent complexes with every IgE binding "its own" antigen and thus antigen-mediated cross-linking between different IgE-bound FcεRIs were lost [292]. SHIP-1 is a haematopoietic signalling molecule with multiple forms that hydrolyses phosphatidylinositol-3, 4, 5 trisphosphate (PIP3), thereby limiting a crucial step between the release of calcium from intracellular stores and the influx of extracellular calcium [293].

The lower basogranulin release detected with sub-optimal concentrations of IgE-dependent stimuli may be a consequence of only a few IgE-bound FcεRIs being cross-linked, resulting in weak activation of downstream signalling pathways and effector functions. At optimal

concentrations there may be a zone of equivalence, with maximal numbers of IgE-bound receptors cross-linked. This results in strong activation of antigen-triggered signalling pathways and maximal induction of effector functions.

Measuring basogranulin in activated basophils present in whole blood or in a nucleated cell fraction was considered as an alternative to studies with purified basophils. However, dot blotting was not practical for measuring basogranulin in whole blood as the membrane could be readily blocked by the cells or macromolecules in plasma. Hence, basogranulin release was measured in whole blood cells after plasma and erythrocytes were removed, and measurements compared between those with a preparation of nucleated cells and purified basophils. It was found that there was less basogranulin release from whole blood cell preparations than was released from highly purified or erythrocyte depleted basophils, whether challenged with anti-IgE, fmlp or *D. pteronyssinus*. The lower basogranulin release with whole blood cells was likely to be a consequence of there being only small numbers of basophils stimulated, and it is not possible to increase numbers in the volume required for the basogranulin assay. Other factors that could explain differences in responsiveness between cell preparations could be alterations in concentrations of stimuli relative to the number of cells present, or effects of non-basophils on the basophils themselves or on the ability to measure basogranulin release.

Accurate measurement of basogranulin continues to represent a major challenge, without there being a well-characterised standard preparation, and just one established specific antibody. The variability of the dot blotting procedure employed for measuring basogranulin in cell lysates had an intra-assay coefficient of variation as high as 20% with a mean of 1.6×10^5 arbitrary unit (96 determinations). This variability may impact the sensitivity and render reliable measurement difficult when little basogranulin is released. On account of the dot blotting procedure being affected by high variability and low sensitivity and being time-consuming, ELISA was considered as an alternative assay.

With BB1 being the only well characterised monoclonal antibody specific for basogranulin, an indirect ELISA procedure was investigated involving coating plates with lysates of purified basophils. BB1 binding was demonstrated, but for increased sensitivity a sandwich ELISA was investigated in which BB1 was used for antigen capture and biotinylated BB1 for detecting basogranulin. However, that assay failed to detect basogranulin in basophil lysate, and this

could be due to the specific epitope of basogranulin already being captured by the non-biotinylated BB1 on the surface of the plate and so that the biotinylated BB1 did not bind to it.

Screening antibodies specific for basogranulin by means of ELISA led to the identification of three promising antibody clones (which were termed 14, 24, and 25). However, high background appeared to be common among all of antibody clones. The high background may be as result of these antibodies being of the IgM class. Elimination of nonspecific binding by diluting the analyte (basophil lysate) in PBS with or without bovine serum had minor effects on detection of basogranulin. Also, elimination of non-specific background using goat serum did not improve the specificity of the test, rather it altered the sensitivity. This might be as a result of the fact that goat serum masked the basogranulin epitopes and, hence, not recognised by corresponding antibodies. Although the ability of these antibodies to detect basogranulin in basophil lysate represents a promising finding, their validity and utility need to be determined. If one of these antibodies is validated as being specific for basogranulin, it can then be used with BB1 for developing ELISA as alternative method for measuring basogranulin. This has important implications in assessing basophil activation *in vitro* and *in vivo* and revealing whether this marker may be of diagnostic and prognostic value for allergic conditions.

Development of a flow cytometric assay for measuring basogranulin expression intracellularly and on the surface of indicates a new means for assessing basophil activation based on this marker. It was decided to avoid using IgE or FcεRIα as gating markers because their expression could markedly change depending on the concentration of serum IgE and because other cells such as monocytes can express these markers [294], and also in expression may be affected in allergic patients [295-298]. In addition, FcεRI crosslinking by allergen induces their internalization, and this can interfere with basophil gating [299].Therefore, CCR3 has been selected to be used as basophil identification marker since it has less inter-individual variability relative to IgE and CD123 [300].

We assessed whether basophil activation is affected by the length of storage time. Storing basophils in whole blood for 24 hours resulted in an apparent decrease in CD63 expression of 71% suggesting that basophil reactivity can decrease over time. This is keeping with previous studies that have indicated decreased expression of CD63 following storage for 24 hours [95, 97]. This is in agreement with the findings of Sturm et al. who reported that a decrease in CD63 and CD203c expression after storage for just four hours was seen only with the lower concentrations of anti-IgE employed for basophil stimulation [95, 97]. Therefore, experimental

activation of basophils should be performed as early as possible after taking the blood sample, preferably within four hours.

We t demonstrated that basophils can be immunostained with BB1 following fixation of the cells with Fix/Perm buffer. Other cells such as eosinophils have not been found to be immunostained with BB1. The staining appeared to be specific for basogranulin since the staining intensity was markedly reduced when an irrelevant antibody (IgG2a) was used as an isotype negative control. Also, the specificity of BB1 for basophils when analysing cells from peripheral blood was suggested by the finding that the vast majority of BB1-positive cells also stained with an anti-CCR3-receptor antibody.

A flow cytometric assay was developed for measuring intracellular basogranulin expression upon cell activation. Flow cytometry of permeabilised "nonstimulated" basophils exhibited high expression of basogranulin confirming the intracellular localisation of this marker. Stores of intracellular basogranulin were depleted following basophil activation, whereas expression of CD63 and basogranulin on the cell surface (in nonpermeabilised basophils) was increased. The extent of basogranulin depletion varied from one experiment to another. Intracellular basogranulin depletion was demonstrated in basophils stimulated with anti-FcεRI. However, we found that levels if intracellular basogranulin did not change in some basophils suggesting that they may be non-responding basophils. CD63 expression was upregulated in activated basophils in an inverse relationship with expression of intracellular basogranulin depleted. The corresponding finding with non-permeablised basophils was that surface basogranulin and CD63 expression increased following stimulation. Intracellular basogranulin stores were depleted completely after the stimulation. This could be due to some basogranulin remaining inside basophils and/or bonded to the surface of basophil, which would be irrespectively counted as basogranulin positive basophils. Interestingly, we found the MFI for intracellular basogranulin expression following stimulation with anti-IgE was reduced by almost an order of magnitude. This may be because MFI reflects median fluorescence for the marker and as such it may provide more accurate assessment for the basophil activation as it is related to the extent of depletion of fluorescence intensity of basogranulin rather than percentage of basogranulin positive basophils. A method that can solely measure expression of intracellular basogranulin, will be valuable as a means for assessing basophil activation *in vitro*, and provide important information about basophil activation in disease.

The assay for measuring intracellular basogranulin stores was optimised for use without a need for basophil purification, making its utility more feasible and time saving particularly in urgent situations. The methods involved taking the advantage of a solution to lyse erythrocytes and permeabilise cells in one step. Detection and measurement of intracellular basogranulin may also provide a reliable means for enumerating basophils in whole blood and be of value in diagnostic haematology.

Monitoring depletion of basogranulin stores may provide valuable information on the course of basophil activation *in vitro* and add to our understating of how basophils can contribute to mediating an allergic reaction. Indeed, basogranulin has been established as a new immunohistochemical staining technique for the detection and enumeration of basophils in routinely processed (formalin-fixed and paraffin-embedded) bone marrow trephine biopsy sections [277].

In parallel with studies of intracellular basogranulin, we developed a reliable flow cytometric assay for measuring surface basogranulin. The assay was initially applied to purified basophils, but it was further optimised for measuring basophil activation in whole blood without the need for purification, as since there is an increased risk of cell loss during the centrifugation steps and excessive handling of the cells may interfere with their responsiveness to activation. We demonstrated stimulation of the FcεRI with anti-FcεRI or anti-IgE antibody, of the fmlp receptor with fmlp, and of allergen receptors with their corresponding allergens all rapidly induced surface basogranulin expression in a concentration dependent manner. Overall, basogranulin expression seemed to be associated with that of CD63, though there was a degree of variability in expression of these markers between individuals.

The heterogeneity of basogranulin expression was observed in basophil populations not only at different time points following stimulation, but at the same time suggesting differences in the degree of degranulation between cells. Fluorescence intensity for activated basophils ranged from about 30 to 3000 relative units of fluorescence, which may reflect the number of granules per basophil and/or in the amount of marker (basogranulin or CD63) per granule.

The MFI of basogranulin in unstimulated basophils was extremely low, but increased significantly up to ten fold in response to stimulation with different concentrations of anti-IgE antibody at 0.1 µg/ml. However, basogranulin MFI decreased gradually upon subsequent higher concentrations of anti-IgE by about 80% at 100 µg/ml. In contrast, CD63 has a narrower

expression range of about 50 to 1200 units. The maximal increase in MFI of CD63 was higher than that of basogranulin (25 times at 0.01 μg/ml), and it decreased by more than 60% upon subsequent increasing doses of anti-IgE at 100 μg/ml. It seems that CD63 staining gives rise to a semi- bimodal distribution whereas basogranulin staining causes a unimodal distribution with a less clear discrimination between activated and nonactivated basophils suggesting a wide range for expression of basogranulin. Similar findings were seen by confocal microscopy as some basophils exhibited disparity of basogranulin staining.

An explanation for the upregulation of basogranulin on the surface of activated basophils is that the granule membrane may fuse with the cell membrane during the degranulation process, and consequently, basogranulin is exposed on the external surface of the cell membrane and not dispersed after secretion from the granules. Another possible explanation is that some of the released basogranulin adhered to the cell surface because of its highly positive charge which would electrostatically favour adsorption to the negatively charged proteoglycans on the membrane of basophils [275] [301]. However, we demonstrated that incubating basophils with a range of concentration of purified basophil lysate (as a source of exogenous basogranulin) for different time points had no effect on expression of basogranulin on the surface of those cells as analysed by flow cytometry. Also, it is possible that the positive charge of basogranulin was neutralised by fragments of DNA or RNA which might be found the basophil lysate. These findings may suggest that upregulation of surface basogranulin is associated with the activation process *per se.* Some basophils exhibited weak expression of basogranulin on the cell surface after activation. A possible reason is that most of the released basogranulin does not bind to the cell surface but end up in the supernatant.

Findings with the new basogranulin-based assay are similar to those with the fluorescent-avidin–based method for staining of anionic proteoglycans from exteriorized basophil granule matrix by cationic fluorescent avidin probes [301, 302]. However, other studies it has been reported that the avidin-based methods produce results that are similar to those of traditional CD63-based basophil activation testing suggesting that both methods are measuring changes in the same cellular compartment. Although it enabled simple and rapid assessment of basophil activation, the avidin-based method might not be superior to a conventional CD63-assay. Also, it is possible that the avidin-based method cannot be applied as a diagnostic test in avidin-sensitized patients because of binding of the probe to anti-avidin specific IgE/FcεRI complexes that are present on the membrane of resting basophils. Nonetheless, basogranulin

expression appears to be under the control of the same receptors and signalling pathways as those for CD63 expression suggesting these markers are co-expressed and under the same control mechanisms. This idea is in accord with the subcellular localisation of basogranulin in the secretory granules. Measuring alterations in their expression should provide a useful means for monitoring basophil degranulation.

By means of co-staining with APC-labelled BB1 and FITC-labelled anti-CD63 antibody, we next monitored differential marker expression on activated basophils. We assessed whether the two methods were identifying the same cell subsets within the population of nonstimulated basophils and in anti-IgE stimulated basophils. Notably, we identified four subsets of CD63+ or basogranulin+ basophils (basogranulin+CD63+, basogranulin+CD63-, basogranulin-CD63+, basogranulin-CD63-). The majority of nonstimulated basophils were negative for both markers (basogranulin-CD63-) though small percentages of basophils were stained with either basogranulin or CD63 alone. Anti-IgE activated basophils were mainly double-positive for marker staining (basogranulin+ CD63 +) though subset of activated basophils expressed C63 only (basogranulin- CD63 +). While the percentage of double positive basophils increases in a concentration dependent manner, and the percentage of CD63 positive basophils decreased accordingly.

We further analysed differential expression of these markers in fmlp or anti-FcεRI stimulated basophils from five donors. Similarly, differential expression of basogranulin and CD63 in fmlp and anti-FcεRI stimulated basophils seemed to be heterogeneously variable among each of the five donors, suggesting individual variability in response. The vast majority of nonstimulated basophils were double negative (basogranulin-CD63-). However, we observed that the proportion of basophils that expressed basogranulin alone (basogranulin+CD63-) were a slightly increased (13%) in nonstimulated basophils. A possible explanation is that basophils were activated *in vivo* with natural exposure to the culprit allergen, in which case basogranulin may be a more sensitive marker for measuring basophil activation in vivo. Another possible explanation could be that this represented spontaneous (basal) expression for that subject. In any donor, variation in basophil numbers, responsiveness, marker expression, shape, size or other features may be affected in the same donor which all can be affected by numerous factors such as hormonal variation, nutritional status, time of basophil sampling and or concentration of allergen. It is not clear whether that increase was as a result of basophil activation *in vivo* or naturally occurring spontaneous expression which varies among

individuals. It will be interesting to determine if the ratio of basogranulin to CD63 expression could provide valuable information on the status of basophil reactivity. This measure could have important implications for measurement of basophil sensitivity, and perhaps prediction of severe allergic reactions. It will be important to explore further the possibility high "baseline" levels of basogranulin positive basophils in allergic patients may help to identify those subjects at risk of exhibiting strong clinical reactions to the offending allergens, including patients who are enrolled in immunotherapy procedures. There is still much to learn about the underlying processes of marker expression (basogranulin and CD63) and how expression of these markers may be controlled by immunotherapy and other pharmacological treatments. Knowledge of novel basophil-dependent pathways in allergy could allow researchers to explore for specific inhibitors that can interfere with basophil activation in allergic diseases.

When we investigated expression of basogranulin and CD63 in anti-FcεRI activated basophils over time, we found that basogranulin expression rapidly increased soon after activation, but then gradually decreased over time of activation. The maximum reduction (20%) has been observed after 60 minutes of activation. Mochizuki et al. reported similar observations regarding the release of basogranulin upon activation with anti-IgE antibody [275]. In contrast, CD63 was shown to gradually increase over time, reaching maximal expression after 10 to 20 minutes, but it remains unchanged afterwards. For this reason, 15 minutes was employed as a standard period for *in vitro* stimulation of basophils.

The maximal expression of double positive basophils (basogranulin+CD63+) was observed at 15 and 30 minutes. However, the numbers in this subset decreased in each of the 5 donors, whereas basogranulin-CD63+ basophils were increased proportionally. The reason that basogranulin expression decreases over time following cellular activation is yet to determined. A possible interpretation is that basogranulin may have a different pathway for elimination that in CD63. Basogranulin may have dissociated from the cell membrane into the supernatant. Another possible explanation is that membrane-bound basogranulin might undergo modification, proteolytic degradation by intrinsic and/or extrinsic factors. BB1 has been found to recognise a native form of the protein that could not be detected under reducing conditions on SDS-PAGE[271, 274]. Also, it is possible that basogranulin dissociates over time into other forms (derivatives) as its natural mechanism of inactivation. Identification of a polymorphic (altered) form of basogranulin should provide important information about the regulation of basogranulin and its fate. The explanation of this phenomenon remains to be

disentangled, but it could be related to negative feedback process. Alternatively, basophils may be desensitised over long exposure to anti-FcεRI. More data are required, as it is not clear whether basogranulin diminution would continue over longer periods of activation, and whether basogranulin expression will return to the basal level of expression at some point. If these findings are confirmed on further studies, then this could be important for understanding the kinetics of basogranulin release, expression and clearance.

Studies of differential expression of basogranulin and CD63 showed that 93% of nonstimulated basophils were double negative (basogranulin-CD63-) and 5% expressed basogranulin alone (basogranulin+CD63-). Fmlp activated basophils indicated that almost a 25% of gated basophils are basogranulin positive whereas almost half of the basophils were negative for both markers. Some two-thirds of basophils were double positive while the remining third compromised the other subsets with some variability (basogranulin+CD63-, basogranulin-CD63+, basogranulin-CD63-). Deferential expression of basogranulin and CD63 could be of value for improving the assessment of basophil activation.

The effect of anticoagulant type on expression of basogranulin and CD63 in nonstimulated and stimulated basophils was investigated by quantifying the percentage of marker expression or MFI. Basophils were found to exhibit expression of basogranulin and CD63 on fmlp, anti-FcεRI or IL-3 stimulated basophils obtained from blood that were collected in either EDTA or heparin. There was not apparent deference between the expression of basogranulin and CD63 in EDTA or heparin following anti-FcεRI stimulation. However, basogranulin and CD63 expression was higher following fmlp stimulation in basophils obtained in heparinised blood than that of EDTA. This may be because of that the chelating effect of EDTA on calcium which plays an essential physiologic role on cell function. This suggests that upregulation of basogranulin requires physiologic extracellular concentrations of calcium, which is consistent with the current understanding that basophil activation is dependent on extracellular calcium and magnesium [303, 304]. The low expression of basogranulin or CD63 in EDTA samples can be compensated for by addition of exogenous calcium/magnesium. Some commercial kits for measuring basophil activation, such as those manufactured by Bühlmann, use EDTA as an anticoagulant and add calcium to permit the stimulus-dependent expression of a CD63 positive basophil population [98, 305]. However, adding exogenous calcium prolongs the procedure and may impact on reliability of results. Also, addition of calcium may induce platelet aggregation, that might have an impact on results. Therefore, we recommend that basophil

activation testing is performed on blood collected in heparinised tubes if basophil activation is to be analysed.

The effect of the type of anticoagulant (EDTA or heparin) was apparent when MFI of basogranulin was measured. Interestingly, MFI of basogranulin and CD63 in response to stimulation with fmlp did not increase, but it did with anti-FcεRI stimulation. This may suggest that the fmlp did not induce full degranulation of basophils and as a result the fluorescent intensities of the markers were relatively low. It is possible that weak expression was a consequence of a suboptimal concentration of fmlp being used. In contrast the expression of activation markers in basophils which were stimulated with anti-FcεRI were significantly upregulated and this was also consistent with their corresponding MFI which were higher than that seen with of fmlp stimulation.

There was a difference between the expression of basogranulin in EDTA and heparin over time, and that difference continued over time in a parallel response for up to 60 minutes, whereas CD63 expression was not affected by the type of anticoagulant used. These findings may suggest that basogranulin is more sensitive, to the changes in the surrounding environment that is influenced by the type of anticoagulant, than CD63. Also, negatively charged heparin may mask positively charged basogranulin, leading to lower antibody binding.

We tested whether immunostaining basophils with BB1 and anti-CD63 before or after the stimulation had an influence on measurements of these markers. It has been found that staining before stimulation enabled higher quantification for basogranulin expression, suggesting better protocol for detection. MFI of both markers was higher in basophils which were immunostained before the stimulation than that immunostained after stimulation. A possible explanation is that staining before the stimulation allowed additional time for antibodies to bind to corresponding antigens. Confirming these findings in further studies should improve the protocol for the newly developed basogranulin based assay.

The patterns of response curve for all of the subtypes of basophils may suggest basogranulin and CD63 may share in some kinetics of expression. A possible explanation is that MFI of both markers showed bell-shaped response compared to semi-sigmoidal response once marker expression was quantified is that MFI seems to indicate the degree of activation because it measures the extent of marker expression among the basophil positive population rather than

quantification of the percentage basophils which stain positive for a marker, regardless of the amount of expression of that marker.

Findings of flow cytometry showed concentration dependent response in surface basogranulin expression in response to stimulation with anti-IgE *verses* buffer. These findings were also confirmed with other stimuli (fmlp or anti-FcεRI) and found that basogranulin rapidly increased on the surface of activated basophils but remained unchanged on the nonstimulated cells. However, there was clearly variation in expression of both basogranulin and CD63 among individuals. It could be that basophil may be activated by different pathways, mediated by FcεRI or other receptors [105].

In some experiments, there was more basogranulin expression than was apparent for CD63 expression in basophils even without stimulation. This may indicate that basogranulin may be a more sensitive marker than CD63. This finding may aid in monitoring the extent of basophil responsiveness for allergic patients. It will be valuable to further explore whether detection of high ‘‘baseline’’ levels of basogranulin on basophils of food-allergic patients may represent potential indication for identifying subjects who are at risk of exhibiting strong clinical reactions to the offending allergens, including patients who are enrolled in immunotherapy protocols. Perhaps the detection of basogranulin positive basophils in specimens incubated only with buffer reflects low ‘‘basal’’ levels of activation even without *ex vivo* antigen stimulation and/or persistent effects of previous, perhaps subclinical, episodes of activation for instance that is related to the oral immunotherapy treatments or because of unintentional exposure to small amounts of offending food allergens, and/or is due to particular intrinsic features of the basophils of some allergic subjects. It also is possible that some of the differences observed at ‘‘baseline’’ in the extent of basogranulin binding versus CD63 expression on basophils from various groups of food allergic subjects may reflect a longer persistence of basogranulin-biding content on the basophil surface in such subjects compared with elevated levels of CD63. These and other possible explanations of our findings should be analysed in future studies of larger groups of subjects. However, whatever the mechanisms accounting for the basogranulin expression observed at baseline in the blood of many of the allergic subjects, such evidence of potential current (or previous) activation was not observed in the same specimens by assessment of CD63.

Basophils exhibited heterogeneity in basogranulin granule content and time of antigen stimulation. Basogranulin was poorly expressed in basophils from some individuals after

stimulation by anti-FcεRI and grass pollen. In each sample, basophils were variably activated and that may be because of their random distribution of the IgE specific for the allergens tested. In routine practice, there are always a proportion of basophils that is not activated. In case they respond, the degranulation was usually rapid and massive with very high and homogenous expression of CD63. It is commonly assumed that basophils can survive strong stimulation, but this may be not necessarily true. In any case, in basophil activation testing, degranulation is only measured on individual basophils which have maintained their shape and identity. This could explain some bell shape of the dose-response curves with progressive response to increasing doses but reduced response to the highest stimulation. Another possibility is that allergen extracts contain additives that are cytotoxic at the highest concentrations used. It is difficult to measure cell survival as basophil absolute count is not possible in basophil activation testing because of their rarity and the multiple steps in sample preparation [306].

There were basophils in which basogranulin and CD63 were not increased and these basophils may be non-responders suggesting a defect in early signalling, particularly spleen tyrosine kinase phosphorylation[81].However, the expression of surface basogranulin and CD63 did not show the same pattern in some basophil preparations following anti-IgE stimulation. In some cases, basogranulin expression was upregulated whereas CD63 expression was not. Similar findings were also seen with IL-3 stimulation. These findings suggest that basogranulin may be a more sensitive marker than CD63, and this may raise the concerns about basophil activation assays based on CD63, with cells being wrongly considered as non-responders. Further studies are, however, required to investigate and confirm whether Syk deficiency impact on basogranulin expression.

A major difference on current findings involved the lower basal expression of basogranulin and the extent of its expression after stimulation in comparison to those presented in earlier studies by Mochizuki et al. [275]. The limitations in their studies could be due to that the use isolated basophils in which their responsiveness could have been impacted throughout purification procedures. Our findings, therefore, seem to be superior because of the advent of the newly developed assay for assessing basophil activation in whole blood without the need for cell purification. However, in some cases, when basophils were stimulated with different dilution of anti-IgE, the expression of basogranulin showed a bell shape response while CD63 did not, which was rather weakly expressed. Our data showed a bell-shaped response curve

for basogranulin expressed on the surface of basophil induced by anti-IgE with maximal expression (88%) observed at optimal concentration of 0.1% compared by with only (35%) for CD63 at anti-IgE concentration 0.001%.

Being an *in vitro* surrogate of the allergic reaction that occurs *in vivo* in patients, basogranulin-based assays can be used to support the diagnosis of various allergic conditions, such as food, drug, respiratory and insect venom allergies, and the assessment of clinical response to allergen-specific immunotherapy treatments. Also, this assay can also be used to explore the mechanisms of allergy and tolerance at the level of the basophil, for instance by studying intracellular signalling cascade in response to allergen. Further, it can also relate to the severity of allergic reactions in that patients with more severe reactions may show a greater proportion of activated basophils and patients reacting to trace amounts of the allergen show a greater basophil sensitivity, meaning their basophils start reacting at lower allergen concentrations. This can predict therapeutic outcomes and monitor treatment responses. Our data also suggest that this method might represent a more specific and sensitive alternative to CD63 for monitoring the activation status of blood basophils, and might even be valuable in measuring the extent to which food allergic subjects are at risk for developing severe clinical reactions to their offending allergen.

The major advantage of the assay is implementation of basogranulin as a novel specific marker in flow cytometric basophil activation testing. In addition, it does not need the use of laborious, expensive, long procedure purification, since small amount of blood can be sufficient for many tests. The test can be performed on whole blood rather than isolated basophils, due both to the simpler and faster manipulation of the method, but also leaving basophils in their natural environment ensures a better functionality. Additionally, it does not require large numbers of basophils as only 500-1000 basophils are required per test that can be readily obtained in 50 to 100 µl of blood per test.

IL-3 has been reported to enhance the upregulation of CD63 [97] [307]and synergise with stimulation through FcεRI to enhance activation of basophils by 30% [308]. IL3 enhanced sensitivity of basophils to FcεRI-mediated activation independently of extracellular calcium [309]. In this study, we tested whether exposure to IL-3 can influence marker expression (basogranulin and CD63) in nonstimulated and stimulated basophils. It has been demonstrated that basogranulin expression was to some extent influenced by the addition of IL-3. The percentage of basogranulin expression increased in basophils which were incubated with IL-3

with optimal expression at 10 and 20 ng/m, though the differences in basogranulin expression were low compared to that in cells without IL-3. In contrast, CD63 was not affected by the presence of IL-3. This may indicate that IL-3 has a role, though limited, in basogranulin signalling pathways. Thus, it will be important to consider exploring the effect of other interleukins on the expression of this markers, and this should expand our understanding about the kinetics of basogranulin.

Stimulating basophils with anti-FcεRI in the presence of IL-3 basophils showed that basogranulin expression increased though fluctuated within narrow. However, CD63 was increased more steadily in concentration dependent manner where the optimal expression was at 10 ng/ml but remained unchanged upon following increasing concentrations of IL-3. There were not important differences in expressions of basogranulin or CD63 at 5 and 20 minutes. However, MFI of basogranulin showed a concentration dependent in response to stimulation with IL-3. Also, MFI of basogranulin was relatively higher in basophils stimulated with anti-FcεRI in response to increasing concentrations of IL-3 for 20 minutes than that at 5 minutes. MFI of CD63 showed concentration dependent response to IL-3 in basophil activated for 5 or 20 minutes. However, we observed that basogranulin expression is slightly decreased in response to high concentration of IL-3 (200 ng/ml), suggesting that this interleukin may have inhibitory effect on basogranulin expression. However, these findings involve limitations of the curves and also the low number of experiments performed. Investigating the effect of higher concentrations Il-3 on basophils should be considered in future studies.

CCR3 expression was occasionally decreased or even undetectable, making basophil identification by flow cytometry a challenge. Therefore, MFI of CCR3 was monitored in nonstimulated basophils and stimulated with anti-FcεRI in presence or not various concentration of IL-3. Findings showed that CCR3 expression is influenced by activation, and also by the presence IL-3. CCR3 was expressed at high levels on nonstimulated basophils, but it was downregulated. IL-3 has been shown to enhance the MFI of CCR3 in a concentration-dependent manner in non-stimulated basophils. Also, prolonging activation time to 20 minutes has increased the MFI of CCR3 to almost double. However, addition of anti-FcεRI simultaneously with IL-3 seems to synergistically decrease the MFI of CCR3 in a concentration response manner. Further, prolonging activation time to 20 minutes showed further decreases in MFI of CCR3.

CCR3 down-regulation seems to be caused by different signalling events. A possible explanation is that the IL-3-induced down-regulation of CCR3 could be due to IL-3-induced release of CCR3 ligands such as eotaxin and RANTES. Also, it is possible that an internalization of CCR3 was induced from the surface of basophils by IL-3 binding to its receptor which may activates kinases such as PI3-kinase, which phosphorylate CCR3 and cause receptor internalization. However, the underlying IL-3 -specific signal transduction pathway leading to CCR3 internalization, an altered transcription of the CCR3 gene and consequently to reduced surface protein expression, should be clarified in further studies. Other possibilities of cellular events leading to the variation of receptor expression include endocytosis, exocytosis, shedding, modifications of the transcription of the gene, etc. These findings can be further validated by studying the expression level of CCR3 after IL-3 stimulation, to confirm whether the amount of CCR3 is diminished by IL-3. Nonetheless, these findings suggest a novel role for the cytokine IL-3 in the activation process of basophils and its marker CCR3.

The MFI of CCR3 exhibited a semi bell-shaped response in basophils stimulated with various concentrations of IL-3 for 5 minutes. Prolonging the stimulation time for 20 minutes increases CCR3 MFI up to almost a fold. However, MFI of CCR3 inversely decreased in basophils stimulated with anti-FcεRI in presence of various concentrations of IL-3 for 5 minutes. Further, the decrease of CCR3 MFI continued when the activation time was prolonged for 20 minutes with maximal decrease was at 200 ng/ml. This may suggest that the crucial role of IL-3 on CCR3 expression kinetics. These findings have important information for interpretation the decrease or disappearance of CCR3 expression after stimulation. Thus, optimisations the system so that CCR3 stability can be preserved, should enable to overcome these drawbacks of this marker, though identifying a more stable identification marker for basophils is still needed.

Using fluorescently- labelled BB1, alterations in expression of surface and intracellular basogranulin was further analysed in purified activated basophils using confocal microscopy to determine whether BB1 could stain basophils stimulated with anti-FcєRI or fmlp in different time points. Consistently with our flow cytometric studies, anti-CCR3 was employed to permit identification for basophils using confocal microscopy.

Intracellular basogranulin was analysed in basophils activated by anti-FcєRI and fmlp at different time points. Intracellular basogranulin was highly condensed within granules in permeablised basophils which were activated for 5 minutes with anti-FcєRI Some basophils exhibited depletion of intracellular basogranulin in basophils in response to stimulation to anti-

FcεRI for 10,20, and 30 minutes although the level of depletion varied from among basophils. A possible explanation is that basogranulin is found in the basophil granule, and degranulation of the cell results in basogranulin release leading to loss of staining. Another explanation for the heterogeneity of intracellular basogranulin expression is that some subsets of basophils may possess different amounts of basogranulin, or that subsets may respond differently to the stimuli. While this might be useful in determining the degranulation state of the cell in certain circumstances, it may result in an underestimation of the number of basophils present in a tissue of reaction. It is possibly related to a change(s) in accessibility of BB1 to the basogranulin stores. Although, the exact reason remains elusive and speculative, these the findings confirm our flow cytometric findings of MFI histograms of basogranulin.

Fmlp stimulated basophils exhibited differences in their expression of intracellular basogranulin. While some showed weak expression of basogranulin, others appeared to contain high levels intracellular basogranulin. However, this method did not permit discrimination between surface and intracellular basogranulin because BB1 bound to basogranulin irrespective to the location making discrimination between membrane and intracellular staining a challenge. This method can be improved by implementation of specific staining for membrane, and this should allow for better distinguishing of subcellular co-localisation of basogranulin.

Another limitation of this method was that the CCR3 was employed as an identification marker for basophils, in which can also be expressed on eosinophils. Thus, it would be better if a more specific marker can be employed for basophil identification in future studies for confocal microscopy analysis. Also, monitoring another independent activation marker such as CD63 or histamine (as internal control) may contribute for better understating of this marker. Another limitation is that visualisation was performed in different basophils in which basogranulin expression and responsiveness may vary between the cells. Basogranulin expression in nonstimulated basophils was not monitored by confocal microscopy because cells were lost during the procedures. Further studies are required to explore the subcellular distribution, function, and molecular structure of the basogranulin in basophils before, after activation. The biologic role of basogranulin must be studied in the context of an extracellular environment containing histamine, acidic proteoglycans, and the other constituents of the basophil secretory granule.

When nonpermeabilised basophils were investigated by confocal microscopy, it was found that nonstimulated basophils did not express basogranulin and that surface basogranulin expression was upregulated following stimulation. However, there was apparent variation in the amount and distribution of basogranulin in stimulated basophils. Basophils which were stimulated with anti-FcεRI for 5 to 30 minutes had variable expression of basogranulin in nonpermeabilised basophils. While some basophils exhibited robust expression of basogranulin on their surface, other basophils showed little expression of basogranulin. Some basophils had basogranulin condensed in their granules and seemed to be adjacent to the cell membrane in nonpermeabilised basophils activated with anti- FcεRI for 5 minutes. This may support the explanation of that granules fuse to the cell membrane during the process of release. It was observed that basogranulin on the surface of anti-FcεRI activated basophils slightly decrease after 20 to 30 minutes of activation, though some basophils showed moderate expression of basogranulin. Basogranulin expression was weakly expressed in basophils stimulated with fmlp for 5 minutes, but significantly increased when stimulation was prolonged to 20 minutes. These findings are in concordance with those analysed by flow cytometry, suggesting different extent of activation within basophil populations from the same donor. Thus, monitoring basogranulin expression over time in living cells (rather than fixed cells) by confocal video microscopy should allow for better analysis of basogranulin expression and that should provide robust information about the basophil activation processes.

6.2 Clinical applications of the newly developed assay for assessing allergic sensitivity

We investigated the validity of basogranulin flow cytometric assays for assessing basophil activation in clinical samples in response to dust mite, grass pollen, cat hair, and various food allergens. Basogranulin release exhibited heterogenous responses to stimulation with dust mites and grass pollen *in vitro*. These findings help to establish the concept that basogranulin is a reliable marker for measuring basophil activation. However, the findings on basogranulin release measurements were undermined by the small number of the experiments performed as a result of the shortcomings of the assays employed. If a better assay can be developed for measuring basogranulin release, it should provide direct evidence for a role for basophils in allergic diseases.

Measuring of intracellular basogranulin expression by flow cytometry was verified in basophils stimulated with grass pollen. While CD63 increase on the surface of activated basophils, there

was a corresponding decrease in expression of intracellular basogranulin, though the extent of depletion of basogranulin was less than that for CD63. A possible explanation is that activated basophils did not release the entire basogranulin contents of their granules after the activation process. Again, a limitation of these findings is that this assay did not discriminate between surface and intracellular basogranulin. In other words, surface basogranulin positive basophils were counted as intracellular basogranulin positive basophils. This can be improved by measuring MFI of the markers. Nonetheless, these interesting results may confirm the concept that basogranulin intracellular depletion can provide a valuable means for measuring basophil activation.

The overall correlation between intracellular basogranulin and surface CD63 was neither strong nor significant. However, the correlation in anti-IgE or an anti-FcεRI stimulation was higher and significant, whereas grass pollen was not. The correlation was analysed based on the marker expression which may not accurately reflect the degree of expression because that BB1 stains any basogranulin positive basophils irrespective to site on the cells (whether the expression is intracellular or on the cell surface). Thus, we anticipate that the correlation could be higher if the basogranulin MFI is considered for analysing basogranulin expression.

We further applied the newly developed assay for measuring surface basogranulin in assessing basophil activation in clinical samples for assessing basophil sensitivity in response to stimulation with various concentrations of different specific allergens. Our findings confirmed that validity of surface basogranulin in assessing basophil activation. Overall, basogranulin expression has been shown to concomitantly upregulate with CD63 on the surface of activated basophils and correlation between the two was strong and highly significant. This correlation is strongly resembling to that of basogranulin and histamine release [275]. Yet, some findings showed that basogranulin expression is even higher particularly in food allergen activated basophils, though CD63 was higher than basogranulin in grass pollen activated basophils. Interestingly, basogranulin has been found to also expressed on the cell surface of basophils following stimulation with low concentrations of allergen including Oyster, crab, *D. pteronyssinus*, shrimp, *D. farinae*. This suggests that basogranulin may be more sensitive marker for basogranulin than CD63, and it seems that basogranulin can provide indication of the early phases of activation whereas CD63 is only upregulate upon relatively high concentrations. Because measuring surface basogranulin is reliable and robust means for

assessing basophil activation in vitro, further applications on a larger scale of samples should provide direct evidence for a role for basophils in health and disease.

Our flow-assisted dual analysis of surface and intracellular basogranulin expression at a single cell level may provide important information that are inaccessible for traditional techniques that require homogeneous cell populations and of which results represent a mean of isolated cells analysed. Measuring intracellular basogranulin expression may mirror its release, and therefore it can be reliable means for assessing basophil activation. Because basogranulin is granular protein, measuring its intracellular expression may serve as a new means to investigate whether degranulated basophils can regranulate during recovery from degranulation in vitro (i.e. whether basogranulin-rich granules are reconstituted in basophils after stimulated secretion). This may establish for a means in monitoring 'basophil recovery' which can provide useful information assessing the clinical status of allergic reaction.

Detection of basal basogranulin expression may improve the sensitivity of basophil activation assay by two factors. First, the recognition of basophils is better with the use of BB1. However, because it is an activation marker, it is absent or weakly expressed on the surface of normal resting basophils, and therefore surface basogranulin seems to be of limited value in nonactivated basophils. However, permeabilising basophils should allow for staining of intracellular basogranulin and this should permit for better identification and quantitation of basophils in tissues including peripheral blood. We know that intracellular basogranulin decrease in activated basophils. However, the extent to which the depletion of this marker impact on the identification and enumeration of basophils is yet to be established.

6.3 Measuring basogranulin release as a means for investigating basophil activation in clinical disease

Assessing the extent to which the activation of basophils *in vivo* may contribute to the symptoms of allergic conditions has been a challenge. Although basophils have the capacity to release several potent mediators of inflammation, their mediators have been reported to be produced also by mast cells, eosinophils or by other cell types that are likely to be involved in inflammation and allergic conditions. Basogranulin is a specific marker for basophils and as such its measurements in biological fluids can provide a direct evidence for assessing basophil activation *in vivo* [275]. In this study, we detected and measured basogranulin in saliva and BAL fluids by BB1 antibody and dot blotting.

The decrease in basogranulin levels in saliva from 83 subjects following oral challenge was unexpected.

The saliva samples were anonymous, and no information was available on which cases had allergen or a control material and therefore these findings represent preliminary data. However, measuring this marker in a study involving more samples with related details should allow measuring basogranulin in these biologic fluids and could be of value in investigating basophil-driven mechanisms of disease and confirming that an allergic reaction has taken place.

We also measured basogranulin in BAL fluids of healthy subjects and patients with atopic and severe asthma. The correlation between the levels of basogranulin levels in the three groups were not significant. Detection of basogranulin even at low levels in BAL fluid for healthy normal people may suggest that basophils may contribute to physiological processes in the airways. However, basogranulin concentrations in BAL fluid from patients with atopic or severe asthma has been found to be higher than that of healthy controls. Possible interpretation is that the basophils infiltrated lung tissue in asthmatic patients, in which their releasability was increased as well. Basophils have been reported to increase in the airway's tissues of asthmatic subjects as these cells can respond to allergic stimuli by migrating and accumulating at sites of allergic inflammation [177]. Moreover, basophils have been reported to be increased in sputum of asthmatic patients [310, 311] as well as in the sputum or BAL fluid during exacerbations or after allergen challenge of asthmatic patients [183],[180, 185, 312],[313]. Furthermore, basophils have been detected in the lungs of patients with fatal asthma [181]. An interesting study showed that BAL tryptase and histamine levels were are higher in asthmatics than in normal subjects although levels of tryptase and histamine in BAL were not correlated suggesting that pulmonary basophils, rather than mast cells, could be the source for histamine in those patients [314]. Unlike histamine which can be produced by various types of cells, BAL basogranulin may indicate the role of basophils in atopic asthma and severe asthma as two examples for pulmonary disorders. If basogranulin concentration per basophil can be determined, then this could be a potential means to indirectly assess the number of basophils infiltrated, and this should have important implication for prediction the extent of the basophil response to a culprit allergen. Also, it will be important to investigate whether *in vitro* basogranulin release is increased in atopic subjects and that the clinical severity of some atopic conditions correlates with this increase.

The recruitment of basophils to the lungs in allergic subjects is not fully understood. Thus, it will be interesting to study the exact mechanism by which increased basogranulin release levels relates to enhanced basophil infiltration. In addition, measuring basogranulin in other biological fluids could provide important information about the underlying processes of basophil recruitment and activation and the mechanisms that result in modulation of the function and their numbers by treatment. Our findings may support the hypothesis that basophils are implicated in asthma and play a major role in the pathogenesis of atopic asthma and severe asthma in agreement with other studies [315, 316]. In conclusion, the levels of basogranulin in BAL fluid suggest that there may be constant degranulation of basophils in the normal lung, but that the process is enhanced in patients with atopic and severe asthma. The increased concentrations of basogranulin have the potential to play an important role in the pathogenesis of these diseases.

Although, there was not a correlation between the levels of basogranulin in saliva from between the three groups, if basogranulin measurements can be applied on a larger number of samples, and be found to be associated with clinical outcomes, then this may confirm a key role for basophils in the development of asthma. The diagnostic value of determining basogranulin levels in BAL fluid or other body's fluids could be used as means for discriminating between allergic and nonallergic asthma, and whether these measuring levels could provide valuable information for monitoring the status of allergy condition is yet to be established. These findings will enhance understanding of the pathogenesis of allergic asthma and provide a basis for diagnosis and perhaps therapeutic targets of allergic asthma.

While basogranulin in saliva and BAL fluid has been shown to be a potential surrogate marker of basophil activation *in vivo*, there is still work that must be completed to fully establish the usefulness of this marker together with mast cells and eosinophils markers in clinical practice. The best methods to measure these cells markers should be employed and made more widely available for application in clinical studies. Further, the diagnostic value of monitoring altered levels of basogranulin deserves attention either in cohort of patients undergoing challenges or those who receive immunotherapy. Also, investigating and analysing the levels basogranulin and compare them with other markers such as tryptase or histamine could provide valuable indications on the course of the disease. Monitoring basogranulin levels can involve measuring the concentration in the body fluids of patients pre and post challenge or before and after treatment. Nonetheless, understanding the mechanisms regulating the release and expression

of markers and will provide practical application in the management of diseases associated with basophil activation.

Summary

We now have confirmed that basogranulin measurements can provide valuable information on assessing basophil activation following exposure to allergen and other stimuli (Figure 6-1). Basophil activation tests using basogranulin as the activation marker can represent robust and reliable means for in vitro assessments of allergy sensitivity. These novel developments provide new insights for research and should allow a better assessment of the true roles of these neglected cells in allergic diseases. The present studies open the way for addressing unmet medical needs in diagnosis of allergic reactions, and perhaps confirming and predicting those at particular risk of reactions. This is an exciting time for basogranulin research, and this marker could in the future be employed in routinely testing in allergy clinics and provide evidence as to what may be the unique contribution of basophils in allergic disease. We expect that the basogranulin will provide novel and exciting information relating to its *in vivo* function not only in allergic reactions but in pathological conditions in which basophils are implicated.

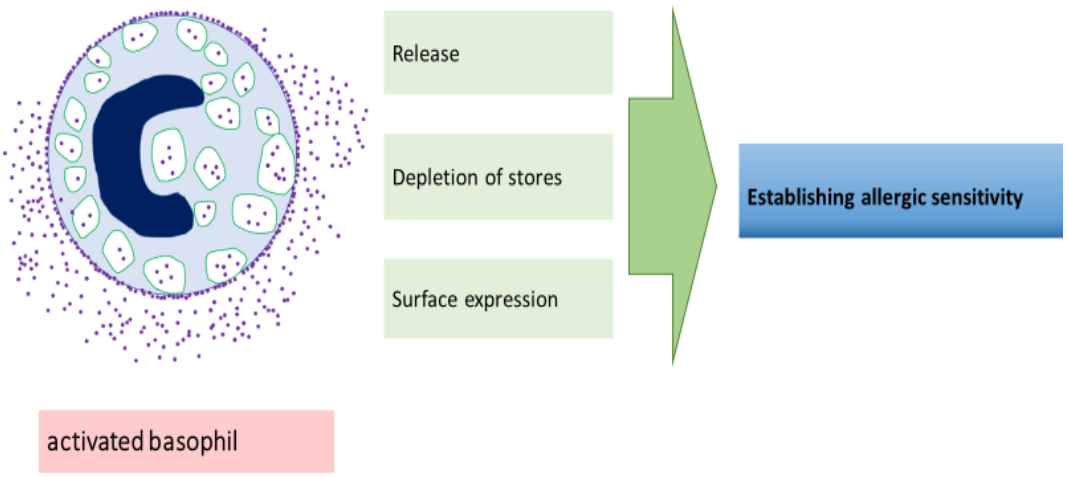


Figure 6-1. A schematic summary for novel approaches of measuring basogranulin as a marker for basophil activation in allergic sensitivity.

Appendices

1) Basophil staining

Basophils stained with alcian blue and Kimura stains coloured cyan and purple respectively (Figure 6-2). Non basophil cells did not stain for Kimura.

A B

Figure 6-2. Staining basophils with alcian blue, Kimura stain:
A) purified basophils stained with alcian blue (cyan), B) basophils stained with Kimura stain (purple). Unstained cells are not basophils. Note that basophils clumped after purification.

2) Confocal microscopy

LAD2 cells were stained with BB1- conjugated to APC and the nucleus stained with DAPI. Scant amount of basogranulin could be visualised in some cells (Figure 6-3 A). basophil stained with isotypes to BB1 and anti-CCR3 were showed no expression for basogranulin or CCR3 suggesting that there was nonspecific cross reaction to these markers (Figure 6-3B).

A B

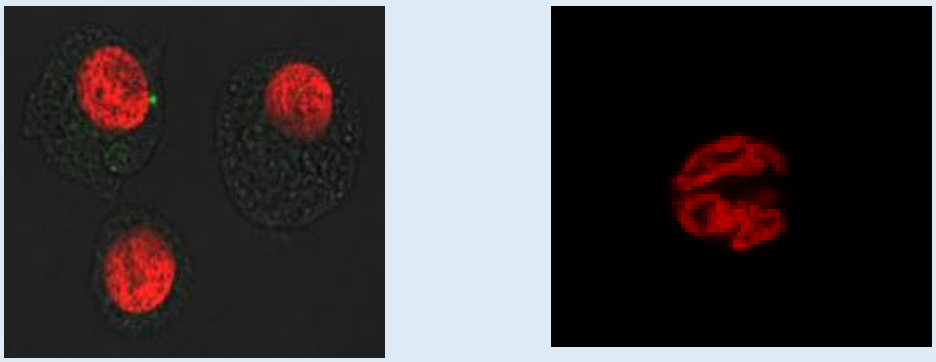


Figure 6-3. Detection of basogranulin in LAD2 cells by confocal microscopy.
(A) LAD2 cells showed small amount of basogranulin (green) as detected by APC-labelled BB1 and (B) single basophil stained fluorescently labelled isotype antibodies for BB1 and anti-CCR3. Nucleus were stained with DAPI (red).

3) Screening monoclonal antibodies for basogranulin

A range of monoclonal antibodies were screened for basogranulin using indirect ELISA. BB1 detected basogranulin when low dilution was employed whereas higher dilutions were not successful. Other monoclonal antibodies showed variability in the optical intensity when diluted.

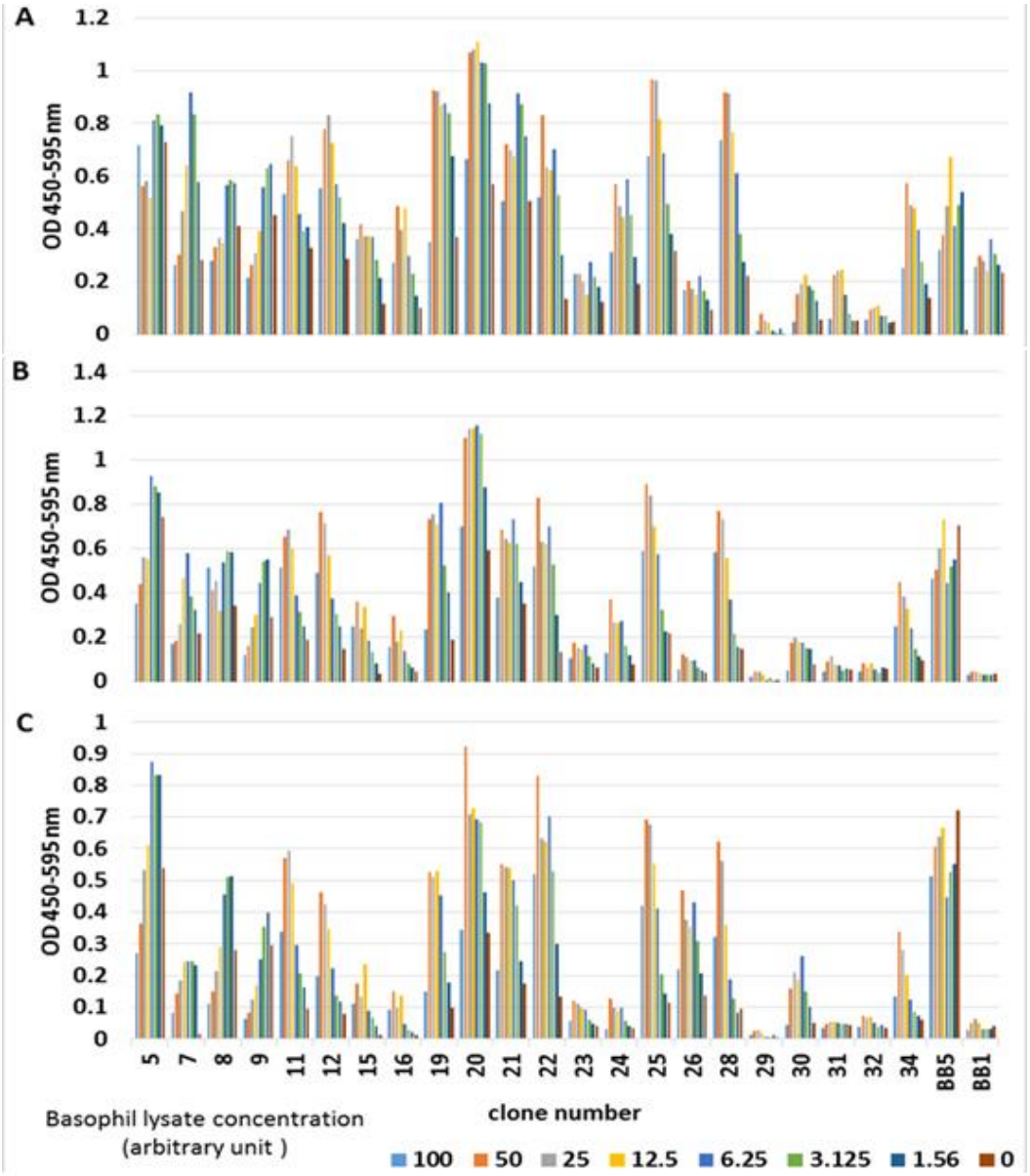


Figure 6-4. Indirect ELISA for screening of new antibodies for basogranulin in different dilution. Monoclonal antibodies (A) 1/5 (B) 1/ 10 and (C) 1/20 diluted hybridoma cell antibody-rich supernatant of numerous clones for detection of partially purified basogranulin coated on high binding plates. A 3% BSA in PBS was used as for dilution antibodies as well as elimination non-specific binding. Peroxidase-conjugated rabbit anti-mouse immunoglobulins were used as secondary antibody. The reaction developed by TMB at 20 ^{o}C. Mean of duplicate determinations are shown.

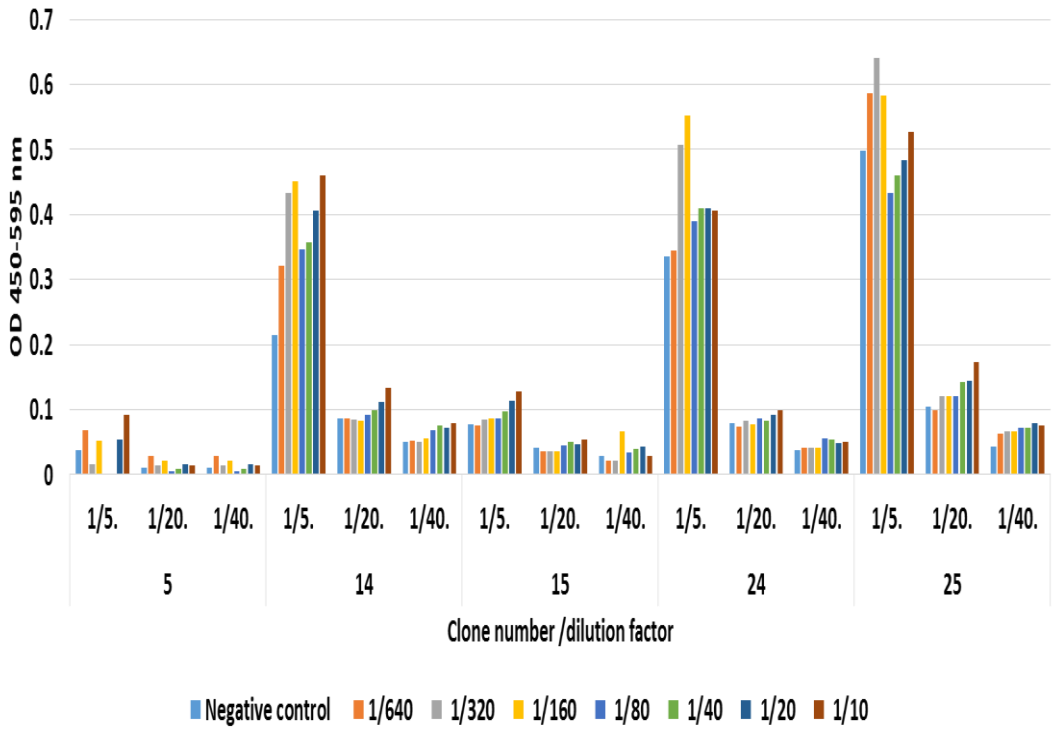


Figure 6-5. Sandwich ELISA for five monoclonal antibodies for detection of basogranulin. Different dilution (1/5, 1/10/ 1/20) of hybridoma cell supernatant for five clones (5,14,15,24,25) were further tested for detection of range of basogranulin concentrations in basophil lysate. BB1 (as a capture antibody). A 3% BSA in PBS was used as for dilution antibodies as well as elimination non-specific binding. Peroxidase-conjugated rabbit anti-mouse immunoglobulins were used as secondary antibody. The reaction developed by TMB at 20 ^{o}C. Mean of duplicate determinations are shown.

Lower limit of quantification for basogranulin in BAL fluids.

Basogranulin levels in 5 BAL fluids were shown to be lower than the LLOQ of basogranulin standard as determined by the FDA bioanalytical validation method guidelines (Figure 6-6).

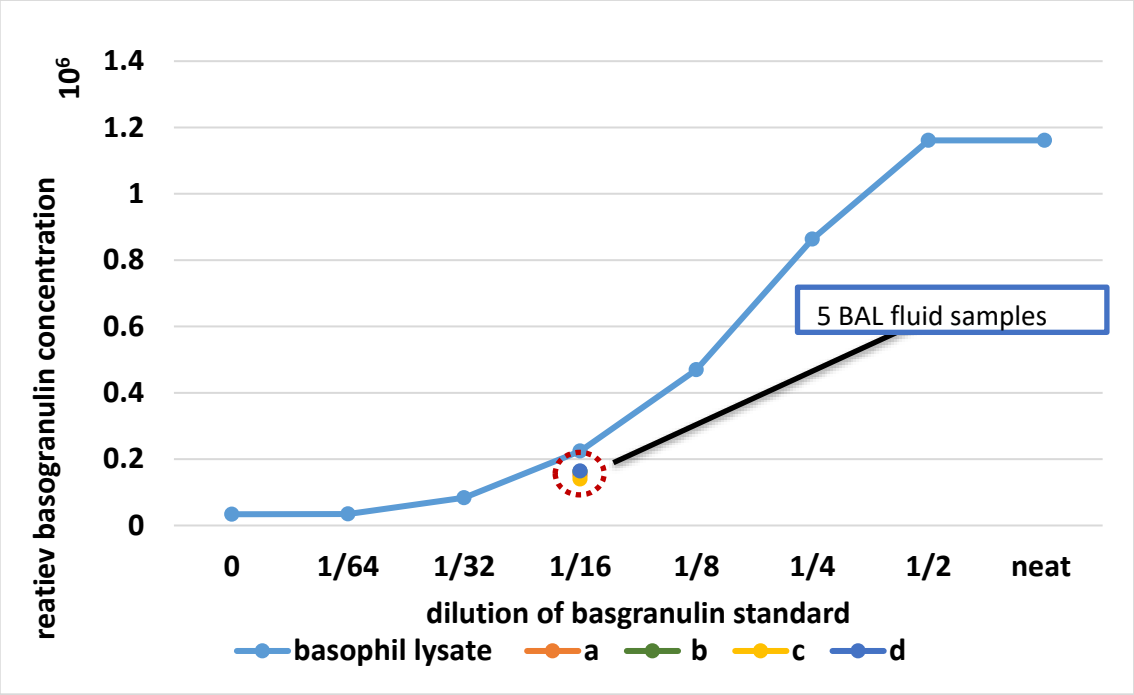


Figure 6-6. Measuring of basogranulin in BAL samples from five subjects.
The basogranulin concentration of BAL fluids was measured serial dilutions of purified basophil lysate (10^5 basophils/ ml) as a basogranulin standard. Dashed line indicates the lower limit of quantification as determined according to the FDA bioanalytical method validation [284].

Appendix A

A.1 Activation of peripheral blood basophils in allergic sensitivity

A.1.1 Information sheet

Study Title

Activation of peripheral blood basophils in allergic sensitivity

LREC ref: 05 / Q1702 / 9

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for reading this.

What is the purpose of the study?

To evaluate new procedures for the assessment of allergic disease. This involves measuring the ability of certain white blood cells called 'basophils' to become activated in the presence of allergen. This project will use basophils to study release of histamine and a protein product, which can be analysed in the laboratory. The project will involve various laboratory tests.

Why have I been chosen?

You have been chosen as you have shown an interest in research. You may have a history of allergies. There will be 40 volunteers involved in this study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide

to take part you are still free to withdraw at any time and without giving a reason. This would not affect the standard of care you receive.

What would happen to me if I take part?

You will be asked to visit the department once for this study. You will have a skin prick test and will be asked to provide 100ml of blood. The skin prick test will test your skin's reactivity to common environmental allergens. This will involve putting a drop of fluid containing allergen onto your skin, then pricking your skin with a needle through the droplet. If you have a reaction to the allergen, a small red lump will appear on your skin which should disappear within a day or two.

What do I have to do?

There are no lifestyle restrictions in this study.

What is the drug or procedure that is being tested?

Blood (100ml) will be taken for studies with basophils and allergic sensitivity to up to 10 common environmental allergens will be investigated by skin testing.

What are the side effects of taking part?

Giving blood can cause some bruising and could cause some soreness or stinging. With the skin prick test, the surrounding skin may itch, redden and swell up if you are allergic to a particular allergen. If you are concerned about your health at all after your visit, please call Dr Andrew F Walls on 023 8079 6151.

What are the possible benefits of taking part?

You will not directly benefit from taking part in this study. However, the research may lead to an increase in the knowledge of the mechanisms of allergies and could lead to the development of further therapies.

What if something goes wrong?

In the unlikely event that you become ill or are injured as a result of taking part in this study then the normal NHS and University complaints procedures will be available. In this case please contact the following researcher:

Dr Walls (tel: 023 8079 6151), Immunopharmacology Group, Southampton General Hospital.

Would my taking part in this study be kept confidential?

All information, which is collected about you during the course of the research, would be kept strictly confidential. All information about you would have your name and address

removed so that you cannot be recognised from it. Your blood sample will remain unidentifiable.

What will happen to the blood sample?

Blood will be kept for further analysis but will be unidentifiable. Several different tests will be performed on the blood within the laboratory. Your sample will be stored (in a way which is not identifiable) and may be used in future research if ethical approval is given for this.

What will happen to the results of the research?

The results of the research will be prepared for publication in a scientific journal, and may be presented at a scientific research meeting or included in a thesis that is examined as part of a medical student's training.

Who is organising and funding the research?

The Thrasher Research Fund, the London Law Trust, Allergy Therapeutics and the University of Southampton are contributing to this research.

Who has reviewed the study?

The Southampton and South West Hampshire Local Research Ethics Committee.

Contact for further information:

Dr Andrew Walls, Tel 023 8079 6151, Email: afw1@soton.ac.uk

Thank you for taking the time to read this.

If you decide to take part in the study, you will be given a copy of the information sheet and a signed consent form to keep.

A.1.2 Consent form

Study Number: Patient Identification Number for this trial:

CONSENT FORM

Title of Project:

Activation of peripheral blood basophils in allergic sensitivity

Name of Researcher:

Dr Andrew F Walls

PLEASE **INITIAL** THE BOXES IF YOU AGREE WITH EACH SECTION:

PART A Consent for the main study

I confirm that I have read the information sheet dated **01/02/2005** for the above study, have had the opportunity to ask questions, understand why the research is being done and any possible risks which have been explained to me	☐
1. I understand that my participation is voluntary and that I am free to withdraw at any time by contacting **Dr Andrew Walls** without giving any reason and without my medical care or legal rights being affected. If I withdraw, I understand that any unused blood will be disposed of	☐
2. I understand that I will be informed if any of the results of the medical tests done as part of the research are important for my health	☐
3. I agree to take part in the above study to collect a sample of blood to use it in studies of allergic sensitivity	☐
4. I understand that the results of this research project may be published in the form of a scientific paper in which I will not be identified	☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

Samples gifted for storage and use in future studies

(The researcher should place N/A in the boxes which are not relevant)

PART B Unlinked Anonymised Samples

5. Provided that specific study protocols have been reviewed and approved by the Local Research Ethics Committee, I indicate my consent for the sample and its derived cells to be stored (potentially for many years) for the following types of studies. I understand that these studies are not for the purpose of directly benefiting my health:

   a. I give permission for the sample to be used for investigations of medical conditions relating to allergic disorders ☐

   b. I give permission for the sample to be stored for use in other unrelated research studies the precise nature of which will depend upon future scientific advances, but excluding genetic engineering and germ-line research ☐

   c. I understand that future research using the sample I give may include genetic research aimed at understanding the genetic influences on inflammation ☐

6. I understand that the sample may be used for commercial development, without financial or other benefit to myself, for the investigation and treatment of medical conditions, potentially leading to new preventative measures against such conditions in keeping with the gift nature of my sample ☐

This section does not need to be signed if the participant does not wish the blood sample to be stored.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

A.2 Testing Food for Oral Immunotherapy

A.2.1 Participant Information Sheet

Testing Food for Oral Immunotherapy

PARTICIPANT INFORMATION SHEET

You are being invited to participate in a study that we hope will help us gain more understanding of seafood and peanut allergy. Information of this kind is important as it will help us develop strategies to treat these allergies in the future. Before you decide to participate it is important for you to understand why the research is being done and what it will involve.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Please read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

Food allergic reactions are common, affecting 4-6% of the population (approximately 3 million patients in the UK alone). Certain foods such as nuts and seafood cause persistent allergy and may cause life threatening reactions. Food allergy is the most common cause of anaphylaxis presenting to emergency departments. No specific treatment is available for food allergy and avoidance, which is not always possible, is the only advice that can be given. This puts patients at risk of an allergic reaction and their quality of life is severely affected.

Oral allergen immunotherapy (OIT) with food is being investigated as a possible long term solution, avoiding the need for life-long avoidance and the need to keep adrenaline auto-

injectors. Before we can develop immunotherapy for seafood and peanut allergy, we need to fully understand exactly which allergens are present in these foods.

Why have I been chosen?

You have been chosen because you have an allergy to peanut or crustacean shellfish (prawn) or because you do not suffer from these allergies and have volunteered to help with the research.

What will happen to me if I take part?

You will be asked to provide a blood sample. If your doctor needs you to have a blood test as part of the management of your allergy, an additional sample will be taken for research at the same time, if possible. The total amount of blood taken will not exceed 30mls (6 teaspoons). You will also be asked to complete a questionnaire which will include questions about your medical history. Some information may be gathered from your hospital notes. Peanut and shellfish contain a variety of proteins which can cause allergy in susceptible people. We intend to identify these proteins and mix them with your blood see how your blood reacts to the different allergens. This may aid us in finding a preparation that can in the future be used as oral immunotherapy for allergic patients.

What do I have to do?

Complete a questionnaire

Provide a blood sample

Allow the study team to review your hospital notes.

What are the possible disadvantages and risks of taking part?

There may be a little discomfort associated with obtaining a blood sample. In the highly unlikely event of any injury, medical care through the National Health Service will be available to you. St. Mary's Hospital NHS Trust and University Hospital Southampton NHS Foundation Trust have indemnified this study.

What are the possible benefits of taking part?

Your participation in this study will help us to identify the precise allergens that are present in peanut and prawn. Although it may not immediately benefit you, you will be helping researchers to get a better understanding of the allergens in these foods that can cause potentially life-threatening reactions and hopefully devise oral immunotherapy which will benefit patients in the future.

Do I have to take part?

You do not have to take part. It is entirely up to you to decide. If you do decide to participate, you will be given this information sheet to keep and asked to sign a consent form. The first part of the consent form (Part A) asks for your consent to participate in the study. The second part (Part B) asks for permission to store any unused blood or other samples for use in future research into allergic diseases. The samples will only be used for studies approved by the Local Research Ethics Committee (an independent committee which oversees any research done within University Hospital Southampton NHS Foundation Trust and the Isle of Wight NHS Trust). The samples will be fully anonymous to the researchers who use them but will contain codes that would allow the clinical study team who collected them to link them back to you. You are free to choose to just sign Part A and not sign Part B. You will receive a copy of the signed consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect your medical care.

Further Information and Contact Details

If you have any questions or concerns about this research study, please speak to a member of the study who will be able to help you, their contact details (during office hours) are:

Dr Shalini Chandel (Southampton):

Telephone: 07901 582558 Email: email@soton.ac.uk (insert once known)

Prof Hasan Arshad (Isle of Wight and Southampton:

Telephone: 023 8120 5232 Email: *sha@soton.ac.uk*

You can also contact the Study Team at The David Hide Asthma and Allergy Research Centre, during office hours on 01983 534113 or iowstudy@iow.nhs.uk.

If you would like some independent advice about whether you should take part, or you would like to discuss this study with somebody outside of the research team, or you would just like some more information about medical research, please contact the Isle of Wight NHS Trust Research and Development Team on 01983 532354.

You may also find the following internet resource useful:

http://www.invo.org.uk/ - INVOLVE is a National advisory group that supports greater public involvement in NHS, public health and social care research

If you are unhappy about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (see contacts for further information above). If you still have questions or concerns, you can contact Alexandra Punter (Research Management & Governance Manger, St Mary's Hospital, Newport, Isle of Wight, PO30 5TG; email alexandra.punter@iow.nhs.uk). Southampton contact?

This completes Part 1 of the Information Sheet. Part 2 will give you more detailed information about the conduct of the study.

Part 2

What if something goes wrong?

In the very unlikely event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against the Isle of Wight NHS Trust or University Hospital Southampton NHS Foundation Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?

The personal information collected in this study will be kept confidential. The data we collect from you will not be labelled with your personal details and will be stored securely. Data collected during the study may be shared with our research collaborators; however, they will not know who the information belongs to as your name and address will only be kept at University Hospital Southampton NHS Foundation Trust or The David Hide Asthma and Allergy Research Centre. Only the study personnel will have access to your personal details. You will not be individually identified in any reports or publications resulting from the study. We will keep your data on file for 25 Years for use in future studies approved by the Research Ethics Committee.

Who will have access to my health records?

Senior Investigators on this project will need to look at your health records to ensure safe conduct of the study procedures.

Involvement of the General Practitioner

We would like your permission to notify your General Practitioner (GP) of your participation in this study.

What will happen to any samples I give?

Samples will be stored securely at the Research Laboratory in Clinical and Experimental Sciences, University of Southampton or The David Hide Asthma and Allergy Research Centre until they are analysed. Only the researchers at both sites will have access to them. The samples will not be labelled with your name or address so that the researchers analysing them will not know that the sample belongs to you. With your permission, we would like to store some blood for use in further studies into asthma and allergic disease. We will only use stored samples for studies approved by the Local Research Ethics Committee.

What will happen to the results of the research study?

We aim to publish the results of the study in medical journals so that other doctors and researchers can make use of them. It will not be possible to identify any individuals involved in this study from these published results.

Who is organising and funding the research?

The researchers at the University of Southampton and The David Hide Asthma and Allergy Research Centre, Isle of Wight, are organising and carrying out this study. The study is being funded by the Asthma, Allergy and Inflammation Research (AAIR) charity.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by London – Bromley Research Ethics Committee.

How long do I have to decide whether I should take part?

Your decision to participate in this study is entirely voluntary. You should take as much time as you need.

Thank you for taking time to read this information sheet.

A.2.2 Patient questionnaire

Allergy Questionnaire

University Hospital Southampton NHS
NHS Foundation Trust

Name: Date of Birth:

1. Ethnicity:

White/British[1]	☐	Black/British Caribbean[2]	☐	Asian/British[3]	☐
White/European[4]	☐	Black/British African[5]	☐	Asian/British other[6]	☐
White other[7]	☐	Black/British other[8]	☐	Chinese [9]	☐
Mixed Background[10]	☐	Other, please state[11]	☐		

2.
   a. Height:
   b. Weight:
3. Occupation (if your work involves contact with fish/shellfish/nuts, please state):

4. Do you have a proven or suspected allergy to any food? Yes[1] ☐ No[0] ☐

(If **NO**, please go to Page 6, Q27)

5.
   a. For the following foods, please tick the appropriate box:

	Eat regularly with no problems [1]	Avoid/unrelated to allergy i.e. dislike[2]	Avoid/proven allergy i.e. previous reaction + positive allergy tests[3]	Avoid/suspected allergy i.e. positive tests + no previous reaction[4]
Fish, type if known:	☐	☐	☐	☐
Crustaceans e.g. - Prawn/shrimp - Crab - Crayfish - Langoustines - Lobster	☐	☐	☐	☐
Molluscs e.g. - Mussels - Oyster - Scallops - Clams - Squid - Octopus	☐	☐	☐	☐
Celery	☐	☐	☐	☐
Milk/ Dairy	☐	☐	☐	☐
Egg	☐	☐	☐	☐
Lupin	☐	☐	☐	☐
Mustard	☐	☐	☐	☐

b. For the following foods, please tick the appropriate box:

	Eat regularly with no problems[1]	Avoid/unrelated to allergy i.e. dislike[2]	Avoid/proven allergy i.e. previous reaction + positive allergy tests[3]	Avoid/suspected allergy i.e. positive tests + no previous reaction[4]
Peanuts	☐	☐	☐	☐
Brazil	☐	☐	☐	☐
Hazelnut	☐	☐	☐	☐
Pistachio	☐	☐	☐	☐
Walnut	☐	☐	☐	☐
Pecan	☐	☐	☐	☐
Almond	☐	☐	☐	☐
Macadamia	☐	☐	☐	☐
Cashew	☐	☐	☐	☐
Sesame	☐	☐	☐	☐
Soya	☐	☐	☐	☐
Wheat	☐	☐	☐	☐
Sulphite sensitivity	☐	☐	☐	☐
Other food additives	☐	☐	☐	☐
Other, please specify:	☐	☐	☐	☐

6. Have you ever required hospitalisation following an allergic reaction? Yes[1] ☐ No[0] ☐

7.

   a. Do you take any medications for your allergies?

      Yes[1] ☐ No[0] ☐

   b. If yes, which of the following medications do you take?

      Antihistamines[1] ☐ Inhalers[2] ☐ Other, please specify

      ☐

8. The next question asks you to provide further information for each of your CURRENT allergies. If you have more than 4 food allergies, please provide details for the 4 most SEVERE/RECENT allergies. In addition, if you are allergic to a number of fish species, group them together as ‘fish’, if you are allergic to a number of shellfish species, group them together as ‘shellfish’, if you are allergic to more than one nut, group them together as ‘nuts’.

	Food 1	Food 2	Food 3	Food 4
Allergen:				
Approximate age diagnosed:				
Symptoms:				
How quickly did the symptoms occur after eating the food?	Immediately[1] ☐ <30 mins[2] ☐ 30 mins - 2 hours[3] ☐ >2 hours[4] ☐ Don't know[5] ☐	Immediately[1] ☐ <30 mins[2] ☐ 30 mins - 2 hours[3] ☐ >2 hours[4] ☐ Don't know[5] ☐	Immediately[1] ☐ <30 mins[2] ☐ 30 mins - 2 hours[3] ☐ >2 hours[4] ☐ Don't know[5] ☐	Immediately[1] ☐ <30 mins[2] ☐ 30 mins - 2 hours[3] ☐ >2 hours[4] ☐ Don't know[5] ☐
How was this allergy diagnosed?	Self-diagnosed[1] ☐ Dr-diagnosed[2] ☐ Oral food challenge[3] ☐ Skin prick test[4] ☐ Blood test[5] ☐ Don't know[6] ☐ Other, please specify[7]:	Self-diagnosed[1] ☐ Dr-diagnosed[2] ☐ Oral food challenge[3] ☐ Skin prick test[4] ☐ Blood test[5] ☐ Don't know[6] ☐ Other, please specify[7]:	Self-diagnosed[1] ☐ Dr-diagnosed[2] ☐ Oral food challenge[3] ☐ Skin prick test[4] ☐ Blood test[5] ☐ Don't know[6] ☐ Other, please specify[7]:	Self-diagnosed[1] ☐ Dr-diagnosed[2] ☐ Oral food challenge[3] ☐ Skin prick test[4] ☐ Blood test[5] ☐ Don't know[6] ☐ Other, please specify[7]:

Fish Allergy

With regard to the **FIRST** allergic reaction you had to fish/shellfish:

9. Which fish/shellfish did you react to?
10. How was it prepared?

Baked[1] ☐ BBQ[2] ☐

Fried [3] ☐ Microwave[4] ☐

Raw/Sushi[5] ☐ Steamed[6] ☐

Smoked [7] ☐ Tinned[8] ☐

Other, please specify:

11. How old were you when this reaction first happened?

12.
    a. Was this the first time you had eaten this food? Yes[1] ☐
       No[0] ☐
    b. If no, how often had you eaten this food before?
       Once only[1] ☐
       Occasionally (less than once a month)[2] ☐
       Regularly (2-3 times a month)[3] ☐
       Frequently (once a week or more)[4] ☐
13. What symptoms did you have at this first reaction to fish/shellfish?

14.
    a. Have you had any further reactions to other types of fish or shellfish? Yes[1] ☐ No[0] ☐
    b. If yes, please provide the following details:

Type of fish/shellfish	How was it prepared?	Age of first reaction to this food

15.

a. Have you ever reacted to the steam or vapour produced by cooking fish/shellfish?

Yes[1] ☐

No[0] ☐

b. If yes, what sort of reaction?

16.

a. Do you tolerate any tinned fish, that you cannot tolerate fresh i.e. fresh tuna vs. tinned tuna?

Yes[1] ☐

No[0] ☐

b. If yes which ones

17. After visiting the allergy clinic with regards to your fish/shellfish allergy, were you given advice regarding avoidance of fish/shellfish?

Yes[1] ☐

No[0] ☐

18. After your diagnosis what did you do with your diet? (For each row, tick one box which best suits your practice)

	Not at all	Sometimes	Most of the time	Always
Avoid all shellfish	☐	☐	☐	☐
Avoid all fish	☐	☐	☐	☐
Avoid all fish, but can eat tinned	☐	☐	☐	☐
Avoid specific fish	☐	☐	☐	☐
Avoid food where the label states 'May contain traces of…'	☐	☐	☐	☐
Avoid eating out in restaurants serving fish/shellfish	☐	☐	☐	☐
Avoid food containing gelatine or fish oil supplements	☐	☐	☐	☐
Other changes, please specify:				

Nut Allergy

With regard to the **FIRST** allergic reaction you had to peanut/tree nut:

19. What type (s) of nut did you react to?
20. How old were you when this reaction first happened?
21.
    a. Was this the first time you had eaten this food? Yes[1]
    ☐ No[0] ☐
    b. If no, how often had you eaten this food before?

Once only[1] ☐

Occasionally (less than once a month)[2] ☐

Regularly (2-3 times a month)[3] ☐

Frequently (once a week or more)[4] ☐

22. What symptoms did you have at this first reaction to peanut/tree nuts?

23. Have you had any further reactions to other types of peanut/tree nuts?

Yes[1] ☐

No[0] ☐

24.

a. Have you ever reacted to inhaled vapour of nuts?

Yes[1] ☐

No[0] ☐

b. If yes, what sort of reaction? ☐

25. After visiting the allergy clinic with regards to your nut allergy, were you given advice regarding avoidance of this?

Yes[1] ☐ No[0] ☐

26. After your diagnosis what did you do with your diet? (For each row, tick one box which best suits your practice)

	Not at all	Sometimes	Most of the time	Always
Avoid all nuts	☐	☐	☐	☐
Avoid all peanuts	☐	☐	☐	☐
Avoid all tree nuts	☐	☐	☐	☐
Avoid only the nuts specified	☐	☐	☐	☐
Avoid food where the label states 'May contain traces of…'	☐	☐	☐	☐
Avoid eating out in restaurants serving nuts or nuts in cooking	☐	☐	☐	☐
Other changes, please specify:				

General Allergy Questions

27. Have you had wheezing or whistling in your chest in the past 12 months?

Yes[1] ☐ No[0] ☐

28. Have you EVER had wheezing or whistling in your chest at any time in the past?

Yes[1] ☐ No[0] ☐

29. In the past 12 months, has your chest sounded wheezy during or after exercise?

Yes[1] ☐ No[0] ☐

30. In the past 12 months, have you had a dry cough at night, apart from a cough associated with a cold or chest infection?

Yes[1] ☐ No[0] ☐

31. Have you EVER had asthma?

Yes[1] ☐ No[0] ☐

32. In the past 12 months, have you had a problem with sneezing, or a runny, or blocked nose when you DID NOT have a cold of the flu?

Yes[1] ☐ No[0] ☐

33. Have you EVER had a problem with sneezing, or a runny, or blocked nose when you DID NOT have a cold or the flu?

Yes[1] ☐ No[0] ☐

34. Have you EVER had hay fever?

Yes[1] ☐ No[0] ☐

35. Have you EVER had an itchy rash, which was coming and going for at least six months?

Yes[1] ☐ No[0] ☐

(if No, go to Q37)

36. Have you had this itchy rash at any time in the past 12 months?

Yes[1] ☐ No[0] ☐

37. Have you ever had eczema?

Yes[1] ☐ No[0] ☐

Thank you for taking the time to complete this questionnaire.

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