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Article type : Original Article

Allergen-specific IgG show distinct patterns in persistent and transient food allergy

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/PAI.13567](https://doi.org/10.1111/PAI.13567)

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Funding: This work was supported by the Medical Research Council (MRC Clinician Scientist Fellowship MR/M008517/1 awarded to A. F. Santos) and the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust.

Conflict of interest: Dr. Santos reports grants and personal fees from Medical Research Council (MR/T032081/1; MR/M008517/1; G0902018); grants from Asthma UK and the NIHR through the Biomedical Research Centre (BRC) award to Guy's and St Thomas' NHS Foundation Trust, during the conduct of the study; grants from Immune Tolerance Network/National Institute of Allergy and Infectious Diseases (NIAID, NIH), grants from Asthma UK; personal fees from Thermo Scientific, Nutricia, Infomed, Novartis, Allergy Therapeutics, Buhlmann, as well as research support from Buhlmann and Thermo Scientific through a collaboration agreement with King's College London. The other authors have nothing to disclose.

Abstract (249 words):

Background: Immediate food allergic reactions are IgE-mediated food, but many individuals with detectable allergen-specific IgE do not react to food. Allergen-specific IgG may interfere with allergen-IgE interaction and/or through intracellular inhibitory signalling to suppress mast cell and basophil response to food allergens. We aimed to understand the role of allergen-specific IgG in food allergy and natural tolerance.

Methods: IgG and IgG isotypes specific to peanut, cow's milk and egg were measured using ImmunoCAP and ELISA, respectively, in samples of children with suspected food allergies. Expression of IgE and IgG and their receptors and of activation markers following allergen

stimulation were measured on basophils and mast cells by flow cytometry, with and without blockade of FcγRIIα or FcγRIIβ receptors.

Results: The levels of peanut-specific IgG, IgG1, IgG2, IgG3 and IgG4 were higher in peanut allergic compared to peanut non-allergic children. No difference in allergen-specific IgG isotypes was observed between allergic and non-allergic children to milk or egg, except for milk-specific IgG4 that was higher in cow's milk non-allergic compared to cow's milk allergic children. Basophils and LAD2 cells expressed IgG receptors, but IgG and IgA were not detected on the surface of either cell type and blocking FcγRIIα or FcγRIIβ did not modify basophil or mast cell activation in response to allergen.

Conclusion: Allergen-specific IgG patterns were distinct in persistent (peanut) versus transient (milk and egg) food allergies. We found no evidence that FcγRIIα or FcγRIIβ receptors affect allergen-induced activation of mast cells and basophils in food allergy or tolerance.

Key Words: food allergy, basophil, IgG, FcγRIIβ, tolerance, mast cell, IgE, peanut, cow's milk, egg

Key messages:

1. Allergen-specific IgG show distinct patterns in persistent (peanut) versus transient (milk and egg) food allergies, which could be a reflection of the different mechanisms of IgE production and dysregulation.
2. Although allergen-specific IgG can compete with IgE for allergen binding and reduce allergen-induced basophil and mast cell activation and degranulation, we have found no evidence that additional suppression could be mediated by inhibitory IgG receptors on the surface of basophils and mast cells in natural food tolerance.

Abbreviations:

BAT, basophil activation test

BSA, bovine serum albumin

CM, cow's milk

CMA, cow's milk allergy

ELISA, enzyme-linked immunosorbent assay

EA, egg allergy

EW, egg white

HRP, horseradish peroxidase

AIT, allergen-specific immunotherapy

MAT, mast cell activation test

OFC, oral food challenge

PE, peanut extract

rIL-4, recombinant Interleukin-4

slgE, specific Immunoglobulin E

Word count: 3678

Tables: 2

Figures: 5

Online Repository: 3 figures

Introduction

Immediate allergic reactions to foods are mediated by allergen-specific IgE (sIgE) bound to FcεRI on the surface of basophils and mast cells. sIgE concentrations in serum can be used with the clinical history to confirm food allergy, but there are many individuals with detectable sIgE who do not react on oral food challenge (OFC)¹. In IgE-sensitised individuals who are tolerant to the food, allergen-specific antibodies of isotypes other than IgE may be involved in the absence of mast cell and basophil reactivity, despite the presence of IgE. One candidate of interest has been IgG4 which is typically produced together with IgE in Th2-type immune responses. Levels of allergen-specific IgG4 have been shown to increase in patients who outgrow food allergies and in patients who undergo allergen-specific immunotherapy (AIT), at the same time that these patients become less sensitive to the allergen during treatment². Previously, we showed that peanut-sIgG4 in the plasma of patients treated with peanut AIT was able to suppress the activation of mast cells and basophils sensitised with plasma from peanut allergic patients and stimulated with peanut allergens³. We confirmed the involvement of IgG4 through the depletion of IgG4 from the plasma of sensitised but tolerant subjects and the consequent reduction in this inhibitory effect. However, this reduction was only partial, despite the complete depletion of IgG4 from the plasma samples, suggesting that other factors may contribute to the suppressed mast cell response to food allergens. We hypothesized that such factors could be allergen-specific IgG antibodies of different subclasses other than IgG4 and that such inhibitory allergen-specific antibodies could suppress allergen-induced mast cell and basophil activation by either competing with IgE for binding to the allergen and/or by co-cross-linking⁴ of receptor-bound IgE and IgG inducing inhibitory signalling inside the effector cells.

In other studies, allergen-specific IgG antibodies have shown to be able to modulate basophil and mast cell activation by acting as blocking antibodies, such as those induced during AIT to respiratory, venom and food allergens and more recently during a trial of monoclonal cat-specific IgGs^{3,5-7}. Monoclonal antibodies of different isotypes but the same specificity could equally suppress allergen-induced basophil activation in an *in vitro* model of grass pollen allergy⁸, thus allergen-specific antibodies of different isotypes can share blocking activity. In *in vitro* studies and in mouse models, co-cross-linking of receptor-bound IgE and IgG led to phosphorylation of

ITIM motifs and resulting inhibitory intracellular signalling dampened the stimulatory signalling downstream of the IgE receptor^{9, 7,10-12}. Using samples from a cohort of cat-allergic individuals treated with AIT, antibodies blocking FcγRIIα and FcγRIIβ increased basophil sensitivity to the allergen¹³. FcγRIIβ blocking antibodies also increased effector cell activation upon food challenge in a murine model of food allergy and in human mast cells derived from humanized mice and sensitised with plasma from peanut allergic patients treated with AIT with and without blocking of FcγRII receptor¹⁴. To our knowledge, the inhibitory mechanisms of IgG have not been explored in the context of natural tolerance to food. We aimed to study the mechanisms by which IgG antibodies modulate mast cell and basophil responses to food allergens in IgE-sensitised children.

Methods

Study population and procedures

Children with suspected allergy to either peanut, egg and/or CM were recruited into one of two diagnostic studies (REC 10/H0802/044 and 17/LO/029). Some patients were assessed for more than one food allergy (Figure E1). Suspected food allergy was defined as: 1. reported history of an immediate allergic reaction upon exposure to the food, 2. absence of regular consumption of the food or active food avoidance and/or 3. evidence of IgE sensitisation to the food, as measured by skin prick test greater than or equal to 1 mm and/or sIgE greater than or equal to 0.10 KU/L. Individuals stopped anti-histamine treatment prior to evaluation, and treatment with immunomodulators, such as AIT or biologics, was an exclusion criteria. Study participants underwent clinical assessment, SPT, blood collection and OFC, as clinically indicated. For instance, allergen-specific IgE was measured to egg white, cow's milk and peanut extracts for children assessed for egg, cow's milk and peanut allergies, respectively: 39 egg allergic, 21 egg non-allergic, 24 cow's milk allergic, 8 cow's milk non-allergic, 44 peanut allergic and 41 peanut non-allergic. Food allergy was defined as a positive OFC in the majority of participants. Patients who did not undergo OFC to foods tested in this study were diagnosed as allergic if they had an unequivocal history of an immediate type allergic reaction to the food in the past year and showed evidence of sIgE on skin prick test and/or serum sIgE; and as tolerant if they had been consuming an age-appropriate amount of the food regularly in the diet without developing any

allergic symptoms. Whole blood was used for basophil activation test (BAT) within 4 hours of blood being taken. Plasma was separated from whole blood and stored at -80°C until use in ELISA or mast cell activation tests (MAT). All participants' parents or carers with parental responsibility provided written consent prior to any study procedures.

Allergen-specific IgG measurements

Food-specific IgG levels were measured using ImmunoCAP following the manufacturer's instructions using Phadia 100 (ThermoFisher, Uppsala, Sweden). Food-specific IgG isotypes were measured by ELISA. Nunc-Immuno™ MicroWell™ 96 well solid plates (Sigma-Aldrich, Poole, UK) were coated with 100µg/ml crude peanut, egg white (EW) or cow's milk (CM) extracts (ALK-Abelló, Hørsholm, Denmark) and incubated overnight at 4°C and washed with wash buffer (1x PBS, 0.05% Tween-20) before blocking with 1% bovine serum albumin (BSA, Sigma-Aldrich, Poole, UK) diluted in PBS for 1 hour at room temperature (RT). Plates were washed and plasma samples diluted 1:8 or 1:16 (depending on dose-finding experiments for each ELISA) were added to the plates and incubated at RT for 2 hours. Plates were washed and anti-human IgG1, IgG2, IgG3 (all Sigma-Aldrich, Poole, UK) or IgG4 (BD Pharmingen, San Jose, Calif.) was added and incubated at RT for 1 hour. For anti-human IgG2 and IgG4 an additional step of incubation with streptavidin-HRP conjugate (R&D Systems, Minneapolis, Min.) was required for 30 min at RT. This was not required for IgG1 and IgG3. After a final wash, TMB-substrate (ThermoFisher, Uppsala, Sweden) was added to the wells and allowed to develop for 10-15 minutes in darkness. The reaction was stopped using 1M H₂SO₄ (Sigma-Aldrich, Poole, UK). Plates were read at 450nm on SpectraMax (Molecular Devices, San Jose, Calif) or HT3 (Anthos Labtec Instruments, Wals, Austria) Microtiter plate readers.

Basophil activation assays coupled with FcγRIIα/β blockade

Blood was collected in lithium-heparin (Greiner Bio One, Kremsmünster, Austria). Whole blood was incubated with 1µg/ml anti-FcγRIIα or anti-FcγRIIβ antibodies (Macrogenics, Rockville, Mary.) for 1 hour at 37°C and stimulated for 5 minutes at 37°C with peanut, egg or CM extracts

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diluted in RPMI medium (Gibco, Paisley, UK) at 100ng/ml. Reactions were stopped using 20mM EDTA (Sigma-Aldrich, Poole, UK) prior to staining of cells at 4°C for 30 min with α CD203c-BV421, α CD63-PECy7, α Fc γ RI-PECy7, α Fc γ RIII-PECF594, α IgG-APC (all BD Biosciences, San Jose, Calif.), α Fc ϵ RI-FITC, α Fc γ RII-APC, α IgE-PE (all BioLegend, San Diego, Calif.), α Fc γ RII α -AF488, α Fc γ RII β -AF488 (Macrogenics, Rockville, Mary.), α IgA-FITC (Miltenyi, Bergisch Gladbach, Germany). Expression of cell surface markers or receptor-bound antibodies was acquired by FACS Cantoll using FACSDiva software (BD Biosciences, San Jose, Calif.). See Figure E2 for gating strategy for the basophil activation assay. Data was analysed using Flowjo software (version 10.6.2; TreeStar, Ashland, Ore).

Mast cell activation assays coupled with Fc γ RII α / β blockade

LAD2 cells were kindly provided by Drs Arnold Kirshenbaum and Dean Metcalfe¹⁵ (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases), and were cultured in StemPro media (Gibco, Paisley, UK) supplemented with 10ng/ml rIL-4 (Peprotech, London, UK) and 100ng/ml SCF (Gibco, Paisley, UK) for 4 days in a Corning Costar 48-well flat-bottomed culture plate (Sigma-Aldrich, Poole, UK). Cells were treated with 1 μ g/ml anti-Fc γ RII α or anti-Fc γ RII β for 1 hour at 37°C and were incubated with neat plasma from allergic or non-allergic individuals overnight at 37°C. On day 5, cells were stimulated for 30 minutes at 37°C with peanut, egg or CM extracts diluted in StemPro media (Gibco, Paisley, UK). Cells were not washed following sensitisation and prior to stimulation. Reactions were stopped using 20mM EDTA prior to staining of cells at 4°C for 30 minutes with α CD203c-BV421, α CD63-PECy7, α Fc γ RI-PECy7, α Fc γ RIII-PECF594, α IgG-APC, α Fc ϵ RI-FITC, α Fc γ RII-APC, α IgE-PE, α Fc γ RII α -AF488, α Fc γ RII β -AF488, α IgA-FITC. See Figure E3 for gating strategy for the mast cell activation assay. Flow cytometry data collection and analyses was performed as above.

B cell isolation from tonsils and staining for flow cytometry

One adult patient undergoing tonsillectomy was recruited and consented at Guy's hospital for use of the discarded tonsil tissue (REC 08/H0804/94). The tonsil tissue was minced mechanically

in a petri dish and washed in RPMI. Supernatant was filtered to yield a single cell suspension and a Ficoll (Gibco, Paisley, UK) density centrifugation separation was performed to isolate peripheral blood mononuclear cells, which were then incubated with an equal volume of sheep red blood cells (Thermofisher, Uppsala, Sweden) at 4°C for 30 min to aggregate T cells and NK cells. Supernatant was centrifuged, the interface was removed and a second Ficoll density centrifugation separation was performed to isolate tonsil B cells. B cells were washed in PBS containing 0.5% BSA and 2mM EDTA and resuspended to a concentration of 1×10^6 cells/ml. B cells were stained at 4°C for 30 minutes with α CD19-PECy7 (Biolegend, San Jose, Calif.), α IgG-APC and/or α IgA-FITC. Flow cytometry data collection and analyses was performed as above.

Statistical analyses

Statistical analyses were performed using GraphPAD Prism v7.0 (GraphPad Software, San Diego, Calif). Normally distributed data was analyzed by two-tailed t tests or paired t tests. Non-normally distributed data was analyzed using two-tailed Mann-Whitney test while non-parametric data was analyzed using Wilcoxon signed ranked tests.

Results

Study population

Seventy one children were assessed for egg allergy (EA), 72 for cow's milk allergy (CMA) and 79 for peanut allergy (PA). **Table 1** represents their clinical and serological features. Overall, their ages ranged between 9 months and 9.75 years old (median=2.58 years) and 68.49% of children were male. Allergen-specific IgE was detectable in all allergic patients and in a subset of non-allergic children (n=18/21 for egg, n=8/8 for cow's milk and 30/41 for peanut). Allergen-sIgE concentrations were higher in allergic compared to non-allergic for patients, but there was an overlap between the groups (Figure 1A-C).

Allergen-specific IgG isotypes were elevated in peanut allergic patients and IgG4 was elevated in children with resolved cow's milk allergy

No statistically significant difference was seen in the concentration of peanut-specific IgG between peanut allergic and peanut non-allergic individuals but there was a trend towards higher levels in PA ($p=0.056$, Figure 1D). Higher levels of peanut-sIgG1 ($p<0.001$), sIgG2 ($p<0.001$), sIgG3 ($p=0.003$) and sIgG4 ($p<0.001$) were observed in peanut allergic compared with peanut non-allergic subjects (Figure 1E-H). EW-sIgG1, sIgG2, sIgG3 and sIgG4 levels were similar in EA and egg non-allergic individuals, with EW-sIgG1 being virtually undetectable in both groups (Figure 1I-L). CM-sIgG4 was present at significantly higher levels ($p=0.034$) in CM non-allergic compared to CMA individuals (Figure 1P), whereas CM-sIgG1, sIgG2 and sIgG3 were detectable but not significantly different between CMA and non-allergic individuals (Figure 1M-O).

Basophils and LAD2 cells differentially express FcγRIIα and FcγRIIβ

Expression of IgE and IgG receptors was assessed by flow cytometry (Table 2). FcεRI was constitutively expressed on LAD2 cells and primary blood basophils (Figure 2A), as expected. FcγRI (CD64) was detected on LAD2 cells (Figure 2B) but was virtually undetectable on basophils. FcγRII (CD32) was constitutively expressed on both LAD2 and primary basophils (Figure 2A+B). FcγRIII (CD16) did not seem to be expressed by basophils or LAD2 cells (Figure 2A+B).

FcγRII has two known subtypes, FcγRIIα and FcγRIIβ, with the latter being implicated in the inhibition of basophil and mast cells activation via ITIM-motifs¹⁴. Efficient binding of FcγRIIα and FcγRIIβ blocking antibodies was confirmed by the loss of detection of panCD32 on LAD2 and basophil surfaces (Figure E4). FcγRIIβ was preferentially expressed on basophils and at greater levels than LAD2 cells ($p<0.001$ Figure 2C+D). Conversely, LAD2 cells expressed FcγRIIα at higher levels than that of FcγRIIβ ($p<0.001$, Figures 2C+D and E5).

Basophils and sensitised LAD2 cells exhibit IgE, but little IgG or IgA, on their surface

The expression of surface-bound IgE was observed in both basophils (Figure 3A) and sensitised LAD2 cells (Figure 3B). The ratio of fluorescence measured for IgE between sensitised and non-sensitised LAD2 cells was not significantly different between peanut allergic and peanut non-allergic children (Figure 3C), indicating similar levels of IgE bound to receptors on the surface of

the effector cells. Conversely, IgA and IgG were not detected on the surface of basophils (Figure 3A) or LAD2 cells (Figure 3B). IgG and IgA were, however, expressed on the surface of B cells isolated from human tonsils which was tested in a parallel control experiment (Figures 3D+E).

Blocking FcγRIIα or FcγRIIβ does not change basophil or mast cell responses to allergen

To assess the role of FcγRIIα or FcγRIIβ during the basophil response to food allergy, primary blood basophils from patients were stimulated with CM, EW or peanut extract (PE) following the incubation with monoclonal blocking antibodies targeting specifically FcγRIIα or FcγRIIβ. Blocking FcγRIIα or FcγRIIβ did not modify the expression of CD63 by basophils of allergic or non-allergic individuals following stimulation with PE, CM or EW (Figures 4A-F and E6).

We performed similar experiments using LAD2 cells, which had the advantage of allowing treatment with blocking antibodies anti-FcγRIIα and anti-FcγRIIβ prior to sensitisation with plasma and binding of IgG to the respective receptors. Blocking the FcγRIIα or FcγRIIβ receptors prior to sensitisation did not attenuate LAD2 cell activation in response to peanut allergen neither in cells sensitised with plasma from peanut allergic patient (Figure 5A), nor in cells sensitised with plasma from peanut non-allergic children (Figure 5B).

Discussion

The absence of clinical reactivity to foods in the presence of allergen-specific IgE may be explained by the interference of allergen-specific antibodies of an isotype other than IgE, either by competing with IgE for allergen binding or by inducing inhibitory intracellular signalling in effector cells. We have previously shown that allergen-specific IgG4 from tolerant patients with IgE directed to the major peanut allergens was able to suppress allergen-induced activation of basophils and mast cells in previous studies of immune tolerance to foods³. As the effect of IgG4 was only partial, we aimed to assess the role of other sIgG isotypes. We found that peanut-specific IgG1, IgG2, IgG3 and IgG4 as measured by ELISA were higher in peanut allergic than non-allergic children whereas levels allergen-specific IgG isotypes also measured by ELISA were comparable in children assessed for CMA and EA, except for milk-specific IgG4 that was higher in

children who had resolved their CMA compared to CM allergic children. Despite the presence of IgG in the extracellular space, we have not found evidence of IgG being able to bind receptors on the surface of mast cells or basophils or to induce inhibitory signalling and suppress IgE-mediated activation of mast cells or basophils following allergen stimulation. We found, however, distinct serologic IgG patterns in persistent (i.e. peanut) versus transient (i.e. cow's milk and egg) food allergies.

Measurement of all IgG isotypes in the plasma of food allergic or non-allergic individuals revealed divergent patterns between persistent and transient food allergies (i.e. peanut versus milk and egg allergies). The presence of sIgGs are, however, not indicative of food allergy and is not recommended for the diagnostic assessment of patients¹⁶. Recent studies have identified a new subset of follicular helper T cells (Tfh13) that are involved in the induction of high-affinity IgE producing B cells¹⁷. Class switch recombination (CSR) to generate sIgE can occur directly from IgM+ B cells, but also sequentially via IgG+ B cells. Sequential CSR generates IgE with a higher affinity than direct CSR¹⁸ and IgG1+ memory B cells can form a reservoir for the generation of IgE+ plasma cells^{19,20}. It is possible that the higher levels of allergen-specific IgG1 in individuals with persistent food allergy compared to children with transient food allergies result from the fact that their IgE is mainly generated through sequential class switching resulting in high affinity IgE for food allergens as recently demonstrated by Gowthaman et al¹⁷, whereas in children with transient food allergies their lower affinity IgE is mainly produced via direct class switching thus not involving the production of high levels of IgG1²¹.

EW-sIgG1 was below the level of detection for many individuals, despite detection of other EW-sIgG isotypes. Egg-sIgG1 has been detected by ELISA in other studies²², but the methodology used was different – for instance, plates were coated with OVA rather than EW extract used in our study (although the EW extract we used contains significant amounts of OVA), and a different clone of IgG1 antibody was used, and there may have been differences in the patients' phenotype. In our study, we used the same plasma samples and the same reagents for the IgG1 ELISA and only changed the allergen extracts when testing to the different allergens and we tested the same samples across IgG isotypes within the same allergenic food, which constitute important controls. Milk-specific IgG4 was expressed at higher levels in non-allergic individuals

compared to allergic patients - these were mostly patients who had resolved their milk allergy and corroborates previous studies²³.

Interactions between IgG isotypes and effector cells are dependent on surface expression of FcγRs. The utilization of novel antibodies capable of differentiating between FcγRII subtypes has identified FcγRIIα as the dominant subtype of CD32 expressed on mast cells, while FcγRIIβ is expressed to a greater level on primary basophils. Despite the expression of receptors capable of interacting with IgGs, no IgG or IgA was detected on the surface of basophils or sensitised LAD2 cells. In contrast, IgG and IgA was detected on the surface of B cells and IgE was detected on the same primary basophils and sensitised LAD2 cells. It is possible, however, that since the affinity of FcγRII for monomeric IgG is very low and immune complexes need to be formed with allergen in order for IgG to be bound to the receptor for sufficient time to allow it to be measured²⁴. If that were the case, we would have seen differences in the BAT and MAT with blocking of FcγRII given that in both assays the cells were not washed prior to stimulation and there were antibodies present around the cells at the time that allergen was added.

Blocking either FcγRIIα or FcγRIIβ during BAT and MAT experiments did not affect the degree of activation of basophils or LAD2 cells, suggesting that these receptors are not implicated in the mechanism underlying natural tolerance to food allergens. In a spontaneous chronic urticaria model, MacGlashan has reported similar findings in that blocking CD32B did not change basophil response²⁵. Our findings do not, however, reflect the observed effect of engineered chimeric molecules that couple binding to both IgE and IgG receptors. Our study is the first of its type to investigate the effects of blocking FcγRIIα or FcγRIIβ in the context of human food allergy and natural food tolerance. However, such mechanism has been shown in mouse models of passive anaphylaxis⁴; however, there is so far no evidence of IgG-mediated anaphylaxis to foods in humans, as demonstrated in mouse models. Burton *et al.*¹⁴ found that FcγRIIβ-deficient mice sensitised to OVA greatly exacerbated the severity of anaphylactic shock in response to allergen challenge. This was accompanied by an increased production of IgE, Th2 cells and a reduced proportion of FoxP3+ Treg cells. The addition of IgG from immunised mice to FcγRIIβ-competent mice during sensitisation greatly reduced allergic responses and this could have occurred by murine IgG performing a similar role to human IgG4. FcγRIIβ, however, was only identified in

humanised murine mast cells, and was not expressed on mast cells derived from skin²⁶. Whether human mast cells at other tissue sites express FcγRIIβ has yet to be elucidated and could contribute to some of the discrepancies observed in reactivity and potential inhibitory mechanisms between mast cell subsets. In one of few human studies, Cady *et al.*¹³ showed that IgG produced during IT for cat allergy reduced basophil activation to Fel d 1 in cat-allergic patients. Furthermore, blocking of FcγRIIβ prior to allergen challenge ablated the protective effects of IgG present in IT plasma. However, this was during IT and the effects of protective IgG and FcγRIIβ were observed at a single concentration of Fel d1, with inhibition of basophil activation lost at higher concentrations.

A key finding from our study, which may explain the inability of these receptors to induce an inhibitory response, is the lack of detection of surface-bound IgG on either basophils or sensitised LAD2 cells. MAT experiments were performed with cells which were not washed following sensitisation with plasma in order to retain IgG and/or allergen-IgG immune complexes bound to FcγRs. The use of unwashed cells would have allowed for low affinity interactions to occur between IgG and FcγRIIα or FcγRIIβ in these in vitro models and should be sufficient to induce inhibitory signalling through SHIP-1²⁷⁻²⁹. Thus, the mechanism of action of IgG in natural food tolerance is unlikely to be suppression of allergen-induced basophil and mast cell activation via intracellular suppression of activatory signalling but simply by competition with IgE for allergen binding. This has previously been demonstrated using the inhibition of mast cell and basophil activation assays, in which effector cells sensitised with plasma from allergic subjects were stimulated with allergen that had previously been incubated with test plasma, and therefore allowing for antibodies present in human serum to compete with allergen prior to exposure to sensitised cells^{3,6}. Furthermore, it is possible that the quality of both of the IgG and IgE antibodies themselves play a role in the differences in effector cell response observed^{30,31}. Although the number of patients tested on BAT to peanut was limited and one out of three peanut allergic patients had basophils who did not respond to allergen, more patients were tested on the MAT to peanut and on the BAT to milk and egg and overall the findings were consistent across allergens and cell type.

The proposed mechanism for intracellular suppression of allergic responses via FcγRIIβ requires cross-linking of FcεRI and FcγRIIβ³²⁻³⁴ and was demonstrated in mouse and *in vitro* studies with monoclonal antibodies and hybrid protein constructs combining the Fc portion of an IgG with allergen^{10,12,33,35,36}. For such mechanism to happen with two independent antibodies, sIgE and sIgG need to recognise different allergen epitopes, as not to compete with each other and to bind in a conformational manner to allow for FcεRI and FcγRIIβ co-cross-linking. A possible explanation for the absence of co-cross-linking between IgE and IgG is that the IgE and IgG antibodies are developed against the same epitopes in any individual subject, thus precluding co-cross linking to happen. A similar pattern of epitope-binding has indeed been reported between IgE and IgG4 of peanut-sensitized subjects and supports such hypothesis^{3,37,38}.

In summary, we have shown that the patterns of allergen-specific IgG isotypes are different in persistent versus transient food allergies, which could reflect the different mechanisms of IgE production and dysregulation. Although allergen-specific IgG can compete with IgE for allergen binding and reduce allergen-induced basophil and mast cell activation and degranulation, we have found no evidence that additional suppression could be mediated by inhibitory IgG receptors on the surface of basophils and mast cells. Allergen-specific antibodies of isotypes different from IgE can however function as blocking antibodies competing with IgE for binding to the allergen. sIgG4 is the most interesting due to its inherent anti-inflammatory properties. sIgGs can thus be important therapeutic tools, if not for inducing tolerance, for protecting against allergic reactions resulting from accidental exposure to food allergens. Investigations should now be directed towards further characterizing the interaction of IgG with allergen, including IgG specificity and avidity for allergen, together with potential IgG-allergen complex interactions with other cells of the immune system, such as IgE+ B cells and dendritic cells, to better understand whether allergen-specific IgGs could be effective, not only in passive immunization, but also in actively inducing a tolerogenic response to food allergens.

Acknowledgments

The authors wish to thank Professors Gideon Lack and George Du Toit and the Paediatric Allergy team at the Evelina London Children's Hospital for their contribution and continued work to

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generate samples vital for this study. The authors also wish to thank Dr. David Fear and Thomas Moore for tonsil-derived B cells.

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Tables

Table 1. Clinical and serological characteristics of the study population. Median and interquartile range shown for age and allergen-specific IgE.

	Egg Allergic (n=42)	Egg non- allergic (n=29)	Cow's milk allergic (n=31)	Cow's milk non-allergic (n=41)	Peanut Allergic (n=44)	Peanut non- allergic (n=47)
Age (y)	3.75 (1.5-6.58)	2.5 (1.63-4.67)	2.83 (1.58-4.6)	2.71 (1.6-6.46)	8.28 (5.6-13.09)	6.09 (2.5-9.02)
Gender – n male (%)	26 (62%)	21 (72%)	21 (68%)	26 (63%)	31 (86%)	25 (71%)
Other food allergies - n (%)	24 (57%)	4 (14%)	21 (68%)	7 (17%)	30 (68%)	28 (80%)
Eczema - n (%)	37 (88%)	26 (90%)	28 (90%)	37 (90%)	15 (34%)	15 (43%)
Allergic Rhinitis – n (%)	23 (55%)	10 (34%)	10 (32%)	22 (54%)	27 (61%)	12 (34%)
Recurrent wheeze/asthma – n (%)	28 (67%)	27 (93%)	22 (71%)	33 (80%)	26 (59%)	8 (23%)
Allergen specific IgE* (KU/L)	4.01 (0.94-73.6)	0.71 (0.24-3.33)	8.61 (1.11-14.1)	0.4 (0.21-5.86)	16.25 (2.09-148)	0.99 (0.04-8.32)

*Allergen-specific IgE was measured to egg white, cow's milk and peanut extracts for children assessed for egg, cow's milk and peanut allergies, respectively: 39 egg allergic, 21 egg non-allergic, 24 cow's milk allergic, 8 cow's milk non-allergic, 44 peanut allergic and 41 peanut non-allergic. All allergic patients had detectable allergen-specific IgE. Tolerance was defined clinically based on the uneventful consumption of the food and allergen-specific IgE was detectable in a subset of non-allergic children (n=18/21 for egg, n=8/8 for cow's milk and 30/41 for peanut).

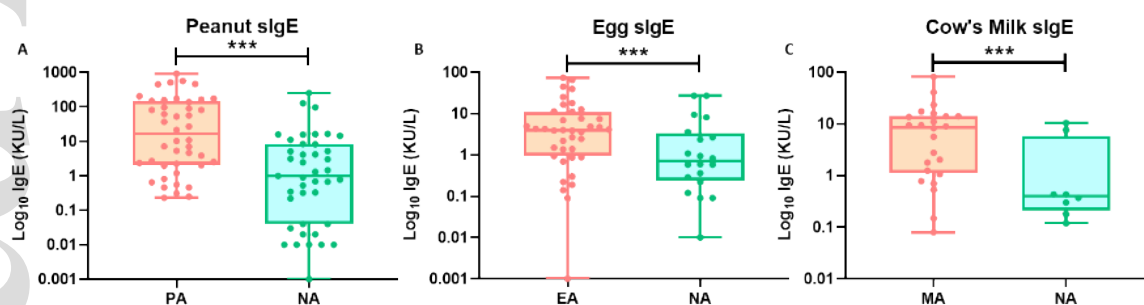
Table 2. Receptor and receptor-bound immunoglobulins on the surface of whole blood human basophils and human LAD2 cells sensitised with patients' plasma. Variables are expressed as percentage of positive cells for the marker of interest and represented as mean and standard deviation(*) or median and interquartile range(ϕ), depending on whether the distribution was normal or not, respectively.

Receptors / Immunoglobulins	Basophils	LAD2 Cells
FcεRI	83.2 ± 6.53%*	97.84±1.894%*
FcγRI (CD64)	3.45 ±1.79%*	17.06±3.99%*
FcγRII (CD32)	81.41 ± 9.81%*	99.33±1.29%*
FcγRIIa (CD32a)	74.54 ± 13.86%*	99.42±1.631%*
FcγRIIb (CD32b)	85.91 ± 8.943%*	55.65±10.24%*
FcγRIII (CD16)	4.12 ± 2.05%*	1.97±1.98%*
IgE	79.05 (62.4-90.15)% ϕ	99.25 (97.58-99.8)% ϕ
IgG	0.42 (0.1625-0.69)% ϕ	0.06 (0-0.458)% ϕ
IgA	2.43 (0.865-3.413)% ϕ	4.68 (1.493-6.585)% ϕ

Figure 1. Circulating allergen-specific immunoglobulin levels. **A.** Peanut-specific IgE; **B.** Egg white-specific IgE; **C.** cow's milk-specific IgE; **D.** Peanut-specific IgG; **E.** Peanut-specific IgG1; **F.** Peanut-specific IgG2; **G.** Peanut-specific IgG3; **H.** Peanut-specific IgG4; **I.** EW-specific IgG1; **J.** EW-specific IgG2; **K.** EW-specific IgG3; **L.** EW-specific IgG4; **M.** CM-specific IgG1; **N.** CM-specific IgG2; **O.** CM-specific IgG3. **P.** CM-specific IgG4. Boxplots express Range, Median and IQR. *** $p < 0.005$, **** $p < 0.0001$ using Mann Whitney U test. Figure 1N-P utilised Unpaired t-tests, in which * $p < 0.05$

Allergen-specific IgE and peanut-specific IgG were measured by ImmunoCAP (Thermofisher): Peanut allergic=43, Peanut non-allergic =41; Egg allergic=39, Egg non-allergic =20; Milk allergic=24, Milk non-allergic =8. Allergen-specific IgG isotypes were measured by ELISA: Peanut allergic=43, Peanut non-allergic =41; Egg allergic=34, Egg non-allergic =23; Milk allergic=23, Milk non-allergic =37.

Abbreviations: OD=Optical Density, EW=egg white.



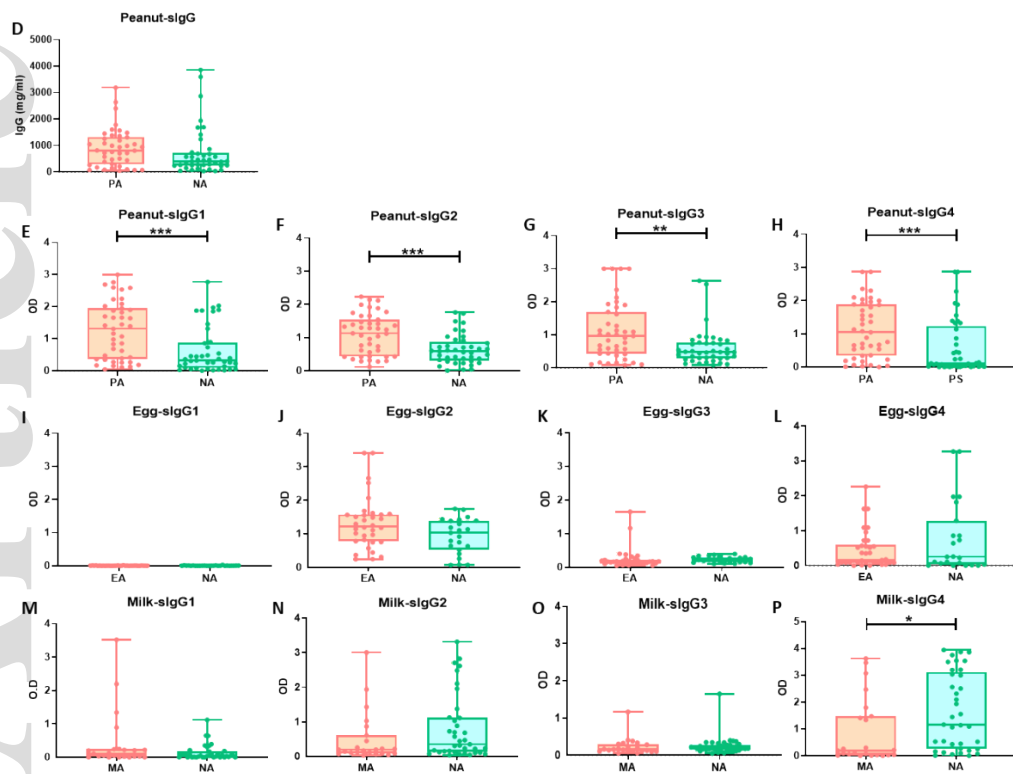


Figure 2. Fc Receptor expression on fresh whole blood basophils and LAD2 cells sensitised with plasma of patients assessed for peanut allergy. **A.** Basophil expression (sMFI) of FcεRI (n=23), CD32 (n=23), CD16 (n=18) and CD64 (n=17); **B.** LAD2 expression of FcεRI (n=21), CD32 (n=19), CD16 (n=16) and CD64 (n=17); **C.** Basophil expression of CD32α or CD32β (n=12) and **D.** LAD2 expression of CD32α or CD32β (n=20). Boxplots express Range, Median and IQR. ***p <0.005, ****p <0.0001 using Mann Whitney U test.

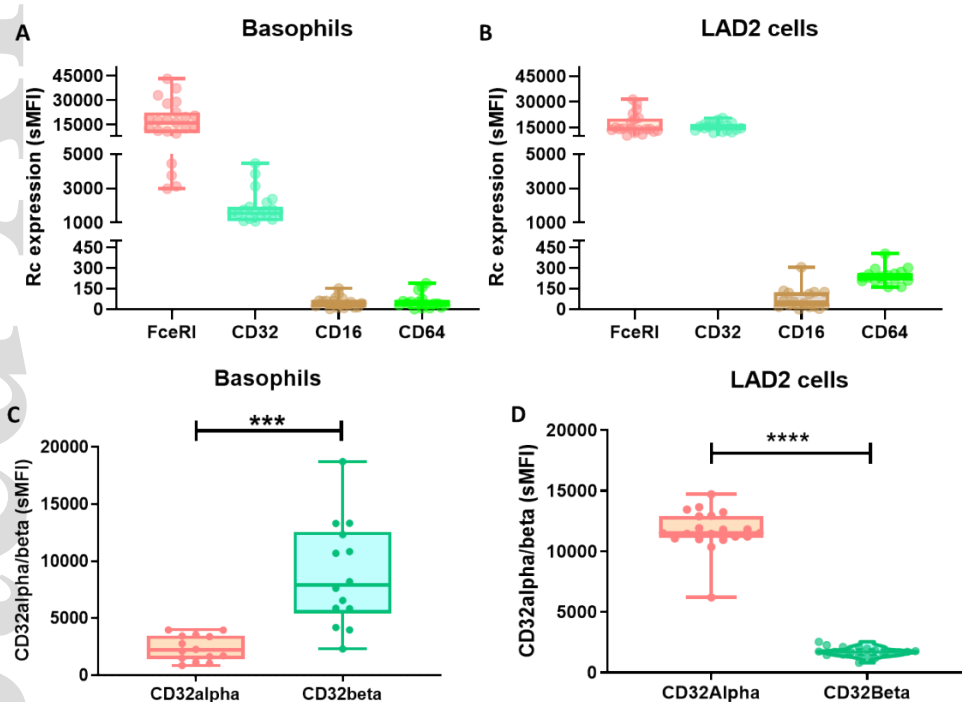


Figure 3. IgE, IgG and IgA surface expression on basophils and sensitised LAD2 cells. **A**, Proportion of basophils (n=18) **B**, and LAD2 cells expressing IgE, IgG and IgA (n = 20). **C**, The stimulation index of LAD2 cells sensitised with plasma in peanut allergic (PA, n=10) and non-allergic (NA, n=10) individuals compared to non-sensitised LAD2 cells. **D**, Representative plot of CD19+IgG+, and **E** CD19+IgA+ B cells used in a control experiment demonstrating efficient binding of IgG and IgA on the surface of tonsil B cells. Boxplots express Range, Median and IQR. A Mann-Whitney U test was performed for Figure 3C but no but no significant differences were observed

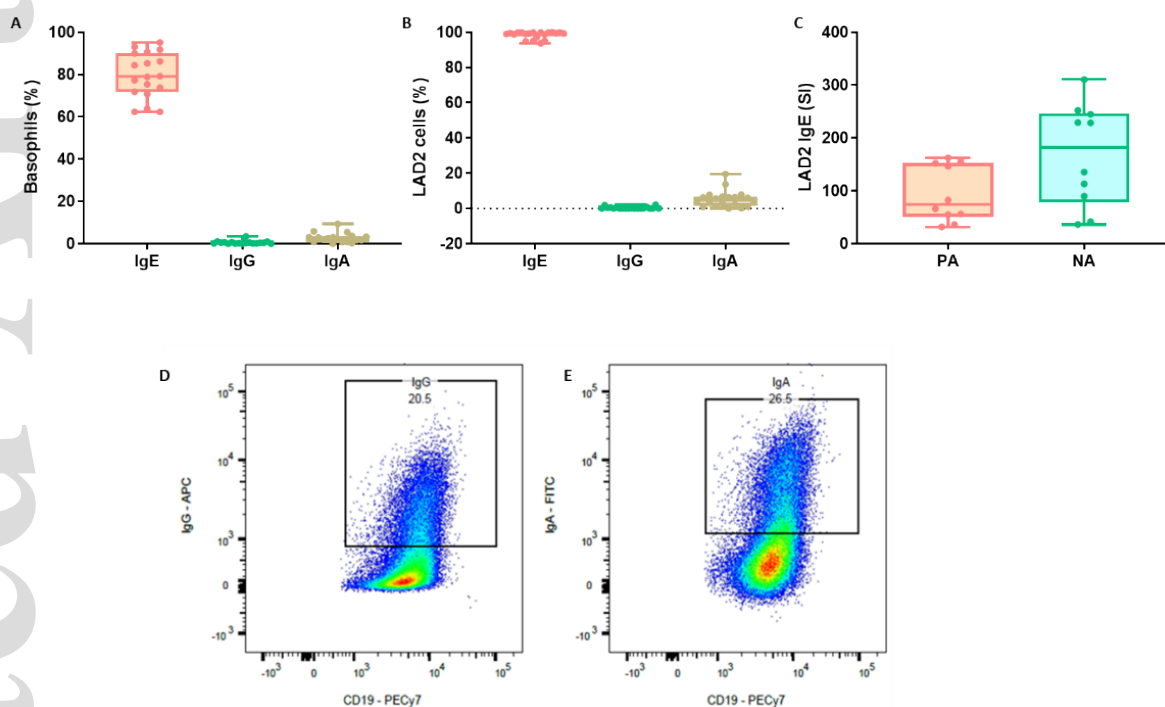


Figure 4. Basophil activation (measured as the proportion of CD63-positive basophils) in whole blood following stimulation with negative control (RPMI), allergen extract or anti-IgE in peanut allergic (A, n=3) or peanut non-allergic (B, n=6); egg allergic patients (C, n=16) or egg non-allergic individuals (D, n=7) with or without anti-FcγRIIα or anti-FcγRIIβ; Cow's milk allergic patients (E, n=11) or cow's milk non-allergic individuals (D, n=7) with or without anti-FcγRIIα or anti-FcγRIIβ. Boxplots express Range, Median and IQR. Wilcoxon signed-rank tests were performed to compare No Blocking, Alpha Blocking and Beta Blocking under all conditions, but no significant differences were observed.

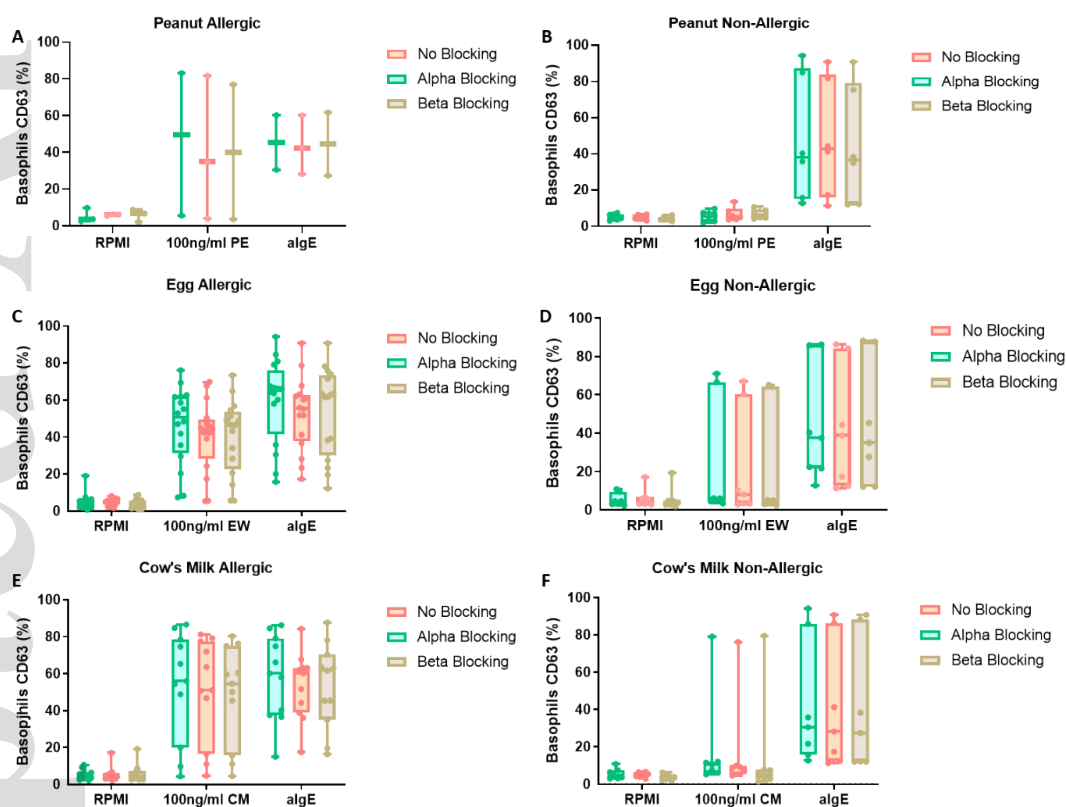
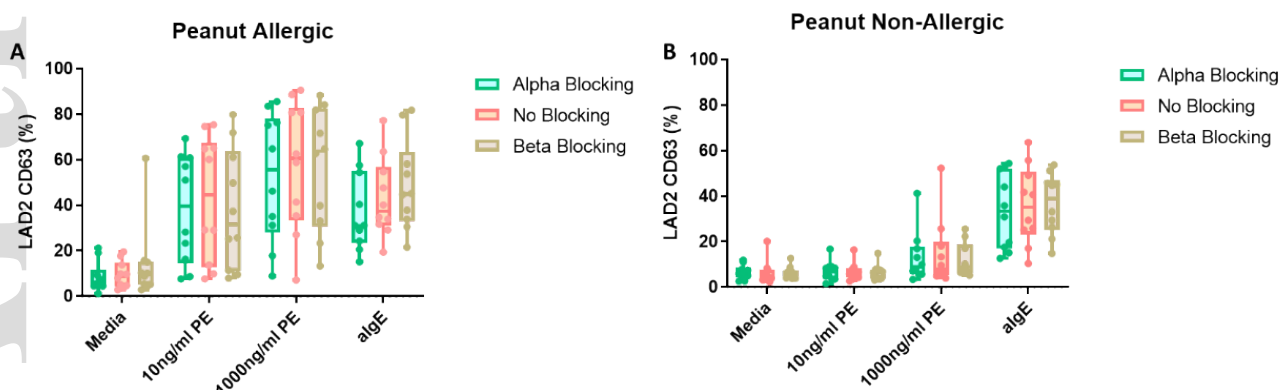


Figure 5. Mast cell activation (measured as proportions of CD63+ LAD2 cells) to peanut allergen after anti-FcγRIIα or anti-FcγRIIβ treatment of LAD2 cells prior to sensitisation with plasma from peanut allergic (A, n = 10) or peanut non-allergic (B, n = 10) children. Boxplots express Range, Median and IQR. Wilcoxon signed-rank tests were performed to compare No Blocking, Alpha Blocking and Beta Blocking under all conditions, but no significant differences were observed.



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Supplementary Material

Figure E1. Number of children tested to more than one food as part of the study.

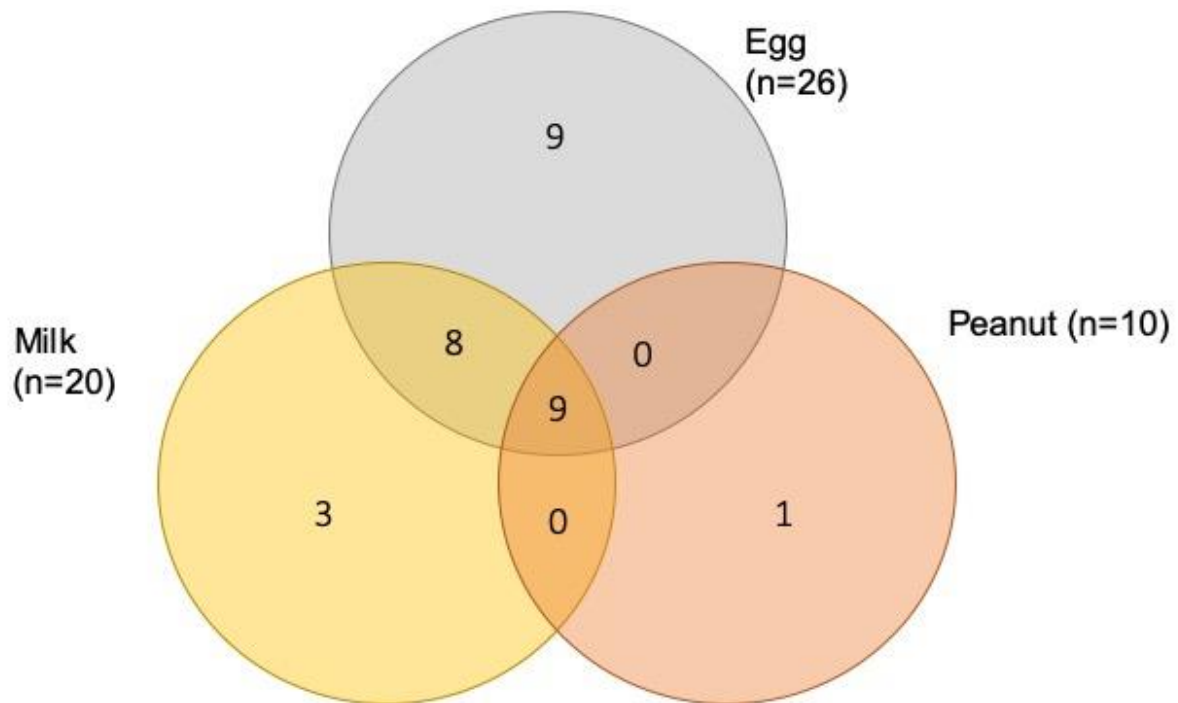


Figure E2. Gating strategy to identify basophils and measure their activation. A, Basophils were gated by size and granularity using FSC vs SSC, **B,** single cells **C,** expression of CD203c. **D,** Untreated basophils expressing baseline levels of CD63. **E,** CD63 expression of basophils stimulated with 100ng/ml peanut extract or **F,** 10ug/ml anti-IgE for 5 minutes at 37°C.

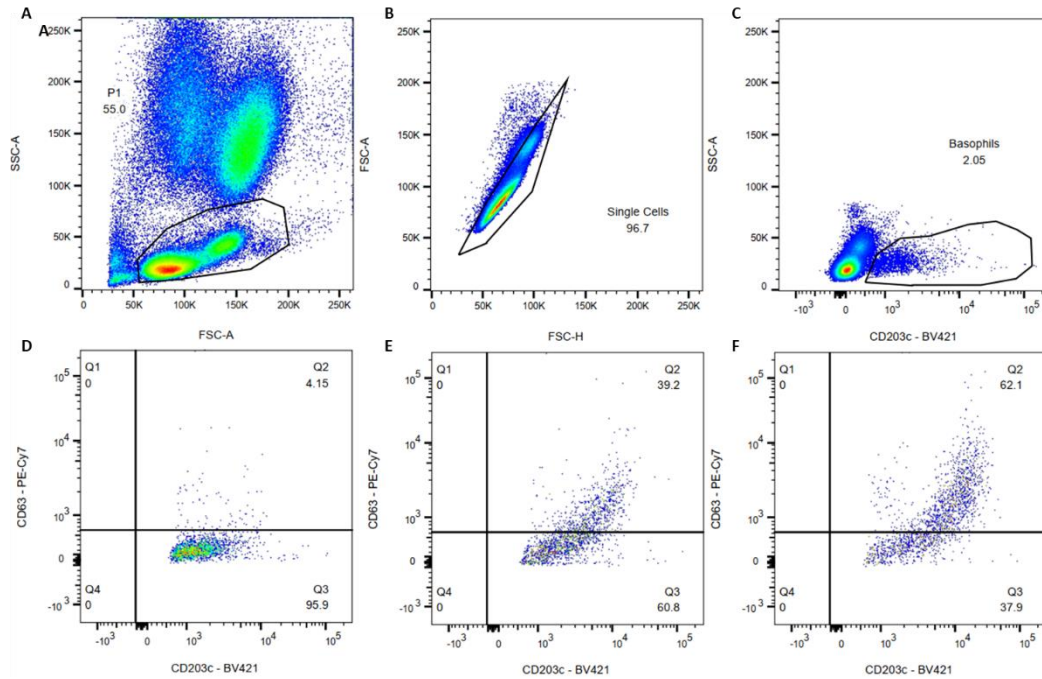


Figure E3. Gating strategy adopted in the mast cell activation test. A, Viable cells were identified by low expression of amine-binding dye. **B**, LAD2 cells were characterised as single cells. **C**, Untreated LAD2 cells expressing baseline levels of CD63. **D**, CD63 expression of LAD2 cells stimulated with 100ng/ml peanut extract or **E**, 10ug/ml anti-IgE for 30min at 37°C.

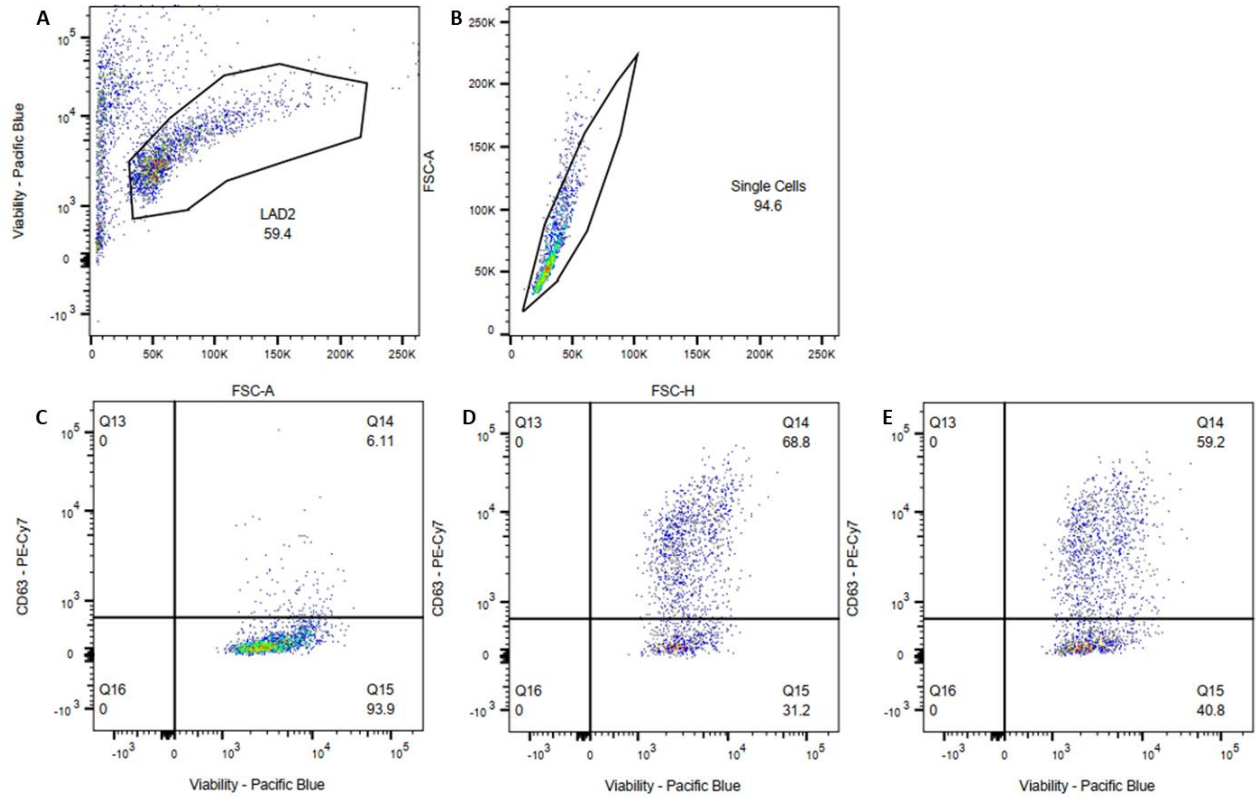


Figure E4. Identification of panCD32 in the presence of anti-FcγRIIα or FcγRIIβ blocking antibodies. **A**, The sMFI of panCD32 of LAD2 cells which were not treated with blocking antibodies, or treated with FcγRIIα blocking antibodies (n = 20). **B**, The sMFI of panCD32 of basophils which were not treated with blocking antibodies, or treated with FcγRIIβ blocking antibodies (n = 18). Boxplots express Range, Median and IQR. ***p < 0.005 using Wilcoxon signed rank test.

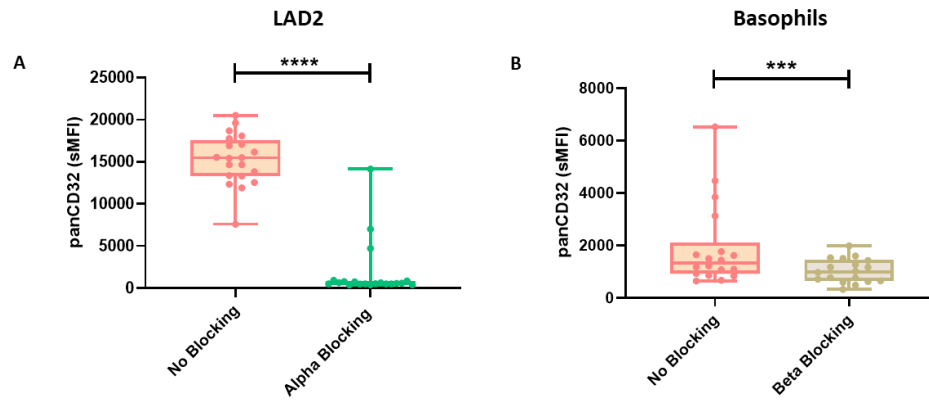


Figure E5. LAD2 Receptor expression. **A**, Isotype control staining or **B**, anti-FcεRI staining of LAD2 cells. **C**, Isotype control staining or **D**, anti-CD32 staining of LAD2 cells. **E**, Isotype control staining or **F**, anti-CD16 staining of LAD2 cells. **G**, Isotype control staining or **H**, anti-CD64 staining of LAD2 cells. **I**, Isotype control staining or **J**, anti-FcγRIIα staining of LAD2 cells. **K**, Isotype control staining or **L**, anti-FcγRIIβ staining of LAD2 cells.

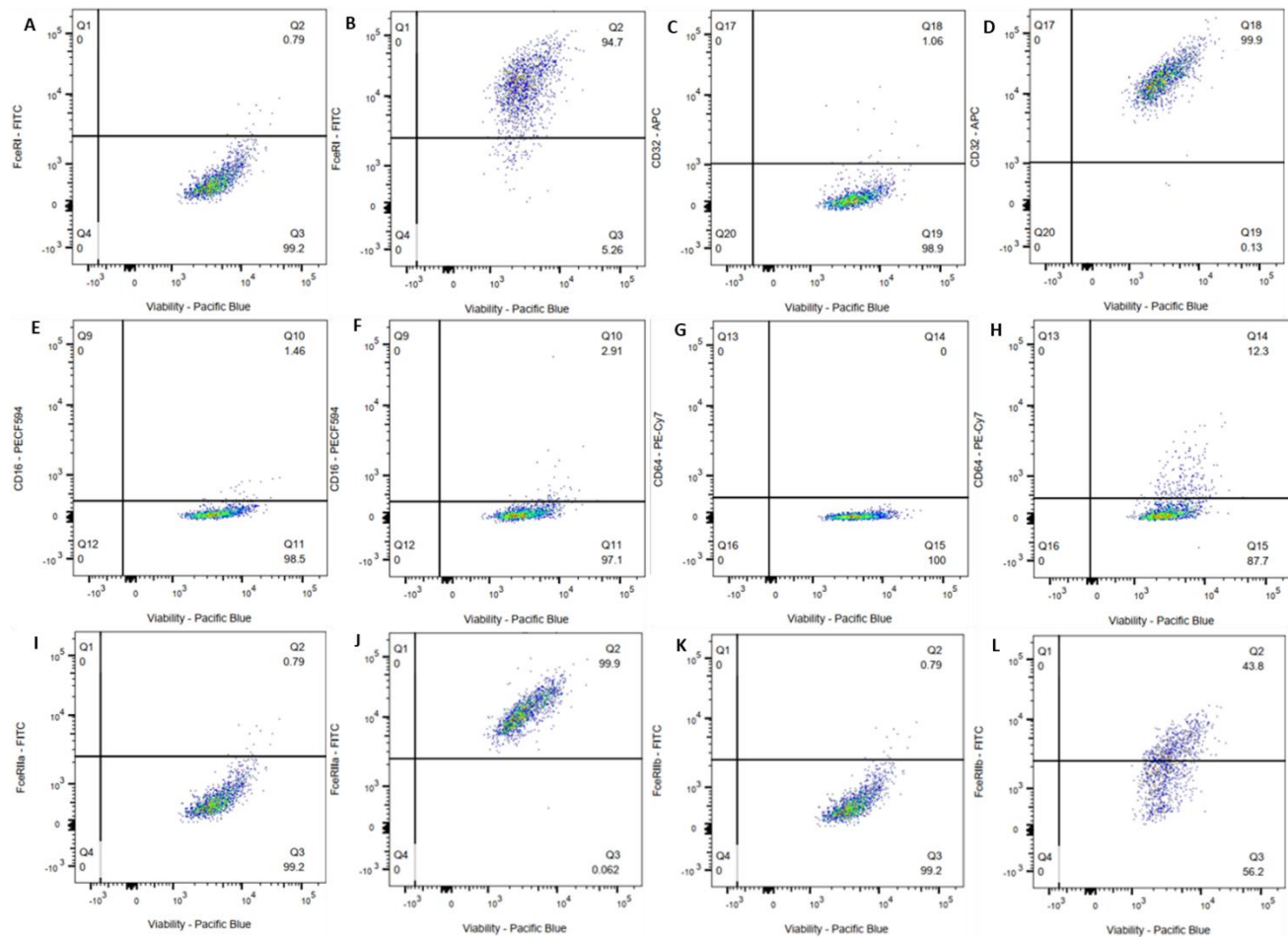


Figure E6. Basophil activation (measured as the proportion of CD63-positive basophils) in whole blood. This Figure shows the data presented in Figure 4 but emphasises the paired nature of the data. (A, n=3) or peanut non-allergic (B, n=6); egg allergic patients (C, n=16) or egg non-allergic individuals (D, n=7) with or without anti-FcγRIIα or anti-FcγRIIβ; Cow's milk allergic patients (E, n=11) or cow's milk non-allergic individuals (D, n=7) with or without anti-FcγRIIα or anti-FcγRIIβ. The effects of FcγRIIα and FcγRIIβ are presented for each individual patient, Wilcoxon signed-rank tests were performed to compare No Blocking, Alpha Blocking and Beta Blocking under all conditions, but no significant differences were observed.

