

IgE-mediated facilitated antigen presentation underlies higher immune responses in peanut allergy

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Abstract

Background: Peanut allergy poses significant healthcare problems, because its prevalence is increasing in many countries, and it is rarely outgrown. To explore the immunological mechanisms that underlie peanut allergy and tolerance, we compared the peanut-specific responses of peanut-allergic (PA) and nonallergic (NA) individuals.

Methods: We measured peanut-specific peripheral blood mononuclear cells (PBMC) proliferation using tritiated thymidine. The frequency of peanut-specific T cells amongst PBMC was determined by carboxyfluorescein succinimidyl ester labelling. The role of IgE-dependent facilitated antigen presentation (FAP) in modulating proliferation was investigated by depleting IgE from plasma with anti-IgE-coated beads and then assessing PBMC proliferation in the presence of IgE-depleted or nondepleted plasma.

Results: We found that peanut-specific PBMC proliferation is higher and peaks earlier in PA than in NA donors. We investigated the immunological mechanisms that could underlie these differences. We found that both PA and NA have memory responses to peanut, but the frequency of peanut-specific T cells is higher in PA than in NA. Facilitated antigen presentation could cause both the higher proliferation and precursor frequency in PA. Facilitated antigen presentation activity *in vitro* was confirmed by showing that IgE depletion decreases proliferation, while adding IgE back restores it.

Conclusion: Our results identify FAP as a mechanism that underlies higher responses to peanut in PA. In these individuals, high levels of peanut-specific IgE could furthermore maintain long-term allergic T-cell responses. We raise the question whether, in the future, therapies targeting IgE such as anti-IgE antibodies may be used to suppress these T-cell responses.

Peanut allergy is a potentially life-threatening condition characterized by allergic reactions that are sometimes triggered even by minute quantities of peanuts and may be very severe (1, 2). The prevalence of peanut allergy has increased in many countries during the last decades, affecting at present 1–2% of children (3, 4).

Abbreviations

APC, antigen presenting cell; CFSE, carboxyfluorescein succinimidyl ester; FAP, Facilitated antigen presentation; NA, nonallergic; PA, peanut allergic; PBMC, peripheral blood mononuclear cells; PF, precursor frequency; Th, T helper; TT, tetanus toxoid.

A better understanding of the processes that lead to peanut allergy or tolerance is necessary to design interventional approaches aimed at preventing peanut allergy and/or inducing its resolution (5). The immunological mechanisms that underlie allergy or tolerance to peanuts are however still unclear. In the past, we have investigated the qualitative differences between the responses of peanut-allergic (PA) and nonallergic (NA) individuals and found significant differences regarding their peanut-specific T-cell cytokine production and Th2-skewing of the cytokine production phenotype in PA (6, 7).

In the present work, we take these findings forward by investigating the immune mechanisms that underlie the quantitative

differences between peanut-specific responses of T cells from PA and NA individuals. For this purpose, we used *in vitro* antigen-specific peripheral blood mononuclear cells (PBMC) proliferation, which is a well-established experimental model for investigating immune responses in peanut allergy (8–13).

Using this model, we initially characterized the intensity, kinetics and dose–response relation of the proliferative responses of PBMC isolated from PA and NA children. We then investigated three potential mechanistic explanations for the quantitative differences that we observed between PA and NA donors. Thus, because the *in vitro* cultures contained equal numbers of PBMC, the observed higher proliferation of PBMC from PA than NA could be explained by:

- *In vitro* PBMC responses from PA being derived predominantly from memory T cells. Nonallergic individuals may have lower responses, because they have predominantly naïve peanut-specific T cells that proliferate less strongly than memory T cells.
- Higher frequencies of peanut-specific circulating T cells in PA compared to NA donors.
- IgE-mediated facilitated antigen presentation (FAP) in PA may amplify peanut-specific T-cell proliferation.

In our study, we investigated these three possible explanations. We thus determined the memory/naïve phenotype of the peanut-specific responses of PBMC from PA and NA donors. We then determined the respective peanut-specific T-cell precursor frequencies and the role of IgE in amplifying peanut-specific proliferation.

Methods

Donors

Forty-five donors – 25 PA and 20 NA participated in this study, which was approved by the Research Ethics Committee at St Mary's Hospital, London, UK. The diagnosis of peanut allergy was based on a characteristic history of immediate hypersensitivity reactions occurring soon after peanut ingestion and at least one of the following diagnostic criteria: peanut skin prick test wheal ≥ 6 mm or peanut-specific IgE ≥ 15 kU/l or a positive peanut challenge (14–16). The NA donors had never reacted to peanut ingestion and were currently consuming peanuts as part of their diets, tolerating the equivalent of at least one peanut butter sandwich.

Reagents

Peanut-defatted extract, kindly provided by Dr Henning Løwenstein (ALK Abellø, Horsholm, Denmark), was added to the cell culture at a final concentration of 100 μ g/ml. Tetanus toxoid (TT; Statens Serum Institute, Copenhagen, Denmark) was used at 10 μ g/ml and was chosen as control antigen, because it is administered as an alum-adsorbed vaccine and therefore induces Th2-skewed responses in children. Ovalbumin and beta-lactoglobulin (Sigma, Poole, UK) were used at 200 μ g/ml in the cultures, while protein purified derivative (PPD) (Evans Laboratories, Liverpool, UK) was used at 20 μ g/ml.

PBMC isolation and cell culture

Citrate dextrose-anticoagulated blood was centrifuged (600 g, 10 min) to separate plasma. Peripheral blood mononuclear cells were further isolated by density gradient separation using Histopaque-1077 (Sigma-Aldrich, Poole, UK). Peripheral blood mononuclear cells, $3 \times 10^6/2$ ml medium/well, were then cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 5% autologous plasma and antigens. The autologous plasma that was added to the cultures was not heat-inactivated to preserve the intact antibodies (including IgE) in it and thus making the system more biologically relevant and decreasing background proliferation. The cell culture system also ensured low background proliferation because of flat-bottom culture plates that limit antigen presenting cell (APC)-Th contact (17).

Assessment of the kinetics of antigen-specific proliferation

After 3, 5, 7 or 9 days of PBMC culture in the presence of peanut or control antigens, 100 μ l aliquots was collected and transferred to 96-well, U-bottom plates (in triplicate). 0.5 μ Ci [^{3} H]-methyl-thymidine was added per well, the plates were incubated for 6 h, and then cells were collected on glass fibre filters using a Filtermate 196 cell harvester (Perkin Elmer, Beaconsfield, UK). Tritiated thymidine incorporation into DNA was measured using a TopCount 9912 Packard microplate scintillation counter (also from Perkin Elmer).

Determination of the naïve/memory phenotype of peanut-responder Th cells

We used magnetic beads (MACS) to isolate the naïve (CD4+ CD45RA+) and the memory Th cells (CD4+CD45RO+) from the PBMC of PA and NA individuals. Thus, we first used the CD4+ T-cell isolation kit II from Miltenyi Biotec (Bisley, UK), according to the manufacturer's protocol, to separate the Th cell fraction from PBMC. The negative Th fraction was further split into memory and naïve cells using CD45RA Microbeads, also from Miltenyi Biotec. The separate population containing dendritic cells, monocytes, B cells etc., was irradiated at 30Gy and used as APC at 10^5 /well in 96-well, U-bottom plates. 5×10^4 memory or naïve Th cells per well were added to the irradiated APC. Proliferation was measured after 5 days of culture in the presence of peanut proteins (100 μ g/ml), by assessing thymidine incorporation into DNA, as previously described.

Determination of the frequency of circulating peanut-specific Th cells

The frequency of circulating peanut-specific Th cells (precursor frequency, PF) was determined using an established method (18–21). We chose an approach based upon the identification of the T cells which proliferate in the presence of peanut antigens rather than a cytokine secretion-based assay, because we found that the T-cell responses of both PA and NA individuals result from mixed Th subsets rather than

'clonally differentiated', pure Th2 or Th1 peanut-specific populations (6). Thus, methods that assess PF by determining a Th2-specific cytokine (for example IL-4 or IL-5) by intracellular cytokine flow cytometry (FACS) underestimate the PF (by missing T cells that do not secrete the cytokine that we stain for). Briefly, after isolation, the PBMC were labelled with 5 μ M carboxyfluorescein succinimidyl ester (CFSE) in a shaking water bath at 37°C, for 10 min. We have previously shown by cloning that the dividing lymphocytes are peanut specific (6). After 7 days, we identified the emergent CFSE^{low} T-cell population that halved their fluorescence after each division and calculated the number of precursor Th cells according to the number of cell divisions they underwent. To correct those values for cell death during culture, we determined the number of Th cells existing at the beginning of the culture using standard count beads (Serotec, Kidlington, UK). At least 10 000 events were collected for each condition, and data were analysed using the WinMDI 2.8 software (from the Scripps Research Institute: <http://facs.scripps.edu/software.html>).

IgE depletion from plasma using magnetic beads

Autologous plasma was filtered through a 0.8/0.2 micron Acrodisc filter (VWR, Lutterworth, UK) to remove any particles. For IgE depletion, plasma was incubated for 1 h at room temperature under gentle rotation with M-450 Tosyl-activated Dynabeads (Dyna, Paisley, UK), coated according to the manufacturers' protocol with goat polyclonal anti-human IgE antibodies (Becton Dickinson, Cowley, UK). $8\text{--}16 \times 10^8$ coated Dynabeads were used per millilitre of plasma for each cycle of IgE depletion. Four rounds of depletion were routinely used to ensure that most of the peanut-specific IgE had been removed. Peanut specificity was determined using UniCAP. IgE-depleted plasma was then collected, sterilized by filtration and added to the cell culture at 5% final concentration. Captured IgE was eluted from the Dynabeads using a 0.02 M acetate buffer pH 2.5. The buffer was then exchanged into RPMI 1640 cell culture medium using a 10-kDa cut-off concentrator (Vivascience AG, Hannover, Germany). The IgE was then added back to control cultures in which IgE-depleted plasma was used as supplement. The concentration of peanut-specific IgE in the depleted plasma and in the eluates was determined using the Pharmacia UniCAP assay (22).

Results

Peanut antigen-driven PBMC proliferation is higher and reaches its peak earlier in PA than in NA donors

We found that PBMC isolated from PA donors have high and early proliferative responses that peak on day 5 and decline by day 7 (Fig. 1). Conversely, the NA donors' responses to peanut reach their maximal values later than PA, usually on day 7. Thus on day 5, the median stimulation indices were 88.35 (range: 13.8–523.26) for PA and 6.07 (range: 1.18–38.8) for NA. On day 7, the corresponding

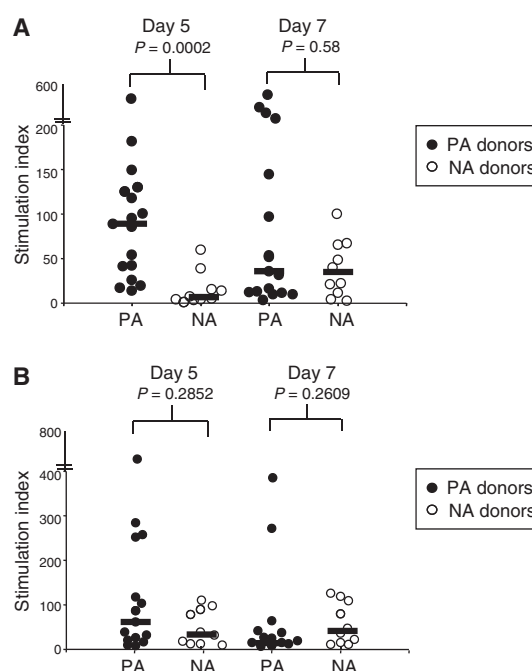


Figure 1 (A) Peanut-specific proliferation of peripheral blood mononuclear cells (PBMC) from $n = 17$ peanut-allergic (PA) donors and $n = 10$ nonallergic (NA) donors. (B) Tetanus toxoid (TT)-specific proliferation of PBMC from $n = 15$ PA donors and $n = 10$ NA donors. Proliferation is expressed as stimulation indices (SI) values representing the radioactivity (counts per minute) ratios between the antigen-stimulated and unstimulated PBMC cultures. SI measured on day 5 and respectively day 7 of culture in the presence of peanut or TT are shown. Points represent values for individual donors, while bars represent median values. P -values were calculated using the Mann–Whitney test.

median values were 35.26 (range: 3.46–428.5) for PA and 34.71 (range: 2.6–239.6) for NA donors.

Hence, owing to the different proliferation kinetics existing in PA *versus* NA donors, only the early measurements (day 5) but not the later ones (day 7) reflect the different clinical phenotypes. Indeed, using the Mann–Whitney U -test, the differences between PA and NA were statistically significant on day 5 ($P = 0.0002$) but not on day 7 ($P = 0.58$) (Fig. 1A). We also assessed PBMC proliferation induced by control antigens TT (Fig. 1B), ovalbumin, lactoglobulin and PPD (data not shown) and found no significant difference between PA and NA donors.

Memory Th cells are a major proliferating subset in both PA and NA donors

Considering that naïve Th cells respond less intensely and only after a prolonged contact with the APC in comparison with memory Th cells (23, 24), we intended to determine whether the lower and delayed peanut-specific proliferation seen in NA was caused by higher levels of naïve Th responses to peanut in these donors. We separated the memory and

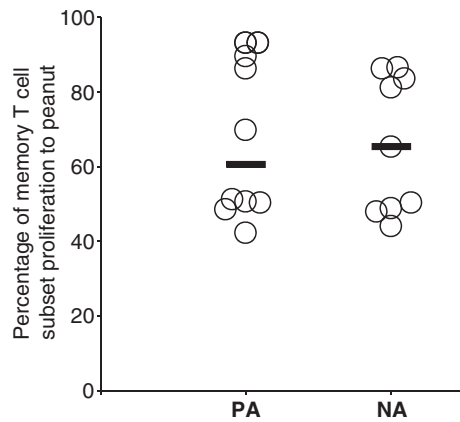


Figure 2 Peanut-specific memory responses in $n = 10$ peanut-allergic and $n = 9$ nonallergic donors. Proliferation of the memory Th cell population is expressed as percentage of the total peanut-specific proliferation (i.e. the sum of memory + naïve proliferation represents 100%). Points represent values for individual donors, while bars represent median values. P -values were calculated using the Mann–Whitney test.

naïve Th subsets by MACS and cultured both subsets in the presence of peanut antigens. We found that the memory Th response to peanuts accounts for more at least half of peanut-specific proliferation in most PA and NA donors investigated (Fig. 2). We did not find any statistically significant difference between the PA and NA response in this respect ($P = 0.603$). Therefore, it appears that memory Th cells are the main peanut-responding subset in both PA and NA donors, and the differences between PA and NA are not caused by a predominantly naïve Th response in NA individuals.

PA donors have higher peanut-specific T-cell frequencies than NA donors

Because for both PA and NA donors, we put equal numbers of PBMC in culture, the higher levels of T-cell proliferation found in PA individuals could be explained by the presence of higher frequencies of peanut-specific lymphocytes amongst their PBMC. We tested this hypothesis by calculating of the frequency of antigen-specific T cells amongst PBMC (18–21). We found that PA individuals have indeed more peanut-specific Th cells amongst their circulating PBMC than NA donors (Fig. 3). The median PF values were 0.608% for PA and 0.076% for NA individuals, and the difference between the two groups was highly statistically significant ($P < 0.001$).

FAP occurs in PA but not in NA individuals

Peanut-specific IgE-mediated FAP could be another explanation for the higher responses seen in PA individuals. As FAP would be revealed by the occurrence of higher responses at limiting concentrations of allergen, we set up dose–response curves to determine the effect of lower peanut protein concentrations

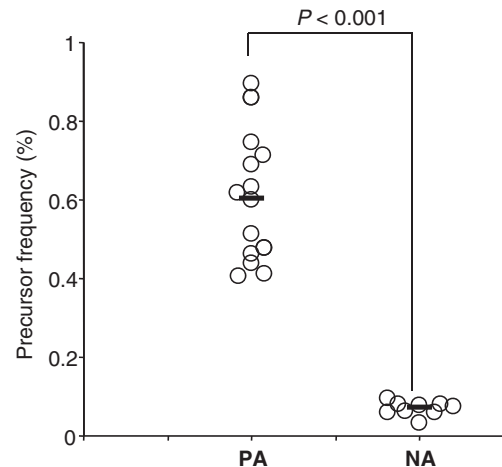


Figure 3 Frequency of peanut-specific circulating CD4⁺ Th cells (precursor frequency, PF) in $n = 14$ peanut-allergic and $n = 9$ nonallergic individuals. PF was calculated according to the number of cell divisions underwent by cells from the CFSE^{low} population that emerged after 7 days of culture in the presence of peanut antigens. Precursor frequency was expressed as percentages amongst the total CD4⁺ Th cells in cultures on day 7, and the values were then corrected for the cell death that occurred during the 7 days of culture. Points represent individual values, while bars represent medians. Statistical significance was calculated using the Mann–Whitney test.

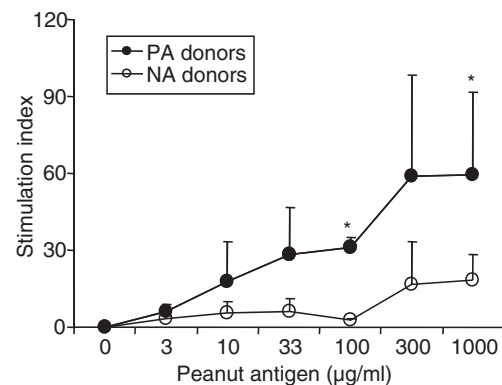


Figure 4 Relation between peanut antigen concentration and peripheral blood mononuclear cells proliferation in $n = 3$ peanut-allergic and $n = 3$ nonallergic donors. Day 5 dose–response SI proliferation curves to different concentrations of peanut antigens were established. Points represent average SI values, and error bars represent standard deviations. Statistical significance was calculated using the Mann–Whitney U -test, and P -values below 0.05 are labelled with *.

upon PBMC proliferation (Fig. 4). We found that these dose–response curves are shifted to the right in the NA donors. Hence, a 20–30-fold higher concentration of peanut protein is required to elicit the same level of proliferation in PBMC from NA, when compared with PBMC from PA donors.

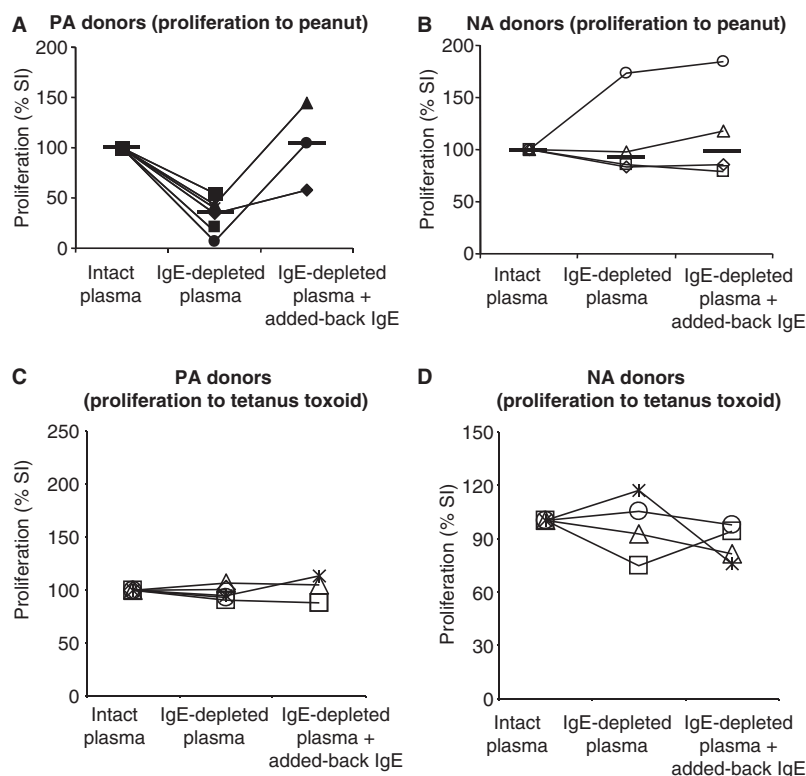


Figure 5 Effect of IgE depletion upon peanut-specific proliferation in peripheral blood mononuclear cells (PBMC) isolated from peanut-allergic (PA) (5A) and nonallergic (NA) (5B) donors. Proliferation to tetanus toxoid, used as a control antigen, is also shown in PA (5C) and NA donors (5D). Peripheral blood mononuclear cells were cultured in RPMI supplemented with 5% of either intact plasma

or IgE-depleted plasma or IgE-depleted plasma to which eluted IgE was added back. Proliferation in the presence of intact plasma is expressed as 100%, and the relative changes in proliferation observed in cultures with IgE-depleted plasma or IgE-depleted + IgE add-back plasma are shown.

These results suggest that PBMC proliferation is higher at relatively lower doses of peanut antigen in PA than in NA donors. As IgE is heat labile, we further confirmed that FAP is IgE mediated by heating the plasma from PA donors at 56°C for 2 h, a treatment that is known to denature IgE antibodies but not other antibody isotypes (25). We found that proliferation to peanut in the PA donors tested was indeed decreased by 46.02–82.71% (median value 69.47% – data not shown).

IgE depletion from plasma using magnetic beads leads to a decrease in peanut antigen-driven PBMC in PA but not in NA donors

To further characterize the role of IgE in FAP, IgE was depleted from the plasma used to supplement PBMC cultures with magnetic beads. When multiple rounds of successive IgE depletion were used, peanut-specific IgE was decreased from a median value IgE = 9.35 kIU/l (range: 0.57–434 kIU/l) to a median value IgE = 3.5 kIU/l (range: 0.39–100 kIU/l). Peripheral blood mononuclear cells culture in the presence of peanut antigens and IgE-depleted plasma led to a significant decrease in peanut-specific PBMC proliferation in PA but

not in NA individuals (Fig. 5A, B). Conversely, proliferation to TT (as control antigen) was not affected, further demonstrating that the decrease in proliferation is allergen specific and IgE dependent (Fig. 5C, D).

Add-back of IgE to the IgE-depleted plasma leads to the restoration of the peanut-specific PBMC proliferation

The attached IgE was eluted from the magnetic beads used to deplete it from plasma. The eluted IgE was then added back to IgE-depleted plasma to be able to compare the peanut-specific proliferation of PBMC cultured in the presence of intact plasma, of IgE-depleted plasma and of IgE-depleted plasma, with IgE added back (Fig. 5).

In a typical PA individual, we observed that IgE depletion (labelled as IgE⁻) led to a decrease in peanut-specific proliferation while adding-back purified IgE (labelled as IgE⁺) restored proliferation (Fig. 6A). Conversely, we did not observe any change in TT-specific proliferation if purified IgE was added to TT-driven cultures (Fig. 6B). This finding further confirms that the observed variations in peanut-specific proliferation are caused by IgE-dependent FAP.

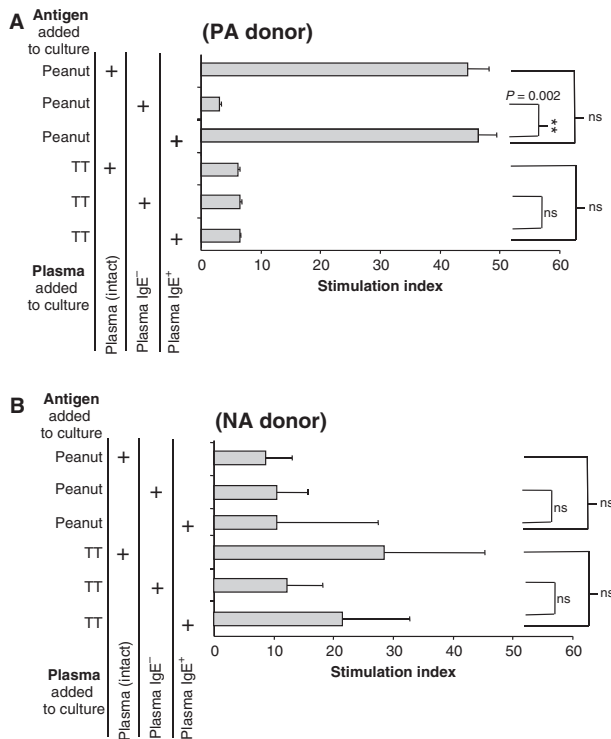


Figure 6 Effect of IgE depletion from the plasma of a typical peanut-allergic and a typical nonallergic donor. peripheral blood mononuclear cells were stimulated with either peanut antigens or with tetanus toxoid as control antigen. Bars represent averages of triplicate determinations with standard deviation values. Statistically significant *P*-values were calculated using Student's *t*-test.

Discussion

The main findings of our study are that peanut-specific *in vitro* PBMC proliferation is increased and has a different kinetic and dose-response profile in PA compared to NA individuals. Our investigation into the underlying immunological mechanisms revealed that both PA and NA donors have predominantly memory responses to peanut antigens, but the PA donors have higher levels of peanut-specific T helper cells amongst PBMC. Furthermore, peanut-specific proliferative responses from PA donors are significantly amplified by plasma IgE.

We demonstrate that FAP is allergen (peanut) specific and characterize its antibody class specificity (IgE). This has been carried out by using well-characterized patients with peanut allergy when compared to control patients who are tolerant to peanuts. We included a NA control patient group and nonallergenic control antigens in our investigation, unlike many published studies on FAP in which only allergic donors and one allergen are analysed.

We have then performed IgE depletion and add-back experiments showing that this only affects proliferation to peanut in allergic and not in NA individuals. Furthermore, the inclusion of a control antigen (TT) demonstrates that the effect of IgE on FAP is antigen specific and is not a

polyclonal IgE effect. This level of characterization of the specificity and functionality of IgE-mediated FAP, to our knowledge, has only been demonstrated as completely in a murine model (26).

Moreover, we have linked the phenomenon of FAP for the first time to the PF of allergen-specific cells if elevated in PA individuals, and this is associated with the phenomenon of IgE-mediated FAP, so that both the initiation and maintenance of the prolonged allergic state to peanut may result from this phenomenon.

Our results concord with those reported by other authors who found high levels of peanut-specific proliferation in PBMC isolated from PA or NA children (8–11). Indeed, de Jong et al. (12) and Hourihane et al. (13) showed significantly higher levels of peanut-specific proliferation in PBMC isolated from PA children, mirroring their PA status.

The mechanistic explanation that could underlie our findings is represented by IgE-mediated FAP. We have indeed observed that plasma containing peanut-specific IgE amplifies peanut-specific proliferation of PBMC from NA individuals. The role of IgE in amplifying peanut-specific proliferation was further demonstrated in IgE-depletion experiments using magnetic beads coated with anti-IgE antibodies. Conversely, plasma IgE depletion decreases peanut-specific proliferation in PA but not in NA individuals, suggesting the involvement of IgE-mediated FAP that may underlie the differences between PA and NA peanut-specific responses. A potential alternative explanation of an IgG-mediated effect that underlies FAP is unlikely because in similar FAP experimental system in atopic dermatitis, it was shown that most of allergen-specific proliferation depends upon IgE (27). Furthermore, in a preliminary experiment, we inhibited peanut-specific but not control antigen-specific responses in PA individuals by heating added plasma at 56°C for 2 h – a treatment that degrades IgE but not IgG.

The current literature on IgE-mediated FAP suggests that allergen presentation to the specific Th cells amongst the PBMC is indeed increased when higher amounts of allergen-derived peptides are present on the surface of APC (28). In this respect, the presence of allergen-specific IgE leads to FAP as a result of a higher rate of allergen capture and internalization because of the endocytosis of IgE-allergen complexes. Mechanistically, it was shown (29–31) that the high-affinity IgE receptors on dendritic cells deliver IgE-bound allergen into vesicles containing MHC class II, HLA-DM and lysosomal proteins (a cathepsin S-dependent pathway of MHC class II presentation). The low-affinity CD23 receptor was also shown to mediate FAP in a mouse model (32).

The relationship between the presence of allergen-specific IgE in the serum of allergic individuals and FAP leading to increased allergen-driven T-lymphocyte proliferation has been clearly established *in vitro* (33) and in experimental models *in vivo* (26). In patients with atopic dermatitis, 59–67% of PBMC proliferation to *Dermatophagoides pteronyssinus* allergens depended upon IgE (27) and APC that bear IgE receptors are likely involved in the pathogenesis of this disease

(34). Mechanistically, it has been shown that antigen endocytosis mediated through the high-affinity IgE receptor turns interferon gamma-treated mouse mast cells into potent APC effectors (35). These results suggest that IgE-mediated allergen endocytosis leads to an increased and possibly qualitatively different presentation because of the activation of signalling pathways in the APC (36), eventually resulting in stronger, Th2-skewed T-cell responses.

The increased proliferation of PBMC from PA donors which we have found in our *in vitro* experimental system arguably results from increased peanut-specific T-cell PF, possibly occurring owing to IgE-mediated FAP. In this respect, our results concord with observations of higher levels of circulating allergen-specific precursor T cells in allergic individuals when compared with NA controls (19–21). The importance of IgE-dependent FAP for the activation of allergen-specific T cell has also been demonstrated with respect to blocking antibodies (37). Furthermore, the addition of humanized monoclonal anti-IgE antibodies prevents the activation of allergen-specific T cells in an *in vitro* experimental model (38).

Therefore, we propose the following interpretation of our findings regarding peanut-specific responses in PA and NA individuals: it is known that persistent food allergy is characterized by high specific IgE levels and high T-cell proliferative responses, whereas resolution of food allergies is characterized by declining IgE levels and decreasing T-cell proliferative responses (39–42). A possible explanation for this is that, in the allergic state, T cells (through the elaboration of pro-allergic cytokines and cognate interaction with B cells) drive the production of IgE. In its turn, IgE production amplifies T-cell proliferative responses through IgE-mediated FAP, leading to a positive feedback loop that maintains the allergy. Indeed, in our study we

found clear evidence of IgE-mediated FAP leading to an increase in allergen-specific T-cell proliferative responses *in vitro*. The role of IgE in stimulating peanut-specific proliferation was further confirmed by our depletion/add-back experiments. Higher levels of proliferative responses lead to an increase in the number of circulating antigen-specific cells, as seen in the case of responses to infectious antigens for example. Because we demonstrate that FAP underlies higher levels of proliferation in PA comparatively with NA individuals, it seems legitimate to speculate that FAP blockade with anti-IgE would be effective to decrease allergic responses in specific immunotherapy (SIT) interventions when high amounts of allergen are administered to patients.

In this respect, anti-IgE blocking antibodies may interfere with the allergic immune mechanisms by not only preventing the IgE-triggered mast cell degranulation that underlies allergic reactions but also FAP that maintains the ongoing allergic immune response. This raises the question whether therapeutic use of anti-IgE in peanut allergy, eventually associated with oral administration of peanut, may lead to long-term resolution of this allergy by interrupting the IgE-dependent pro-allergic feedback loop caused by FAP.

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