

IgE⁺ B cells are scarce, but allergen-specific B cells with a memory phenotype circulate in patients with allergic rhinitis

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Keywords

allergic rhinitis; B cells; grass pollen; immunoglobulin E; immunoglobulin E B cells.

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Abstract

Background: Despite the critical role of immunoglobulin E (IgE) in allergy, circulating IgE⁺ B cells are scarce. Here, we describe in patients with allergic rhinitis B cells with a memory phenotype responding to a prototypic aeroallergen.

Methods: Fifteen allergic rhinitis patients with grass pollen allergy and 13 control subjects were examined. Blood mononuclear cells stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) were cultured with Bahia grass pollen. Proliferation and phenotype were assessed by multicolour flow cytometry.

Results: In blood of allergic rhinitis patients with high serum IgE to grass pollen, most IgE^{hi} cells were CD123⁺ HLA-DR⁺ basophils, with IgE for the major pollen allergen (Pas n 1). Both B and T cells from pollen-allergic donors showed higher proliferation to grass pollen than nonallergic donors ($P = 0.002$, and 0.010 , respectively), whereas responses to vaccine antigens and mitogen did not differ between groups. Allergen-driven B cells that divided rapidly (CD19^{mid} CD3⁺ CFSE^{lo}) showed higher CD27 ($P = 0.008$) and lower CD19 ($P = 0.004$) and CD20 ($P = 0.004$) expression than B cells that were slow to respond to allergen (CD19^{hi} CD3⁺ CFSE^{mid}). Moreover, rapidly dividing allergen-driven B cells (CD19^{mid} CFSE^{lo} CD27^{hi}) showed higher expression of the plasmablast marker CD38 compared with B cells (CD19^{hi} CFSE^{mid} CD27^{lo}) that were slow to divide.

Conclusion: Patients with pollen allergy but not control donors have a population of circulating allergen-specific B cells with the phenotype and functional properties of adaptive memory B-cell responses. These cells could provide precursors for allergen-specific IgE production upon allergen re-exposure.

Immunoglobulin E (IgE) responses to allergen in patients with allergic disease may develop in ways beyond the classical T-cell-driven, IgG B-cell response arising in germinal centres [reviewed in (1)]. Typically, adaptive B-cell responses to T-dependent protein antigens develop in germinal centres of secondary lymphoid organs. This germinal centre pathway of

activation and differentiation involves follicular T-cell help, isotype switch (recombination of Ig heavy chain), somatic hypermutation of their immunoglobulin variable domains and selection for high-affinity antibody by competition for antigen presented by follicular dendritic cells. The prevailing opinion since 2007 has been that IgE⁺ B cells spend little time engaged in germinal centre activities (2–4). Two separate recent IgE reporter mouse models with high sensitivity of detection of IgE⁺ cells *in vivo* have recently shown that IgE⁺ B cells can be found in the germinal centre, but they preferentially differentiate into plasma cells (5, 6). An inference from the murine models is that persistence of memory IgE⁺ B cells is uncommon and that IgE⁺ B cells predominantly exist as plasma cells.

Abbreviations

BaGP, Bahia grass pollen; bio-Pas n 1, biotinylated Pas n 1; CFSE, carboxyfluorescein succinimidyl ester; GPA, Grass pollen allergic; IgE, immunoglobulin E; IgG, immunoglobulin G; NA, Non-pollen-allergic; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell.

These findings now need to be confirmed in humans with allergic disease, and in studies of the specific B-cell response to clinically important allergens. However, circulating IgE⁺ B cells are rare in human patients with allergic diseases (7–9). If most IgE-producing cells are plasma cells rather than B cells (8, 10), then memory allergen-specific B-cell precursors would exist in patients with allergic disease that can then switch to IgE upon subsequent allergen exposure. To provide functional evidence for the presence of circulating allergen-specific memory B cells, we examined B-cell responses in allergic patients using a prototypic pollen allergen source. Grass pollens are the major outdoor aeroallergen globally and are recognized in 45–67% of patients with atopic disorders (11, 12). As patients with grass pollen allergy in our region show higher allergic sensitivity to subtropical grass pollens than temperate grass pollens (13), we investigated allergen-driven responses to Bahia (*Paspalum notatum*) grass pollen (BaGP). We examined (i) the presence of circulating allergen-specific IgE⁺ cells, (ii) cellular proliferation in response to a prototypic aeroallergen and (iii) the phenotypic qualities of B cells responding to allergen in allergic rhinitis patients with grass pollen allergy.

Methods

Patient recruitment

Patients diagnosed with a clinical history of allergic rhinitis and control subjects were recruited from the Respiratory and Immunology Clinics at the Princess Alexandra Hospital, Queensland, with informed consent as approved by the Metro South Human Research Ethics Committee. Allergy status was assessed by positive skin prick test wheal diameter ≥ 3 mm with a panel of ten common aeroallergens including BaGP extract (1/10 weight/volume; Hollister Stier, Spokane, WA, USA) by standard techniques as described (13). Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by density gradient using LymphoPrep (Vital Diagnostics, Braeside, Vic, Australia) and cryopreserved. Plasma samples were stored at -20°C .

IgE and IgG ELISA

BaGP-specific IgE levels were measured in plasma samples (14). Briefly, 96-well plates were coated with 50 μl /well of 5 $\mu\text{g}/\text{ml}$ BaGP extract (Greer, Lenoire, NC, USA) or with coating buffer only. Wells were washed 5 times in phosphate-buffered saline (PBS) with 0.05% Tween-20 and blocked with 200 μl /well of 1% skim milk powder in PBS with 0.05% Tween-20. Plasma (diluted 1/40) or culture supernatants were incubated in duplicate wells at room temperature with shaking for 90 min. IgE binding was detected with polyclonal rabbit anti-human IgE (Dako, Glostrup, Denmark) diluted 1/2000 and then anti-rabbit IgG conjugated to horseradish peroxidase (Promega, Madison, WI, USA) at 1/500. IgG binding was detected in plasma, diluted 1/40, with sheep anti-human IgG conjugated to horseradish peroxidase (Millipore, Billerica, MA, USA) at 1/2000. IgE and IgG reactivity in supernatant or plasma measured at 490 nm was determined after background

levels in wells with no antigen were subtracted from the optical density levels of wells with BaGP.

Whole-blood staining

Red cells from fresh heparinized whole blood were depleted with lysis solution (BD Biosciences, San Jose, CA, USA). Leucocytes were stained with anti-IgE-PE (Abcam, Cambridge, UK), anti-CD19-APC-eF780 (eBiosciences, San Diego, CA, USA), anti-CD123-FITC (Biolegend, San Diego, CA, USA) and HLA-DR-APC (BD Biosciences). Cells were also stained in a separate tube with the purified major allergen of Bahia grass pollen, Pas n 1 (15) labelled with biotin (bio-Pas n 1, 4 $\mu\text{g}/\text{ml}$) in the presence of affinity-purified normal goat IgG (Sigma-Aldrich, St Louis, MO, USA) and PBS 1% foetal calf serum (FCS; Bovogen Biological, Melbourne, Vic, Australia) as a primary staining cocktail. To confirm binding specificity, excess unlabelled Pas n 1 (84 $\mu\text{g}/\text{ml}$) and an irrelevant antigen ovalbumin (OVA; 84 $\mu\text{g}/\text{ml}$) were added to the primary cocktail. After incubation and washing with PBS 1% FCS, a secondary staining cocktail of streptavidin (SA)-APC, anti-IgE-PE, anti-CD4-PerCP-Cy5.5 and anti-CD19-APC-eF780 was added. In additional assays, cells were also stained with CD1c-PE-Cy7 (Biolegend), CD303-FITC (Miltyeni, Bergisch Gladbach, Germany), CD38 eFluor 650NC (eBiosciences) and CD138-PE Cy7 (eBiosciences).

PBMC culture and CFSE labelling

Peripheral blood mononuclear cells were labelled with 1 μM of the cytoplasmic dye; carboxyfluorescein diacetate succinimidyl ester [CFSE, (16)]-stained PBMCs (2×10^6 cells/ml) were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Invitrogen, Carlsbad, CA, USA) with penicillin (100 U/ml)–streptomycin (100 $\mu\text{g}/\text{ml}$)–glutamine (292 $\mu\text{g}/\text{ml}$; Invitrogen) and supplemented with 5% autologous plasma. Antigen titration studies and cultures incubated for 5 and 7 days were performed to optimize culture conditions. Cells were distributed to quadruplicate wells of a 96-well round-bottom plate at 2×10^6 per ml and cultured in the presence of 32 $\mu\text{g}/\text{ml}$ of filter-sterilized and dialysed extract of BaGP, 0.45 $\mu\text{g}/\text{ml}$ trivalent influenza vaccine (2011, Commonwealth Serum Laboratories, Melbourne, Vic, Australia), 1 $\mu\text{g}/\text{ml}$ phytohaemagglutinin (PHA; Sigma-Aldrich) or without antigenic stimulation.

Flow cytometry

After 5-day culture, cells were analysed by flow cytometry using CFSE, CD3-PE (eBiosciences) or CD4-PerCP-Cy5.5 (BD Biosciences), CD19-APC-eF780 (eBiosciences), CD20-PerCP-Cy5.5 (Biolegend), CD27-PE-Cy7 (BD Biosciences) and a fixable viability dye e660 (eBioscience). In subsequent experiments, CD38-PE (eBiosciences), a plasmablast marker, was assessed and CD20-PerCP-Cy5.5 was omitted.

Dead cells were excluded, and live B-cell (CD19⁺CD3[−]) or T-cell (CD19[−]CD3⁺) populations were identified. Actively proliferating cells were distinguished by excluding cells with high CFSE. Lymphocyte proliferation responses were

calculated as a stimulation delta value (percentage of stimulated CFSE^{lo} cells minus CFSE^{lo} cells without stimulation).

Statistical analysis

Data were tested for normal distribution by the Kolmogorov-Smirnov test. Differences in median responses between groups were assessed by Mann-Whitney *U*-test, and differences between paired samples were assessed using a Wilcoxon signed rank test. The difference in frequencies of traits between BaGP-allergic and nonallergic individuals was examined by Fisher's exact test. Statistical significance was considered as $P < 0.05$.

Results

Grass pollen allergy status of participants

A total of 28 subjects were recruited, 15 pollen-allergic patients with allergic rhinitis and 13 non-pollen-allergic controls (Table 1). BaGP SPT and the sum of four grass pollen SPT diameters (Bahia, Johnson, Bermuda and Ryegrass) were significantly increased in pollen-allergic patients compared to the non-pollen-allergic control group.

BaGP-allergic patients show elevated plasma IgE and IgG levels

Consistent with their clinical status, pollen-allergic patients showed higher levels of serum IgE specific for BaGP than nonallergic control subjects (Fig. 1A). BaGP-specific IgG levels were also elevated in the plasma of pollen-allergic compared to non-pollen-allergic individuals (Fig. 1B). There was a trend towards a positive correlation between the allergen-specific IgG and IgE ($r = 0.546$, $P = 0.088$), but this was not significant (data not shown).

Circulating IgE⁺ cells of pollen-allergic patients specifically bound the major grass pollen allergen, Pas n 1

IgE⁺ cells were readily detected in blood of pollen-allergic patients and healthy controls, but very few IgE⁺ cells co-expressed the B-cell marker CD19 (Fig. 2A). The majority of

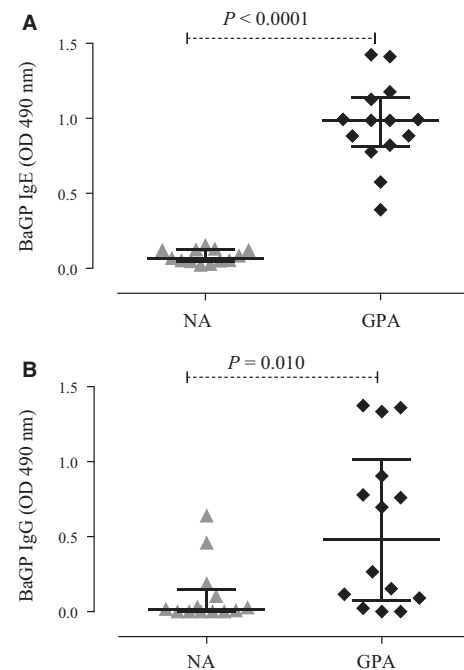


Figure 1 Plasma IgE and IgG reactivity with grass pollen is elevated in grass pollen-allergic donors. (A) Plasma IgE and (B) plasma IgG reactivity with BaGP in 14 grass pollen-allergic (GPA; black diamond) and 13 non-pollen-allergic donors (NA; grey triangle) were analysed by ELISA. Individual values, median and IQR marked. Difference between groups determined by Mann-Whitney *U*-test.

IgE^{hi} CD19^{neg} cells showed a phenotype consistent with basophils based on positive IL-3 receptor (CD123) and negative HLA-DR staining (Fig. 2A). These IgE^{hi} cells from patients but not control donors bound the biotin-labelled group 1 allergen of BaGP, Pas n 1, as detected by streptavidin staining (Fig. 2B). To confirm that Pas n 1 binding was allergen specific, excess unlabelled Pas n 1 was shown to block bio-Pas n 1 binding, whereas the addition of an equal amount of the irrelevant antigen, OVA, did not diminish bio-Pas n 1 staining (Fig. 2C,D). Any Pas n 1 binding detected on the surface of CD19⁺ B cells could not be blocked by unlabelled

Table 1 Demographics of study population

Parameters	Grass pollen-allergic	Non-pollen-allergic	<i>P</i> -value
Subjects (<i>n</i>)	15	13	
Age, median (IQR)	36.0 (24.0–48.0)	34.73 (26.6–51.2)	ns*
Gender: female (<i>n</i>)	9	11	ns†
Total SPT, median (IQR)	54.5 (43.5–68.0)	7 (4–11)	<0.0001*
BaGP SPT, median (IQR)	10 (6.5–12.5)	0 (0–0)	<0.0001*
Combined grass SPT, median (IQR)	30.0 (23.0–64.5)	3.5 (0–5.5)	0.0001*
Allergy symptoms in previous 12 months‡	13	5	0.0163†
Doctor-diagnosed asthma	7	4	ns†

n, number; IQR, interquartile range.

*Mann-Whitney *U*-test for difference in median values between groups ($P < 0.05$ cut-off).

†Fisher's exact test for difference in frequency between groups ($P < 0.05$ cut-off).

‡Some non-pollen-allergic subjects had allergic sensitivity to house dust mite or cat dander allergens.

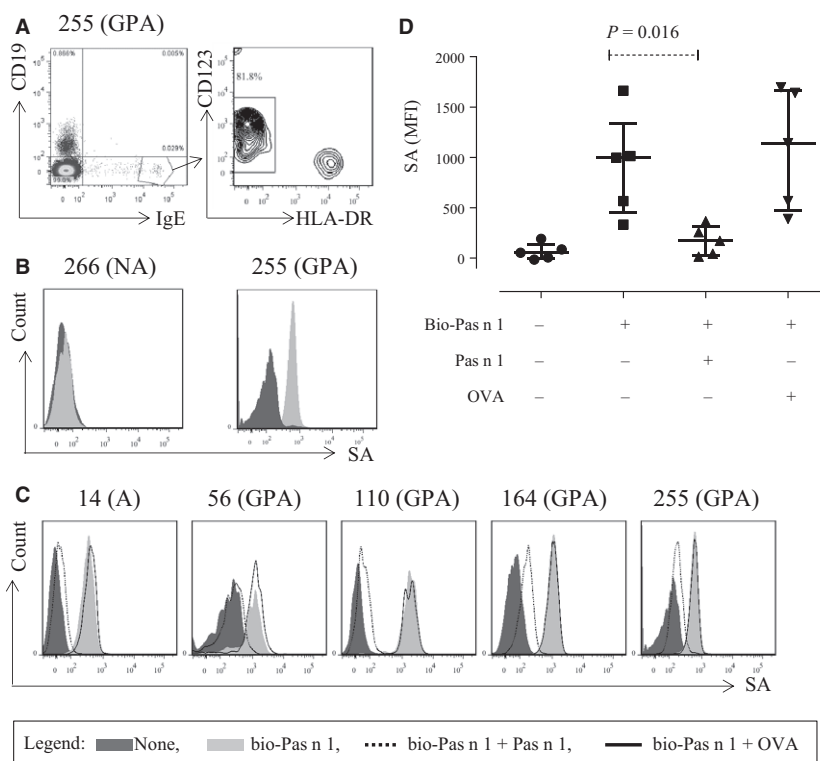


Figure 2 Circulating allergen-specific IgE⁺ cells in patients with allergic rhinitis. (A) CD19 and IgE staining of lymphocytes of a grass pollen-allergic (GPA) donor (exemplary data from five patients). CD19⁺ IgE^{hi} cells showed staining with CD123 and HLA-DR consistent with basophils. (B) IgE^{hi} cells in whole blood stained with (light grey) and without (dark grey) biotinylated (bio)-Pas n 1 and detected by streptavidin (SA)-APC in exemplary non-pollen-

allergic (NA) and GPA donors. (C) Blocking of bio-Pas n 1 binding to IgE^{hi} cells in five GPA donors by excess unlabelled Pas n 1 but not ovalbumin (OVA). (D) Intensity of bio-Pas n 1 staining blocked with Pas n 1 or OVA. Individual values, median and IQR marked and difference assessed by Wilcoxon sign ranked test for paired data. Two GPA donors were used to show IgE⁺ cells were CD123⁺ HLA-DR⁺ basophils.

allergen and was deemed to be nonspecific (data not shown). Whole-blood staining of four allergic donors showed that the IgE^{int}, CD19⁺ population were mostly (median 93%, range 88–97%) HLA-DR⁺ cells, of which a median of 36% (range 26–48%) were CD1c⁺, CD303⁺ myeloid dendritic cells and a median of 18% (range 13–26%) were CD1c⁺, CD303⁺ plasmacytoid dendritic cells (data not shown). Circulating CD3⁺, CD27^{hi}, CD38⁺ (plasmablasts) or CD138⁺ (plasma cells) were not found in either the IgE^{int} or IgE^{hi} populations (Fig. S1).

Allergic donors showed increased allergen-driven B-cell proliferation

To ascertain the presence of allergen-specific B cells in blood, PBMCs were stained with CFSE and assessed for their capacity to proliferate in response to BaGP allergen. Exemplary data for an allergic donor show response of both B (CD19⁺ CD3⁺) and T (CD19⁺ CD3⁺) cell populations in cultures stimulated with BaGP or the T-cell mitogen PHA (Fig. 3A). PBMC from pollen-allergic donors showed higher B- and T-cell proliferation to BaGP than nonallergic individuals (Fig. 3C), whereas responses to PHA (Fig. 3B) and

influenza vaccine antigen (Fig. 3D) did not differ between pollen-allergic donors and nonallergic donors. Heat inactivation of BaGP-specific IgE in autologous plasma in culture media did not reduce B-cell proliferation in response to BaGP (Fig. S2).

B cells from allergic donors that rapidly respond to allergen display a memory phenotype

Within the proliferating allergen-driven B cells, a distinct population (CD19^{mid} CFSE^{lo}) underwent more divisions in response to pollen allergen than B cells that retained high CD19 surface expression (CD19^{hi} CFSE^{mid}) (Fig. 4A). The precursor frequency of rapidly dividing B cells (CD19^{mid} CFSE^{lo}) had undergone between five and seven divisions and arose from up to 3 per 10⁴ precursor B cells. After 5 days, B cells that entered division early showed significantly lower expression of CD19 and CD20 and higher expression of the memory marker CD27 (Fig. 4B) than B cells that were slower to respond to allergen. This was similar to a population of rapidly dividing B cells with high CD27 and low CD20 and CD19 expression that was observed in B cells responding to influenza antigen (Fig. 4C).

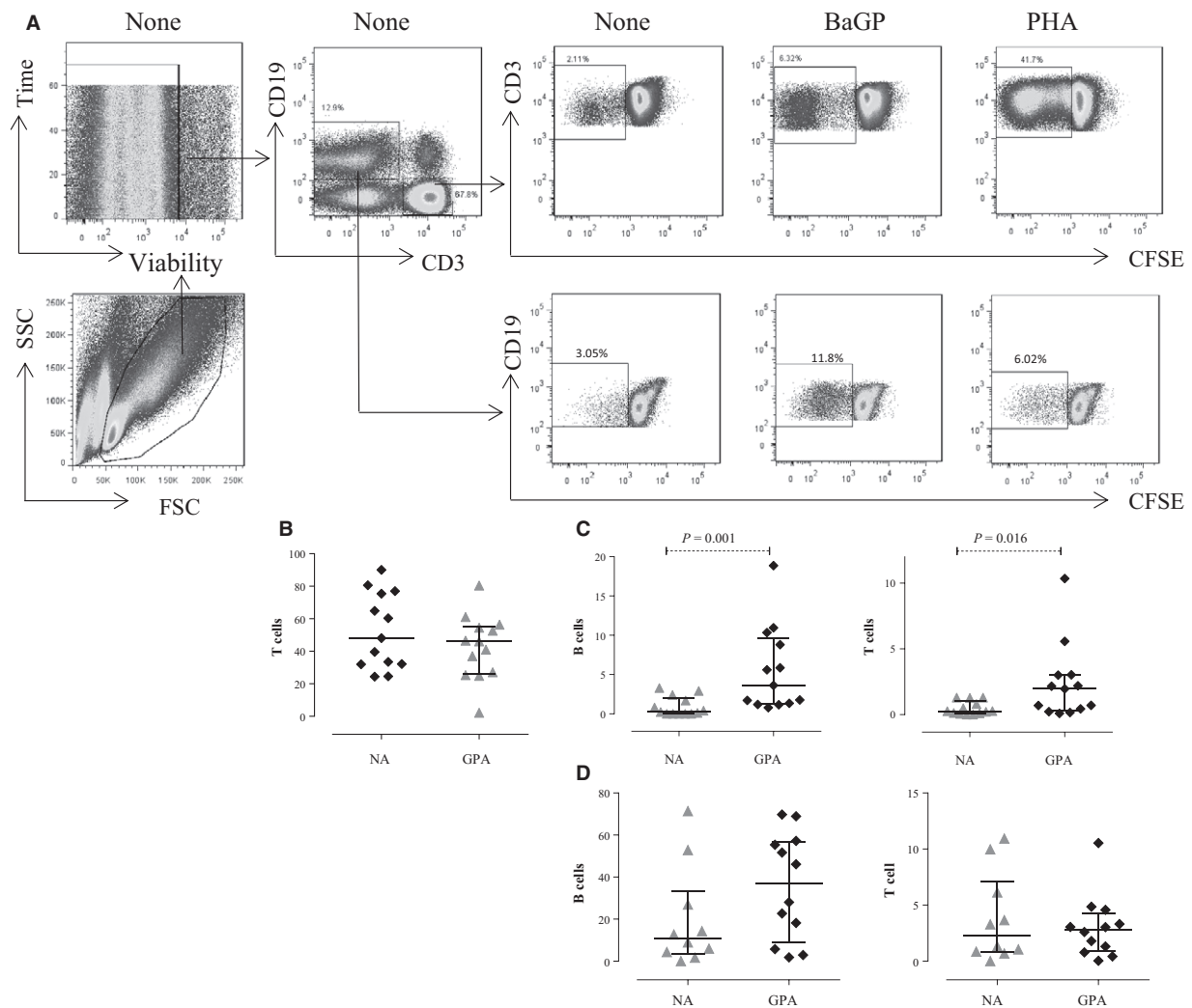


Figure 3 Allergen-driven B- and T-cell proliferation to grass pollen is increased in grass pollen-allergic donors. (A) Forward and side scatter plot, viability stain and live CD19⁺ CD3⁺ B-cell and CD3⁺ CD19⁺ T-cell gating are shown on left. Right panels show CFSE dilution in B and T cells of PBMC cultured with PHA, BaGP or no stimulus (none). Individual T-cell responses of non-pollen-allergic (NA) and grass pollen-allergic (GPA) to PHA (B). Individual B- and

T-cell responses to BaGP (C) and influenza antigen (D). Data are expressed as the percentage CFSE¹⁰ cells with stimulation minus the percentage CFSE¹⁰ cells without stimulation with the median and IQR marked. Significant *P*-values determined by Mann–Whitney *U*-test. (T cells of two NA and two GPA donors were identified with CD4 instead of CD3 staining).

In ten of the pollen-allergic donors, PBMC showed strong proliferative responses to BaGP in Fig. 4, allowing investigation of the expression of CD38, a plasmablast marker of late germinal centre stage B cells (Fig. 5). B cells rapidly dividing in response to BaGP that were CD19^{mid} CD27^{hi} expressed significantly higher CD38 than B cells that entered into division more slowly.

Discussion

A seasonal increase in serum IgE levels boosted by recurrent allergen exposure (17) exemplifies the IgE memory response. Such a boost to serum IgE could arise from re-activation of

resident bone marrow allergen-specific IgE⁺ plasma cells. Alternatively, serum IgE could be increased by re-activation of allergen-specific memory B cells. The existence of circulating memory IgE⁺ B cells appears to be unlikely in humans despite their recent discovery in murine models (5, 6). Our study demonstrates that strong *ex vivo* allergen-driven B-cell responses can be detected in PBMC of patients with grass pollen allergy, but not non-pollen-allergic control donors. Furthermore, the allergen-driven B cells of untreated grass pollen-allergic patients showed characteristics typical of adaptive memory B-cell responses, namely rapid *in vitro* expansion and upregulated maturation and plasmablast differentiation markers.

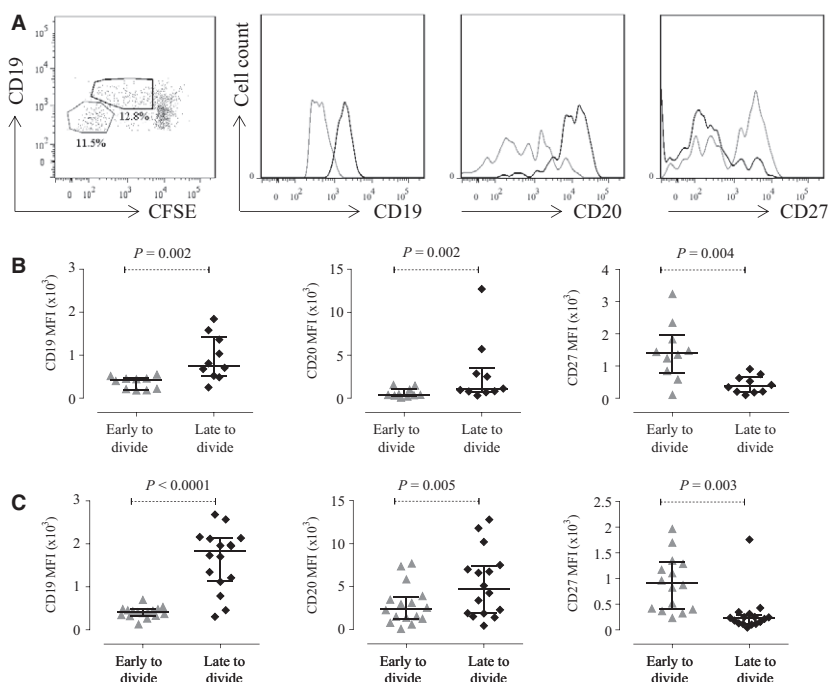


Figure 4 B cells of grass pollen-allergic subjects rapidly dividing in response to grass pollen acquire a memory B-cell phenotype. (A) Exemplary data showing B cells early-to-divide (CD19^{mid} CFSE^{lo}, grey trace) in response to BaGP compared with late-to-divide B cells (CD19^{hi} CFSE^{mid}, black trace). Mean fluorescence intensity

(MFI) for CD19, CD20 and CD27 with individual values, median and IQR marked for grass pollen-allergic patient PBMC responses to BaGP (B). Individual responses of non-allergic and pollen-allergic donor PBMC to influenza antigen (C) (Wilcoxon signed rank test for paired data).

Previously, studies in asthma showed a significant relationship between serum IgG and IgE specific to ryegrass pollen and its major allergen, Lol p 1 (18). It might have been reasonable to expect in our study that nonallergic individuals would show some B-cell response to allergen, as exposure to pollen is likely to be similar for all subjects. Instead, the non-allergic group in our study appeared to be 'ignorant of the allergen' and failed to mount significant allergen-specific antibody or B-cell response (19). That IgG and IgE to BaGP were elevated in the allergic donors and not in the control group is consistent with the observation that only the sensitized group showed B-cell proliferation to allergen.

Flow cytometric staining for IgE in whole blood of allergic and nonallergic individuals did not show significant IgE on CD19+ B cells above isotype control (data not shown), in keeping with prior studies (8, 10). We identified that most of the IgE^{int} CD19- cells stained positively for HLA-DR as well as the myeloid dendritic cell marker (CD1c) or the plasmacytoid DC marker (CD303c). The majority of allergen-specific cells observed in blood of allergic rhinitis patients with grass pollen allergy and not nonallergic controls were IgE^{hi}, CD19⁻, CD123^{hi} and HLA-DR⁻ cells consistent with basophils. That specific Pas n 1 staining on CD19+ B cells was not clearly detected is consistent with observations of Irsch and colleagues (7) who showed in bee venom-allergic donors that only a low frequency of the

phospholipase A₂ allergen-specific cells in blood was B cells, whereas the majority of phospholipase A₂ allergen-specific cells were basophils.

Despite the lack of detectable allergen-specific IgE+ B cells, we have provided functional evidence of a population of B cells in allergic donors at a low precursor frequency consistent with cognate antigen recognition capable of responding to allergen. The rapid proliferation of allergen-activated B cells in PBMC from BaGP-allergic donors is consistent with early expansion of adaptive memory B-cell response (20). Moreover, elevated expression of the CD27 and CD38 markers on rapidly dividing B cells responding to allergen is a hallmark of highly activated memory B cells (21, 22). These cellular characteristics indicate the presence of a population of circulating, pre-existing allergen-specific memory B cells in allergic patients that were not detected in nonallergic control subjects. Future studies will focus on properties of these early-to-divide allergen-specific B cells, as only this population appears to have characteristics of memory B cells, whereas the late-to-divide B cells may depend on T-cell help for activation. That most of the IgE+ cells circulating in blood were basophils or dendritic cells rather than those of the B-cell lineage (e.g. B cells or plasmablasts) suggests that the CFSE^{lo}, CD19^{lo}, CD27^{hi} B cells rapidly responding to allergen have acquired the plasmablast phenotype of the CD27^{hi}, CD38+ *in vitro*.

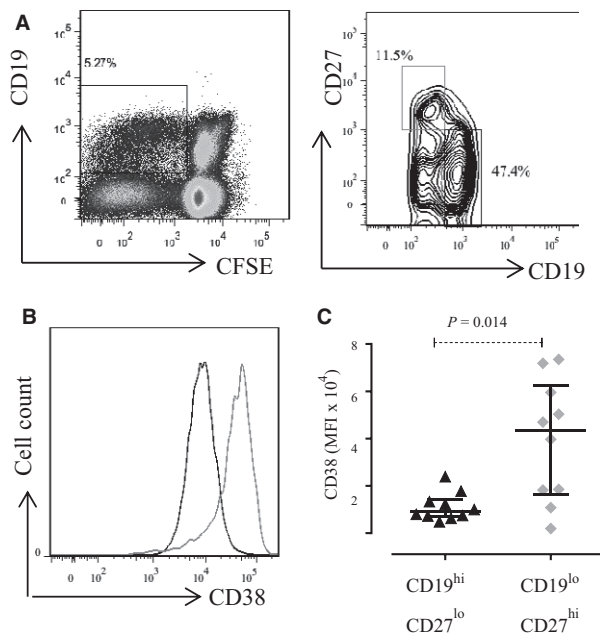


Figure 5 B cells of pollen-allergic subjects rapidly dividing in response to BaGP show a plasmablast phenotype. (A) Exemplary data from a pollen-allergic individual showing CD19 and CD27 staining of CFSE^{lo} CD19⁺ BaGP-driven B cells. (B) Expression of CD38 for CD19^{hi} CD27^{lo} (black) and CD19^{lo} CD27^{hi} (grey) B-cell populations dividing in response to BaGP. (C) Individual values, median and IQR for CD38 expression of BaGP-driven B cells from ten pollen-allergic donors (Wilcoxon signed rank test for paired data).

Murine studies show that IgE-switched B cells have a predilection to rapidly differentiate into IgE-secreting plasma cells (2–6). Earlier studies in humans showed that the majority of circulating IgE⁺ cells constituting a median of 0.29% of lymphocytes appeared to be CD38⁺ plasmablasts and not CD19⁺ B cells (8). That IgE mRNA transcripts can be amplified from blood of allergic donors and individuals from parasite endemic regions (23, 24) is consistent with circulation of IgE⁺ plasmablast. Data from IgE reporter mice indicate that IgE⁺ plasma cells were largely short-lived tissue-resident plasma cells (5, 6). Whilst the persistence and location of plasma cells in humans is not readily investigated, nasal challenge with pollen allergen molecules boosted IgE and IgA responses in pollen-allergic patients (25). This observation may in part reflect the contribution of local mucosal IgE isotype switching and secretion (26, 27).

This research described herein increases our understanding of the phenotypic characteristics of circulating allergen-driven B cells, which have not been previously described in human subjects with a prototypic aeroallergen. Whilst T-cell proliferation to peanut allergen has been demonstrated previously using CFSE, the B-cell response to allergen has primarily been considered in the context of B-cell presentation of antigen to T cells, an important mechanism for perpetuating

allergic disease (28). It is reasonable to speculate that both the memory and the maturation status of allergen-driven B cells will affect IgE-facilitated allergen presentation to T cells (29). However, B-cell response to BaGP was not decreased by heat inactivation of plasma in which allergen-specific IgE and IgG were reduced. An improved understanding of allergen-driven B cells in patients with allergic disease is also likely to be relevant in the context of induction of IgG-blocking antibodies, a mechanism thought to mediate the effectiveness of allergen-specific immunotherapy (30). The existence of a memory population of allergen-specific B cells has implications for our understanding of the pathways mediating IgE switching. Allergen-specific memory B cells of an IgG subclass, possibly IgG4 (31) or even IgG1 (32), could engage in germinal centre activities of somatic hypermutation and affinity maturation in this pre-IgE stage, whereas IgE⁺ B cells appear to bypass germinal centre activities. Somatic hypermutation and affinity maturation of allergen-specific Ig prior to switching to IgE could allow for generation of high-affinity allergen-specific IgE as previously indicated in some studies (23, 33). However, examination of IgE transcripts in other studies indicated a low accumulation of somatic mutations in some situations (24, 34).

Herein, we have provided evidence that a population of grass pollen allergen-specific, circulating memory B cells is present in patients with allergic rhinitis, despite the absence of detectable IgE⁺ B cells. Although we did not detect IgE⁺ memory B cells *ex vivo*, consistent with earlier findings of other laboratories (7–9), it remains possible that a very rare IgE⁺ memory population exists. However, our findings are consistent with the hypothesis that IgE memory is maintained by an allergen-specific, non-IgE-expressing precursor memory B-cell population.

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Author contributions

KW performed most of the experimental studies, analysed data and prepared the manuscript. VT prepared pollen extracts, purified and biotinylated allergens and contributed to manuscript preparation. YX provided expertise in flow cytometry and assisted with data analysis. JWU was the respiratory physician responsible for clinical assessment and recruitment of subjects and has provided input to data analysis and manuscript preparation. AC provided input to study design, data analysis and manuscript preparation. JMD was responsible for the overall design, execution, data analysis and reporting of the study.

Conflicts of interest

KW, VT, YX and AC have no conflict of interests to declare. JWU has received speaker fees for purposes not directly relevant to this research from Novartis, Boehringer Ingelheim, Astra Zeneca and GSK. JMD is an inventor on one patent and three patent applications under examination. JMD has received consultancies and contracted research funds for other pollen allergy research from Stallergenes, Antony France, untied co-sponsorship for a pollen aerobiology workshop from Merck Sharp and Dohme, and speaker fees from GlaxoSmithKline (Australia) and Stallergenes. Thermofisher (Sweden) has provided ImmunoCAP reagents free of charge,

and Sullivan Nicolaides Pathology (Queensland, Australia) has provided in-kind phlebotomy and pathology services to JMD for other research.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

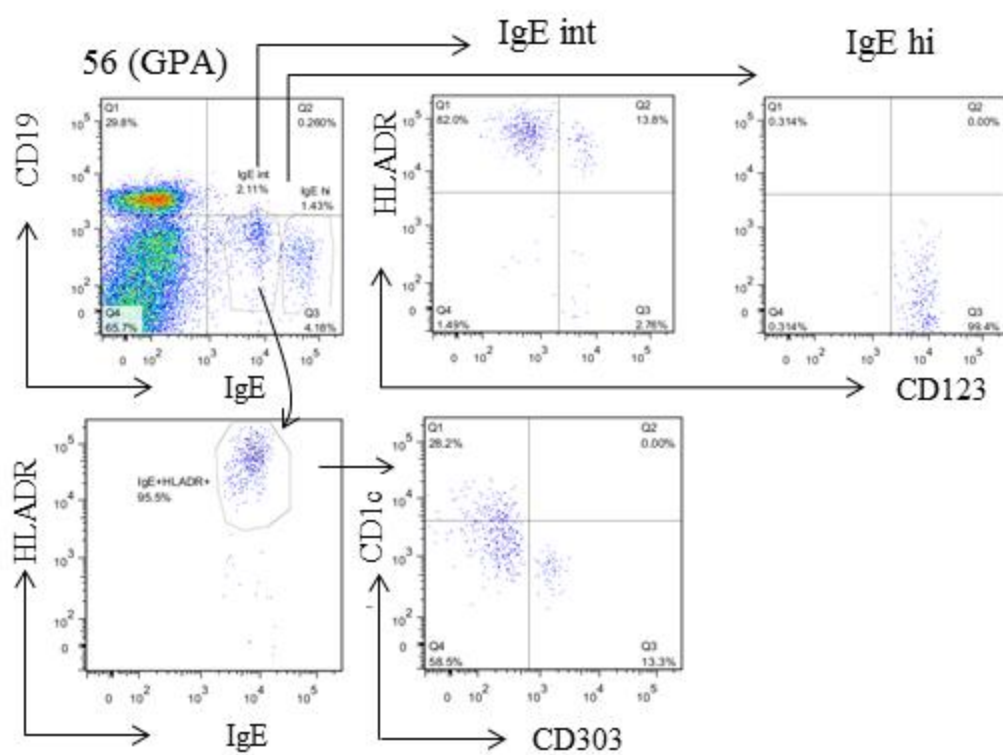
Figure S1. Identification of IgE intermediate cells in whole blood.

Figure S2. Heat inactivation of plasma IgE does not diminish proliferation to BaGP allergen.

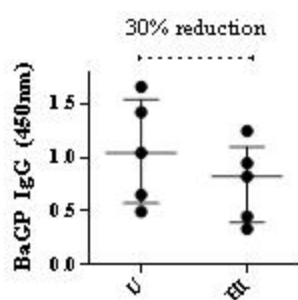
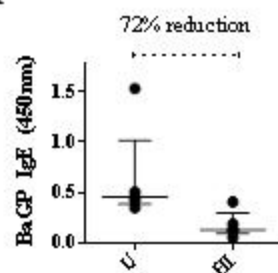
References

- Davies JM, Platts-Mills TA, Aalberse RC. The enigma of IgE+ B cell memory in humans. *J Allergy Clin Immunol* 2013;**131**:972–976.
- Erazo A, Kutchukhidze N, Leung M, Christ AP, Urban JF Jr, Curotto de Lafaille MA et al. Unique maturation program of the IgE response in vivo. *Immunity* 2007;**26**:191–203.
- Wesemann DR, Magee JM, Boboila C, Calado DP, Gallagher MP, Portuguese AJ et al. Immature B cells preferentially switch to IgE with increased direct S μ to S ϵ recombination. *J Exp Med* 2011;**208**:2733–2746.
- Xiong H, Dolpady J, Wabl M, Curotto de Lafaille MA, Lafaille JJ. Sequential class switching is required for the generation of high affinity IgE antibodies. *J Exp Med* 2012;**209**:353–364.
- Talay O, Yan D, Brightbill HD, Straney EE, Zhou M, Ladi E et al. IgE(+) memory B cells and plasma cells generated through a germinal-center pathway. *Nat Immunol* 2012;**13**:396–404.
- Yang Z, Sullivan BM, Allen CD. Fluorescent in vivo detection reveals that IgE(+) B cells are restrained by an intrinsic cell fate predisposition. *Immunity* 2012;**36**:857–872.
- Irsch J, Hunzelmann N, Tesch H, Merk H, Maggi E, Ruffilli A et al. Isolation and characterization of allergen-binding cells from normal and allergic donors. *Immunotechnology* 1995;**1**:115–125.
- Donohoe PJ, Heddle RJ, Sykes PJ, Fusco M, Flego LR, Zola H. IgE+ cells in the peripheral blood of atopic, nonatopic, and bee venom-hypersensitive individuals exhibit the phenotype of highly differentiated B cells. *J Allergy Clin Immunol* 1995;**95**:587–596.
- Mitre E, Norwood S, Nutman TB. Saturation of immunoglobulin E (IgE) binding sites by polyclonal IgE does not explain the protective effect of helminth infections against atopy. *Infect Immun* 2005;**73**:4106–4111.
- Horst A, Hunzelmann N, Arce S, Herber M, Manz RA, Radbruch A et al. Detection and characterization of plasma cells in peripheral blood: correlation of IgE+ plasma cell frequency with IgE serum titre. *Clin Exp Immunol* 2002;**130**:370–378.
- Scala E, Alessandri C, Bernardi ML, Ferrara R, Palazzo P, Pomponi D et al. Cross-sectional survey on immunoglobulin E reactivity in 23 077 subjects using an allergenic molecule-based microarray detection system. *Clin Exp Allergy* 2010;**40**:911–921.
- Bauchau V, Durham SR. Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J* 2004;**24**:758–764.
- Davies JM, Li H, Green M, Towers M, Upham JW. Subtropical grass pollen allergens are important for allergic respiratory diseases in subtropical regions. *Clin Transl Allergy* 2012;**2**:4.
- Davies JM, Dang TD, Voskamp A, Drew AC, Biondo M, Phung M et al. Functional immunoglobulin E cross-reactivity between Pas n 1 of Bahia grass pollen and other group 1 grass pollen allergens. *Clin Exp Allergy* 2011;**41**:281–291.
- Drew A, Davies JM, Dang TD, Rolland JM, O'Hehir RE. Purification of the major group 1 allergen from Bahia grass pollen, Pas n 1. *Int Arch Allergy Appl Immunol* 2011;**154**:295–298.
- Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 1994;**171**:131–137.
- Mothes N, Heinzkill M, Drachenberg KJ, Sperr WR, Krauth MT, Majlesi Y et al. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin Exp Allergy* 2003;**33**:1198–1208.
- Erwin EA, Hosen J, Pollart SM, Reid MJ, Platts-Mills TA. High-titer IgE antibody specific for pollen allergens in northern California is associated with both wheezing and total serum IgE. *J Allergy Clin Immunol* 2009;**123**:706–708.
- Aalberse RC. Specific IgE and IgG responses in atopic versus nonatopic subjects. *Am J Respir Crit Care Med* 2000;**162**:S124–S127.
- Tangye SG, Avery DT, Deenick EK, Hodgkin PD. Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. *J Immunol* 2003;**170**:686–694.
- Avery DT, Ellyard JI, Mackay F, Corcoran LM, Hodgkin PD, Tangye SG. Increased expression of CD27 on activated human memory B cells correlates with their commitment to the plasma cell lineage. *J Immunol* 2005;**174**:4034–4042.
- Shlomchik MJ, Weisel F. Germinal center selection and the development of memory B and plasma cells. *Immunol Rev* 2012;**247**:52–63.
- Davies JM, O'Hehir RE. VH gene usage in immunoglobulin E responses of seasonal rhinitis patients allergic to grass pollen is oligoclonal and antigen driven. *Clin Exp Allergy* 2004;**34**:429–436.
- Wang Y, Jackson JL, Chen Z, Gaeta BA, Siba PM, Pomat W et al. IgE sequences in individuals living in an area of endemic parasitism show little mutational evidence of antigen selection. *Scand J Immunol* 2011;**73**:496–504.
- Niederberger V, Ring J, Rakoski J, Jager S, Spitzauer S, Valent P et al. Antigens drive memory IgE responses in human allergy via the nasal mucosa. *Int Arch Allergy Immunol* 2007;**142**:133–144.
- Durham SR, Gould HJ, Hamid QA. Local IgE production in nasal allergy. *Int Arch Allergy Immunol* 1997;**113**:128–130.
- Gevaert P, Nouri-Aria KT, Wu H, Harper CE, Takhar P, Fear DJ et al. Local receptor revision and class switching to IgE in

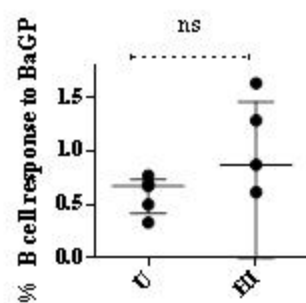
- chronic rhinosinusitis with nasal polyps. *Allergy* 2013;**68**:55–63.
28. Turcanu V, Stephens AC, Chan SM, Rance F, Lack G. IgE-mediated facilitated antigen presentation underlies higher immune responses in peanut allergy. *Allergy* 2010;**65**:1274–1281.
29. Shamji MH, Wilcock LK, Wachholz PA, Dearman RJ, Kimber I, Wurtzen PA et al. The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses. *J Immunol Methods* 2006;**317**:71–79.
30. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN et al. Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. *J Allergy Clin Immunol* 2011;**127**:509–516.
31. Aalberse RC, Van Milligen F, Tan KY, Stapel SO. Allergen-specific IgG4 in atopic disease. *Allergy* 1993;**48**:559–569.
32. Custovic A, Soderstrom L, Ahlstedt S, Sly PD, Simpson A, Holt PG. Allergen-specific IgG antibody levels modify the relationship between allergen-specific IgE and wheezing in childhood. *J Allergy Clin Immunol* 2011;**127**:1480–1485.
33. Kerzel S, Rogosch T, Struecker B, Maier RF, Zemlin M. IgE transcripts in the circulation of allergic children reflect a classical antigen-driven B cell response and not a superantigen-like activation. *J Immunol* 2010;**185**:2253–2260.
34. Pratt E, Collins AM, Sewell WA, Harvey RJ. Antigen selection in IgE antibodies from individuals with chronic rhinosinusitis with nasal polyps. *Am J Rhinol Allergy* 2010;**24**:416–421.



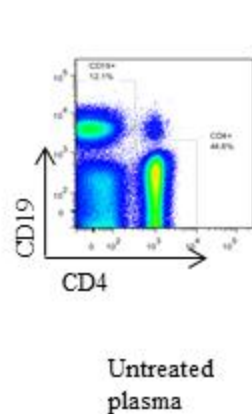
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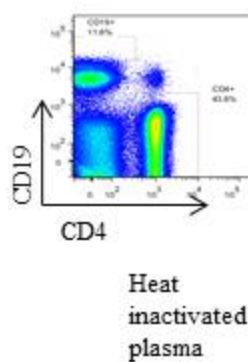
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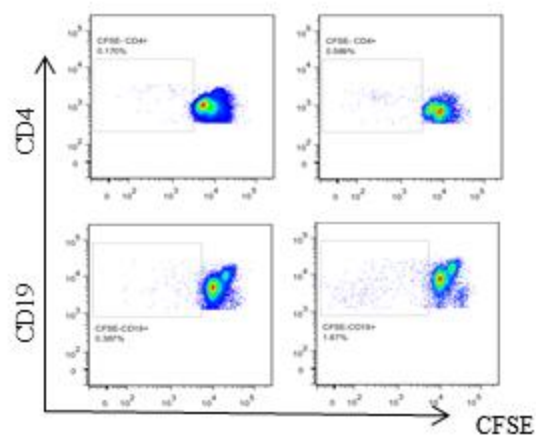
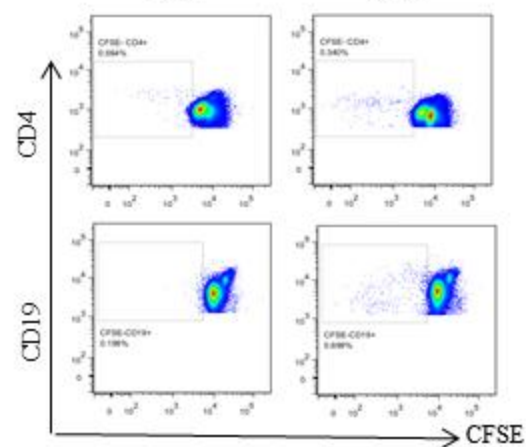


C



None

BaGP



Supplementary Figure Legends

Supplementary figure 1. Identification of IgE intermediate cells in whole blood.

Exemplary data from one of four atopic donors. The lymphocytes and granulocytes (based on SSC and FSC) of whole blood were selected and CD3⁺ cells were excluded (not shown). CD19 and IgE staining (right top). HLA-DR and CD123 staining of CD3⁻, CD19⁻, IgE Int (top middle) and CD3⁻, CD19⁻, IgE Hi populations (top right). CD1c and CD303 staining (bottom right) of the HLA-DR4⁺, IgE Int population (bottom left).

Supplementary figure 2. Heat inactivation of plasma IgE does not diminish

proliferation to BaGP allergen. A. Plasma was heat inactivated for 56°C for 30 and 60 minutes. After thirty minutes, BaGP-specific IgE was reduced by a median of 72 % (IQR 63.63-80.8%) and IgG was reduced by 30% (23-33%). BaGP-specific IgE and IgG were not significantly further decreased by incubation for an additional 30 minutes. **B.** CFSE- labelled PBMC of five grass pollen-allergic donors were cultured as described in media supplemented with either 2% untreated (normal conditions) or 2% plasma heat inactivated for 30 minutes with none, BaGP, Flu or PHA stimuli. Exemplary data from one of five grass-pollen allergic donors shows B cell response to BaGP for PBMC cultures with untreated (U) plasma (**B**) and heat inactivated (HI) plasma. **D.** No significant difference in response to stimuli was observed for the five donors.