Ontogeny of human IgE-expressing B cells and plasma cells

F. Ramadani1,3, H. Bowen1,3, N. Upton1,3, P. S. Hobson2,3, Y.-C. Chan1,3, J.-B. Chen4, T. W. Chang4, J. M. McDonnell1,3, B. J. Sutton1,3, D. J. Fear2,3* & H. J. Gould1,3*

1 Randall Division of Cell and Molecular Biophysics; 2 Division of Asthma, Allergy and Lung Biology, King’s College; 3 Medical Research Council and Asthma UK Centre in Allergic Mechanisms in Asthma, London, UK; 4 Genomics Research Center, Academia Sinica, Taipei, Taiwan

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Abstract

Background: IgE-expressing (IgE⁺) plasma cells (PCs) provide a continuous source of allergen-specific IgE that is central to allergic responses. The extreme sparsity of IgE⁺ cells in vivo has confined their study almost entirely to mouse models.

Objective: To characterize the development pathway of human IgE⁺ PCs and to determine the ontogeny of human IgE⁺ PCs.

Methods: To generate human IgE⁺ cells, we cultured tonsil B cells with IL-4 and anti-CD40. Using FACS and RT-PCR, we examined the phenotype of generated IgE⁺ cells, the capacity of tonsil B-cell subsets to generate IgE⁺ PCs and the class switching pathways involved.

Results: We have identified three phenotypic stages of IgE⁺ PC development pathway, namely (i) IgE⁺ germinal centre (GC)-like B cells, (ii) IgE⁺ ‘plasmablasts’ and (iii) IgE⁺ PCs. The same phenotypic stages were also observed for IgG1⁺ cells. Total tonsil B cells give rise to IgE⁺ PCs by direct and sequential switching, whereas the isolated GC B-cell fraction, the main source of IgE⁺ PCs, generates IgE⁺ PCs by sequential switching. PC differentiation of IgE⁺ cells is accompanied by the down-regulation of surface expression of the short form of membrane IgE (mIgE), which is homologous to mouse mIgE, and the up-regulation of the long form of mIgE (mIgEL), which is associated with an enhanced B-cell survival and expressed in humans, but not in mice.

Conclusion: Generation of IgE⁺ PCs from tonsil GC B cells occurs mainly via sequential switching from IgG. The mIgEL/mIgE ratio may be implicated in survival of IgE⁺ B cells during PC differentiation and allergic disease.

Keywords allergy; germinal centre; human B cells; IgE class switching; plasma cell.
maturation and selection of the B cells of highest affinity for antigen, or allergen in the case of IgE (7, 8). The selected cells may recycle via the T-cell compartment or differentiate into memory B cells and PCs to enter the circulation (9, 10).

Recent studies in the mouse revealed that the fate of IgE+ B cells is dramatically different from that of IgG1+ B cells, which express the most abundant and most thoroughly investigated isotype (11–16). It was shown that although CSR to IgE is initiated in GCs, most of IgE+ cells exhibited a PC phenotype and were excluded from the GCs (14). Likewise, other studies of IgE in the mouse showed that IgE responses are more transient than those of IgG1 and were predominantly directed into the PC lineage (13). It was also reported that CSR pathway leading to IgE+ B cells determined their ultimate fate (16). Direct switching gave rise to IgE+ GC cells with an impaired B-cell receptor (BCR) signalling, due to the low expression of the BCR, leading to cell death (16). This switching pathway was associated with the secretion of low-affinity IgE antibodies (16, 17). In contrast, sequential switching generated IgE+ PCs with elevated BCR expression and was associated with the secretion of high-affinity IgE antibodies (16, 17). It was inferred that the inheritance of SHM and affinity maturation from IgG1+ B cells are needed for the generation of a memory IgE response (16, 17).

The relevance of results in the mouse to human allergy has been questioned (18). For example, human IgE+ B cells express two forms, one short and one long form, of mIgE, mIgES and mIgEL (19, 20). These mIgE isoforms arise from the alternative splicing of a common mRNA precursor, with mIgEL containing a longer extra-membrane proximal domain (EMPD) region, an additional 52-amino acid residue between the C-terminal Ig domain, Cε4 and the transmembrane M1 domain (19–21). Although nothing is yet known about the mechanisms that govern the relative expression of the two mIgE isoforms, there is evidence that the longer EMPD confers greater resistance to BCR-induced apoptosis (21, 22).

We have previously characterized the capacity of various tonsil B-cell subsets to undergo CSR to IgE ex vivo (23). Using this ex vivo tonsil human B-cell culture system, we have now investigated the ontogeny of human IgE+ PCs. We point out many similarities, but also important differences from studies in the mouse models that may illuminate the mechanisms in allergy.

**Methods**

**Isolation of human tonsil B cells**

With informed written consent and ethical approval from Guy’s Research Ethics Committee, we obtained human tonsils from donors undergoing routine tonsillectomies. Mononuclear cells were separated according to the density on a Ficoll gradient (GE Healthcare, Buckinghamshire, UK), and B cells were isolated using 2-aminoethylisothiouronium bromide-treated sheep red blood cells (TCS Biosciences Ltd, Buckingham, UK). B cells were >95% CD19+ as determined by flow cytomtery analysis.

**Cell cultures**

To induce CSR to IgE, B cells were cultured as previously described (23). Briefly, 0.5 × 10⁶ freshly isolated tonsil B cells were stimulated with IL-4 (200 IU/ml unless otherwise stated; R&D Europe Systems Ltd, Abingdon, UK) and anti-CD40 antibody (0.5 μg/ml unless otherwise stated; G28.5; American Type Culture Collection, Manassas, VA, USA) for up to 12 days.

**FACS analysis**

Surface and intracellular staining of IgE+ cells was performed as previously described (23). A detailed account of FACS analysis, cell sorting, RNA isolation, qRT-PCR and switch circle transcript PCR experiments can be found in the supplemental methods (available on the Allergy website).

**Statistical analysis**

Statistical analysis was performed using the one-way ANOVA, with Bonferroni correction, unless otherwise stated. A value of \( P < 0.05 \) was considered significant (*\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \)).

**Results**

**Human IgE+ cells have three successive stages of differentiation into PCs**

To induce CSR to IgE, we cultured freshly isolated human tonsil B cells with IL-4 and anti-CD40 antibody. When staining for intracellular IgE, we consistently observe two IgE+ cell populations (Fig. 1A). We designate these IgElo and IgEhi cells. Similarly, we observe two populations of IgG1+ cells, IgG1lo and IgG1hi cells. The ratio of IgEhi to IgElo rose from day 7 to day 12 in culture, whereas that of IgG1hi to IgG1lo cells remained constant (Fig. 1A and B). On profiling the surface markers of these cells, by FACS, we observed that both IgEhi and IgG1hi express lower levels of CD20, Fas, IL-4R and Bcl-6 and higher levels of CD38, CD27 and Blimp-1 than IgElo and IgG1lo counterparts (Fig. 1C), indicating a more highly differentiated phenotype (24).

Two IgE+ cell populations were observed after the immunization of mice and were characterized as IgE+ GC cells and IgE+ PCs (13). However, unlike in mice, when staining the stimulated human B cells for CD138, a surface marker for the fully differentiated PCs, we observed three IgE+ cell populations: IgEloCD138−, IgEhi CD138− and IgEloCD138+ cells (Fig. 2A).

To characterize in more detail the IgE+ cell populations along their differentiation pathway into PCs, we used a fixation and permeabilization FACS procedure (25) to isolate RNA from sorted IgEloCD138−, IgEhi CD138− and IgEloCD138+ cells. For comparison, we also sorted the IgG1+ cell counterparts. cDNA generated from these cells was used to quantify the relative expression levels of selected transcription factors known to be involved in maintaining the GC reaction and the B-cell identity of the cells, or in
inducing B-cell differentiation towards PCs (7, 26). Figure 2B compares these expression levels with those on the unstimulated tonsil GC B-cell controls. Bcl-6 and Pax-5, important for maintaining the B-cell identity and the GC phenotype (7, 26, 27), are abundantly expressed in the GC B cells and the IgEloCD138− and IgG1loCD138− cells, but strongly down-regulated in both the IgEhi (IgEhiCD138− and IgEhiCD138+) and IgG1hi (IgG1hiCD138− and IgG1hiCD138+) cells (Fig. 2B). The opposite pattern is seen for Blimp-1 expression, an important factor of PC differentiation and function (7, 26, 28). As the FACS-derived phenotype of these cells predicts, expression of the PC marker, CD138, is seen only in IgEhiCD138+ and IgG1hiCD138+ cells (Fig. 2B). There were, moreover, no discernible phenotypic differences between IgE+ and IgG1+ cells at different stages of differentiation. In addition, we find that as IgE− and IgG1− cells differentiate, they down-regulate Ki-67 (Fig. S1), a marker of proliferation, and their cell cycle progression declines, with the majority of the IgE− and IgG1− PCs being at the quiescent G0 stage of the cell cycle (Fig. S2). In sum, these observations, consistent with those on the cell surface markers, indicate that IgEhi (IgEhiCD138+) cells have a phenotype with GC B cell-like characteristics, whereas the IgElo cells represent a later stage of differentiation into a PC-like (IgEhiCD138+) ‘plasmablast’ phenotype, and only a small proportion appear to be fully differentiated PCs (IgEhiCD138+).

Maintenance of GC B cells contributes to increased yields of IgE+ PCs

In IL-4- and anti-CD40-stimulated tonsil B-cell cultures, B cells from the GC compartments are the main sources of IgE+ cells (23). By comparison with naive and memory B cells, these cells have very high rates of cell death, but also display an elevated expression of IL-4R and CD40 (23). We investigated the significance of these differences by stimulating human tonsil B cells with different IL-4 and anti-CD40 concentrations. Reducing the IL-4 concentration from 200 IU/ml (the level customarily used to stimulate CSR to IgE) to 100 IU/ml reduced the percentage of IgE+ cells (Fig. 3A, B), revealing that CSR to IgE is sensitive to the IL-4 concentration within this range of
concentration. Increasing the concentration of IL-4 from 200 to 400 IU/ml caused no further accretion of IgE+ cells, but engendered a threefold increase in the proportion of IgE+ PCs (Fig. 3A,B,C). In contrast, these changes in IL-4 concentrations did not affect IgG1+ cells (Fig. 3A,B,C). Neither IgE+ nor IgG1+ cells evinced any response to changes in anti-CD40 concentration (Fig. 3A,B,C). We also noted a significant reduction in the percentage of live cells cultured with 100 IU/ml of IL-4, and a marked increase in cultures with 400 IU/ml of IL-4 (Fig. 3D).

Figure 2 Human IgE+ and IgG1+ cells have three distinctive stages of differentiation into PCs. (A) On day 10 of total B cultures, the cells were surface-stained for CD138 followed by fixation/permeabilization and intracellular staining for IgE and IgG1. The FACS dot plots show three distinct IgE+ and IgG1+ cell populations: IgE+CD138−, IgE+CD138+ and IgE−CD138+ and the respective IgG1+ cell counterparts. (B) The three IgE+ and IgG1+ cell populations were FACS-sorted into RNA processing buffer. The isolated RNA from these sorted cells was then used to determine the expression levels of the GC B-cell markers, Pax5, Bcl6, and PC markers, Blimp1 and CD138, by qRT-PCR. As controls, we used RNA cells of the FACS-sorted tonsil GC B cells. Data represent the mean ± SEM and are derived from four different experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

This observation led – because GC B cells are the main source of IgE+ cells (23) – to the conjecture that the action of IL-4 may be restricted to the B cells from the GC compartments. To address this, we sorted tonsil B cells into naive (CD27−CD38−CD77−), memory (CD27−CD38−CD77−), early GC (CD27−CD38−CD77−) and GC (CD27−CD38−CD77−) B cells (23) (Fig. S3) and cultured these cells with IL-4 and anti-CD40 (Fig. 3E). Again, unlike anti-CD40, IL-4 exerted a concentration-dependent effect on GC-derived B-cell cultures, but not on naive and memory B-cell cultures (Fig. 3E).
The percentage of live cells in GC B-cell cultures rose when the concentration of IL-4 was increased from 200 to 400 IU/ml (Fig. 3E). We conclude that IL-4 contributes to the maintenance of the GC B cells and that the maintenance of these cells in culture results in higher yields of IgE+ PCs.

Switched IgE+ cells from GC B-cell cultures undergo rapid differentiation into IgE+ PCs

The above results suggest that the GC environment may favour the generation of IgE+ PCs. To confirm this, we...
cultured tonsil naïve, memory, eGC and GC B cells with IL-4 and anti-CD40, with unfractonated total B cells as controls, and determined the IgE$_{bi}$/IgE$_{bo}$ cell ratio as a measure of the rate of differentiation of the newly switched human IgE$^+$ cells. We found that this ratio was lowest in naïve (0.19 ± 0.14) and highest in GC B-cell cultures (2.24 ± 1.1), whereas the memory (0.95 ± 0.59), eGC (1.18 ± 0.64) and total B-cell cultures (0.67 ± 0.42) had IgE$_{bi}$/IgE$_{bo}$ cell ratios intermediate to that in the naïve and GC B-cell cultures (Fig. 4A). IgG$^+$ cells, by contrast, despite their similar pattern of differentiation throughout the different B-cell cultures (Fig. 4A), were predominantly IgG$_{bo}$(0.06 ± 0.06–0.44 ± 0.19).

Examining the expression of Blimp-1 and Xbp-1, two important factors in PC differentiation and function (7, 26, 28), before culture, we found that eGC and GC B-cell fractions expressed significantly higher levels of Blimp-1 (Figs 4B, 28), before culture, we found that eGC and GC B-cell fractions expressed significantly higher levels of Blimp-1 (Fig. 4B) and Xbp-1 (Fig. 4B) than the other fractions. We hypothesized that the higher levels of Blimp-1 and Xbp-1 expression in eGC and GC B cells might predispose newly switched IgE$^+$ cells, derived from these cells, towards the PC lineage. We therefore examined the PC differentiation of IgE$^+$ cells in each of the sorted B-cell cultures (Fig. 4C).

To account for the differences in CSR to IgE in these four different tonsil B-cell fractions and the variability among the different tonsil B-cell cultures (23), we measured the yields of IgE$^+$ PCs as a fraction of all IgE$^+$ cells in each culture (Fig. 4D). We found that the GC B-cell culture yielded the highest proportion of IgE$^+$ PCs and that naïve B-cell cultures yielded the lowest (Fig. 4C,D). Yet, despite the higher expression of Blimp-1 and Xbp-1 in eGC compared to the memory cells, the two cell cultures yielded similar proportions of IgE$^+$ PCs (Fig. 4C,D). The yields of IgE$^+$ PCs in the unfractonated B-cell cultures were between those in naïve and memory/eGC B-cell cultures (Fig. 4C,D). These results show that elevated Blimp-1 and Xbp-1 expression cannot fully account for the rapid rate of PC differentiation.

The difference in the yields of IgG$^+$ PCs between the various B-cell cultures showed a similar pattern to the yields of IgE$^+$ PCs (Fig. 4C,D). However, despite this, we find that the yields of IgG$^+$ PCs were much lower than those of IgE$^+$ PCs. This is also evident in naïve B-cell cultures, which have no IgG$^+$ cells at the start of the culture (23), where the yields of IgE$^+$ PCs are twice those of IgG$^+$ PCs (Fig. 5A). The data demonstrate that IgE$^+$ B cells have a much higher frequency of PC differentiation than IgG$^+$ cells.

IgE$^+$ PCs can be generated by both direct and sequential CSR

Previous studies in the mouse demonstrated that sequential CSR to IgE from an IgG$^+$ B cell is required for the generation of high-affinity IgE antibodies (17). A follow-up report suggested that direct CSR from IgM to IgE generates IgE$^+$ GC cells and sequential CSR from IgG to IgE leads to IgE$^+$ PCs (16). To determine the relative importance of the CSR pathways in the generation of IgE$^+$ cells in our total B-cell cultures, we examined switch circle transcripts (SCTs) by a nested PCR. Analysis of SCTs shows that both IgM to IgE (Ic-Cµ) and IgG to IgE (Ic-Cγ) SCTs were present in IgE$^b$/CD138$, IgE$^b$/CD138$^+$ and IgE$^b$/CD138$^+$ cells (Fig. 5A), revealing that both direct and sequential CSR can give rise to IgE$^+$ GC B cells and IgE$^+$ PCs.

Next, we investigated the CSR pathways that generate IgE$^+$ PCs in the cultures of B cells from the GC and naïve compartments. We found that in GC B-cell cultures, both Ic-Cµ and Ic-Cγ SCTs were present in CD138$^+$ cells, but only Ic-Cγ SCTs in the CD138$^+$ cells (Fig. 5B). In contrast, in naïve B-cell cultures, we detected both Ic-Cµ and Ic-Cγ SCTs in the CD138$^+$ and CD138$^-$ cells (Fig. 5C). Overall, these data demonstrate that in human tonsil B-cell cultures, both direct and sequential CSR can give rise to IgE$^+$ cells at all stages of differentiation. However, the generation of IgE$^+$ PCs from the GC-derived B cells appears to be selective, favouring sequential CSR to IgE. This may reflect the process of affinity maturation in IgG$^+$ GC B cells in vivo, leading to greater survival of cells expressing high-affinity B cells, coordinated with CSR, and resulting in apoptosis of the less-fit GC cells during ex vivo culture (9, 16, 17, 29, 30).

Modulation of surface IgE expression along the differentiateation pathway of IgG$^+$ cells

In mouse IgG$^+$ GC B cells, surface IgG1 is expressed at a 20-fold higher level than in IgG$^+$ PCs, whereas the opposite is true for the IgE$^+$ cells (13, 16). When examining this phenomenon in human B cells, it is necessary to recall that there are two isofoms of human mlgE, mlgE$_A$ and mlgE$_S$, the latter being homologous to the one in the mouse. Stimulated human B cells express a preponderance of the mlgE$_A$ isoform (19–21). To determine the surface expression of the two mlgE isoforms along the differentiation pathway of human IgE$^+$ B cells, we used a combination of different anti-IgE antibodies. Staining with either a polyclonal anti-IgE, which recognizes all forms of IgE, or the anti-IgE omalizumab, which recognizes only free IgE (31), revealed that surface mlgE expression was higher in IgE$_{bo}$ than in IgE$_{bi}$ cells (Fig. 6A). The similarity of staining by these two antibodies excludes the possibility that free exogenous IgE is binding to the low-affinity IgE receptor, CD23, expressed on the B-cell membrane, consistent with our observation that surface expression of membrane CD23 is lower on IgE$_{bi}$ cells relative to IgE$_{bo}$ cells (Fig. 6B).

Next, we determined the surface expressions of mlgE and mlgG1 at successive stages of IgE$^+$ and IgG$^+$ cell differentiation, with the aid of, respectively, anti-IgE omalizumab and a polyclonal anti-IgG1 (Fig. 6C,D). It can be seen that surface mlgE is down-regulated, and surface mlgE is up-regulated in the course of PC differentiation in humans, as in mouse (13, 16). Importantly, however, staining with anti-mlgE$_A$, which recognizes only the mlgE$_A$ (32), reveals a reduction in the surface mlgE on IgE$_{bi}$ cells relative to that on IgE$_{bo}$ cells (Fig. 6A). This result was confirmed by the concomitant reduction in surface mlgE$_A$ along the differentiation pathway of IgE$^+$ cells into PCs (Fig. 6E,F).

In sum, our data provide evidence that the down-regulation of surface mlgE$_A$ is compensated by the up-regulation of
surface mIgE, bearing in mind the elevated total surface mIgE expression.

Discussion

Most of the recent insights into the biology of IgE have come from studies in the mouse (13–17, 33). The primary aim of our work was to elucidate the developmental pathway of three different experiments. (C) FACS dot plots show the CD138 expression on IgE+ cells (top panels) and IgG1+ cells (bottom panels) after 10 days of culture with IL-4 and anti-CD40. (D) Yields of IgE+ PCs (IgE+CD138+) and IgG1+ PCs (IgG1+CD138+) as a percentage of the total IgE+ and IgG1+ cells, respectively. *P < 0.05, **P < 0.01, ***P < 0.001.

IgE+ PCs in the human system. A secondary aim was to compare the results obtained using tonsil B cells with those reported recently from various mouse models.

The developmental pathway of human IgE+ cells can be resolved into three easily distinguishable stages, characterized as (i) IgE+ GC-like (IgEhiCD138lo), (ii) IgE+ PC-like “plasmablasts” (IgEhiCD138lo) and (iii) IgE+ PCs (IgEhiCD138hi).

The same developmental sequence was seen to prevail in the
human tonsil IgG1+ cells. In contrast, mouse in vivo and ex vivo studies show the existence of only two distinct IgE+ (IgEhiCD138+ and IgEloCD138+) and IgG1+ (IgG1loCD138+ and IgG1hiCD138+) cell populations (13).

In the mouse, nascent IgE+ cells appeared to differentiate more swiftly into PCs than IgG1+ cells (13, 14), although this may have been their only route to survival (16, 33). Indeed, PC differentiation was the predominant fate of the mouse IgE+ cells in vivo as well as ex vivo (13, 14). Similarly, we show that in the human system, a greater proportion of IgE+ cells that differentiated into PCs. The mechanisms contributing to the apparent propensity of IgE+ cells to differentiate into the PC lineage remain unclear.

The transgenic Blimp-1-deficient mouse B cells undergo CSR to IgE, but fail to differentiate into PCs (13), confirming that Blimp-1 is not required for the CSR, but only for PC differentiation. However, Blimp-1 expression in our IgE+ and IgG1+ cells is similar and therefore does not account for their differences in PC differentiation.

Both mIgG1 and mIgE have cytoplasmic tails that contribute to their enhanced signalling capacity (34–38). However, mIgE has a unique motif (YANIL-motif) within its cytoplasmic tail, which is not found in the cytoplasmic tails of IgG isotypes, and binds proteins such as HS-1 and HAX-1 (39). This could potentially explain the enhanced PC differentiation of IgE+ cells. We have previously shown that CD23 plays an important role in IgE synthesis (1). Therefore, the propensity of IgE+ cells towards the PC lineage could also result from the activity of CD23.

An important way in which the mouse and human systems diverge is that whereas mouse IgE+ cells are mainly IgE+ PCs (13, 14), only a minor fraction of IgE+ cells in total tonsil B cells become CD138+ PCs. It is possible that in these cultures, IgE+ PCs are generated, but fail to survive. However, we have demonstrated that increased IL-4 concentration improved the yields of IgE+ PCs by maintaining the GC B cells. This implies that GC B cells are important for the generation of IgE+ PCs and that IL-4 is essential not only for CSR to IgE but also for the maintenance of GC B cells, which have very high rates of cell death (23).

The high proportion of IgE+ cells that differentiated into IgE+ PCs at the end of the GC B-cell cultures demonstrates the importance of GCs in the IgE+ PC generation. Yet, despite the different levels of Blimp-1 and Xbp-1 expression, eGC and memory B-cell cultures display similar IgE+ cell propensity to PC differentiation, implying that other factors are important, for example the response to cytokines that affect the balance between cell division and cell death. In contrast, the majority of IgE+ switched cells in naive B-cell cultures, which undergo similar levels of CSR to IgE (23) and express similar levels of Blimp-1 and Xbp-1 to memory B cells, undergo a lower rate of PC differentiation. In vivo memory B cells are generated in GCs following SHM and affinity maturation, resulting in cells with high-affinity BCRs (9, 10). The expression of a high-affinity BCR is associated with the capacity for PC differentiation in the mouse (29, 30).

A novel observation in the mouse was the inheritance of IgE memory from IgG+ cells (13, 14, 16) and the different effects of direct and sequential switching on the fate of IgE+ cells (16, 17). The kinetics of B-cell development revealed the relatively poor survival of IgE+ GC cells, attributed to their low level of mIgE expression, and impaired BCR signalling (13, 16). The conclusion was that IgE+ GC cells fail to undergo the canonical B-cell differentiation programme that potentiates IgG1 memory immune responses (16). The fate of the mouse IgE+ cells was determined by their switching pathway; direct switching from IgM+ cells generated only a transient population of IgE+ GC cells, whereas switching from IgG1+ cells generated IgE+ PCs, accompanied by the up-regulation of mIgE (16).

In contrast to the results in the mouse, we observe that both direct and sequential CSR can give rise to IgE+ GC B

**Figure 5** Both direct and sequential switching generate IgE+ GC B cells and IgE+ PCs. (A) RNA from the sorted IgEhiCD138+ and IgEloCD138− and IgEloCD138− was used for the detection of Ilc-Cμ SCT (167 bp; direct switching) and Ilc-Cγ SCT (202 bp; sequential switching) by nested PCR. The PCRs were standardized by using equal amounts of RNA for the cDNA synthesis. CD138− and CD138+ cells were sorted from the IL-4- and anti-CD40-stimulated cultures of enriched GC (CD38+) B cells (S) and naïve B-cell cultures (IC), and RNA was isolated and used for the analysis of IgE switching pathway as above. As a positive control, cDNA from an IL-4- and anti-CD40-stimulated B-cell culture that yielded high percentages of IgE+ cells was used, and as a negative control, dH2O. (M+ marker). Data are representative of four different experiments.
Figure 6  Surface mIgE expression along the differentiation pathway of IgE+ cells. (A) The FACS dot plot shows the day 12 cultured cells surface-stained for IgE with a polyclonal anti-IgE, anti-IgE omalizumab and anti-mIgE_L (4B12), followed by intracellular staining of IgE. Data are representative of five different experiments. (B) To determine the levels of membrane CD23 expression on IgE_lo and IgE_hi cells on day 12 of the culture, we surface-stained for CD23, followed by fixation, permeabilization and intracellular staining of IgE. The expression level of CD23 is shown as the fold change in CD23 median fluorescence intensity (MFI), within the gated IgE_lo and IgE_hi cells, relative to the CD23 MFI on IgE_lo cells. (C) The histograms show the surface levels of mIgE and mIgG1 expression IgE_lo CD138−, IgE_hi CD138−, IgE_lo CD138+, and IgE_hi CD138+, respective. (D) Summarized surface expression levels of mIgE and mIgG1 at different stages of differentiation into PCs. Expression levels (MFI) were made relative to the levels on the IgE_lo and IgG1_lo-gated cells. (E) The histogram shows the surface expression levels of mIgE_L on IgE_lo CD138− (black line), IgE_hi CD138− (blue line) and IgE_hi CD138+ (red line). (F) Summarized surface expression levels of mIgE_L made relative to the expression levels on IgE_lo CD138−-gated cells. Data represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA, Dunnett’s test).
cells and IgE+ PCs in our tonsil B-cell cultures. This is supported by studies on a chimeric mouse model containing the human M1' sequence inserted into a murine ε gene reporter construct (12, 15). Their IgE+ GC B cells were longer-lived than in other mouse models and able to differentiate into both IgE+ memory B cells and IgE+ PCs. Memory IgE+ B cells and IgE+ PCs can also be detected in the peripheral blood of humans (40). We and others have attributed this to the M1' sequence, which protects against apoptosis (11, 12, 15, 18).

Furthermore, we have also presented evidence of only sequential CSR in IgE+ PCs generated from the human GC-derived B-cell cultures. The major implication of sequential CSR is the probability of affinity maturation of the IgG+ B-cell precursors, by analogy to observations in the mouse (14, 16, 17). It may follow that sequential CSR is the predominant route to IgE in allergic disease (41). This is supported by the relative frequency of Iε-Cγ and Iε-Cμ transcripts in nasal biopsies from allergic rhinitis patients (42) and bronchial biopsies from asthma patients (43).

Our experiments also reveal the modulation of the surface mlGE1 and mlGE2 expression during PC differentiation. Earlier studies have shown that mlGE1 and mlGE2 are the predominant isoforms in the IL-4- and anti-CD40-stimulated and unstimulated peripheral blood lymphocytes, respectively (19–21). Therefore, the expression levels of the two mlGE isoforms during the PC differentiation may reflect the levels of signalling and proliferation induced by the IL-4 and anti-CD40 stimulations. Nonetheless, the above considerations suggest that the regulation of mlGE1 expression is important for IgE homeostasis in the human system. Expression of this isoform is unique to humans and could well be one reason why allergic disease occurs naturally in humans, but apparently not in mice (18). These data are of particular interest because the EMPD of surface mlGE1, is a validated antibody target for immunotherapy of allergic disease, now under development in several laboratories with a view to therapeutic application (32, 44, 45).

In summary, using a tonsil ex vivo human system, we have investigated the ontogeny of IgE+ B cells and IgE+ PCs. We believe that our results have a direct relevance to the discovery of novel targets for the treatment of allergy.

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Author contributions
F.R. designed and performed experiments, analysed data and wrote the paper. H.B., N. U., P.S.H. and Y.C.C. performed experiments and analysed data. J.B.C. and T.W.C contributed vital new reagents and critically reviewed the manuscript. J.M.M. and B.J.S. provided guidance and critically reviewed the manuscript. D.J.F provided guidance, analysed data and critically reviewed the manuscript. H.J.G. designed experiments, analysed data and wrote the manuscript. All authors reviewed the final manuscript.

Conflict of interest
The authors declare that they have no conflict of interests.

Supporting Information
Additional Supporting Information may be found in the online version of this article:
Data S1 Supplemental Methods

References
Developmental pathway of human IgE-expressing plasma cells

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