Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

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Allergy

ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Ontogeny of human IgE-expressing B cells and plasma cells

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To cite this article: Ramadani F, Bowen H, Upton N, Hobson PS, Chan Y-C, Chen J-B, Chang TW, McDonnell JM, Sutton BJ, Fear DJ, Gould HJ. Ontogeny of human IgE-expressing B cells and plasma cells. *Allergy* 2017; **72**: 66–76

Keywords
allergy; germinal centre; human B cells; IgE class switching; plasma cell.

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Accepted for publication 5 April 2016

DOI:10.1111/all.12911

Edited by: Hans-Uwe Simon

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^+PC-like '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 ($mIgE_S$), which is homologous to mouse mIgE, and the up-regulation of the long form of mIgE ($mIgE_L$), 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 $mIgE_L/mIgE_S$ ratio may be implicated in survival of IgE^+ B cells during PC differentiation and allergic disease.

IgE antibodies mediate the activation of IgE effector cells and antigen-presenting cells by allergen and hence are central to allergic disease (1, 2). The increasing prevalence of allergic disease is alarming, yet little is known about the mechanisms of IgE regulation. The sparsity of IgE^+ B cells *in vivo* has hindered the attempts to investigate their development, particularly in the human system, while reliance on the results from mouse models often fails to predict the outcome of proposed therapies (3).

It is well established that T-cell helper type 2 (Th2) cytokines, IL-4 and/or IL-13, in association with CD40 cross-linking on B cells, promote class switch recombination (CSR) to IgE, which may be direct, from IgM to IgE, or sequential, via IgG (4). *In vivo,* CSR occurs in lymphoid tissues and at sites of inflammations (5, 6). In lymphoid tissue, B-cell–T-cell interactions lead to B-cell proliferation and the formation of GCs, in which CSR is accompanied by somatic hypermutation (SHM) in the variable regions, culminating in affinity

Abbreviations
BCR, B-cell receptor; CSR, Class switch recombination; eGC, Early germinal centre; EMPD, Extramembrane proximal domain; FACS, Flow cytometry or fluorescence-activated cell sorting; GC, Germinal centre; $mIgE_L$, Long-form membrane IgE; $mIgE_S$, Short-form membrane IgE; PB, Plasma blast; PC, Plasma cells; SHM, Somatic hypermutations.

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, $mIgE_S$ and $mIgE_L$ (19, 20). These mIgE isoforms arise from the alternative splicing of a common mRNA precursor, with $mIgE_L$ 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 cytometry analysis.

Cell cultures

To induce CSR to IgE, B cells were cultured as previously described (23). Briefly, 0.5×10^6 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 IgE^{lo} and IgE^{hi} cells. Similarly, we observe two populations of $IgG1^+$ cells, $IgG1^{lo}$ and $IgG1^{hi}$ cells. The ratio of IgE^{hi} to IgE^{lo} rose from day 7 to day 12 in culture, whereas that of $IgG1^{hi}$ to $IgG1^{lo}$ cells remained constant (Fig. 1A and B). On profiling the surface markers of these cells, by FACS, we observed that both IgE^{hi} and $IgG1^{hi}$ express lower levels of CD20, Fas, IL-4R and Bcl-6 and higher levels of CD38, CD27 and Blimp-1 than IgE^{lo} and $IgG1^{lo}$ 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: $IgE^{lo}CD138^-$, IgE^{hi} $CD138^-$ and $IgE^{hi}CD138^+$ 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 $IgE^{lo}CD138^-$, $IgE^{hi}CD138^-$ and $IgE^{hi}CD138^+$ 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

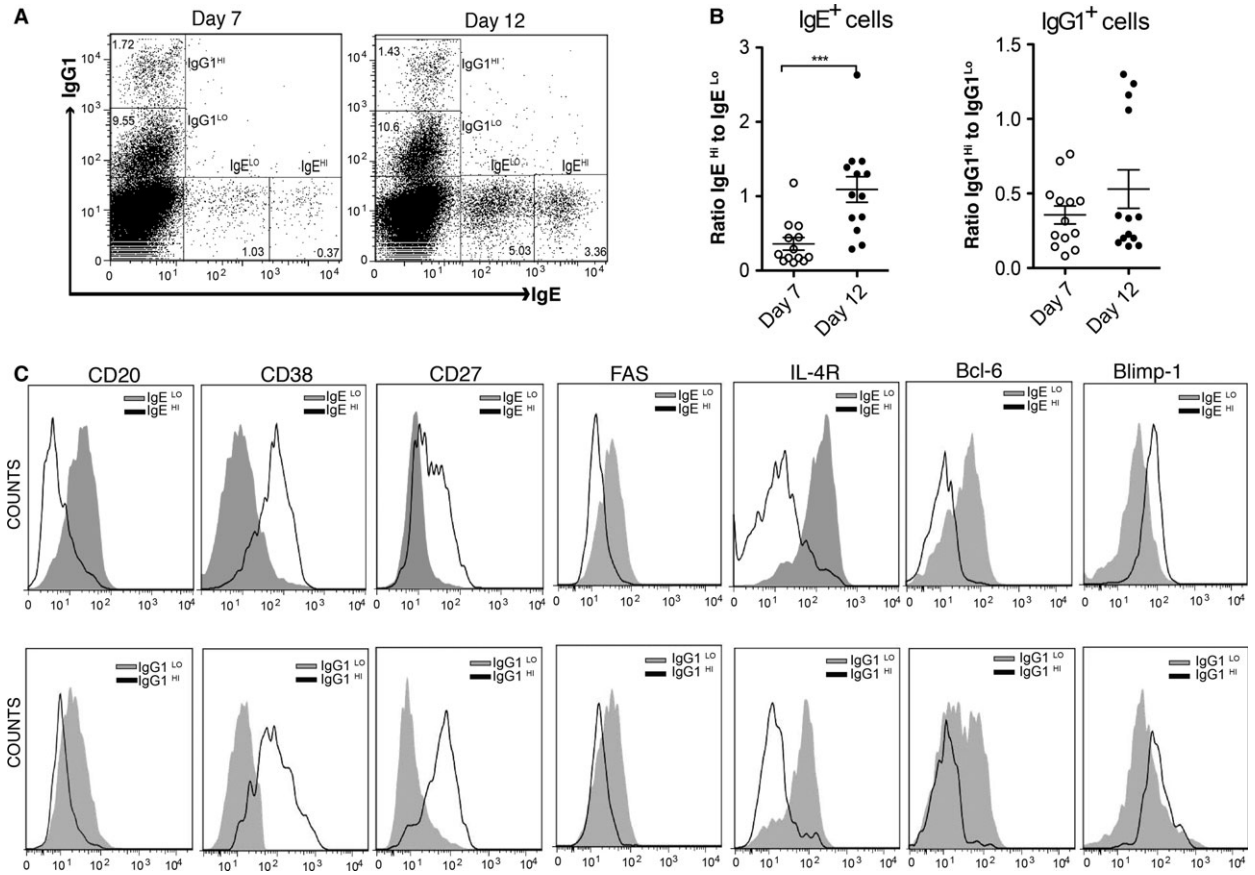

Figure 1 IgE^{hi} cells have a more differentiated phenotype than IgE^{lo} B cells. (A) Intracellular detection of two IgE^{+} and IgG1^{+} cell populations in our CSR to IgE cultures. Data are representative of five experiments. (B) The ratio of IgE^{hi} to IgE^{lo} and IgG1^{hi} to IgG1^{lo} cells was calculated using the frequency of these cells on days 7 and 12 of culture. Data represent the mean ± SD. ***$P < 0.001$ (two-tailed *t*-test). (C) Expression levels of CD20, CD27, CD38, Fas, IL-4R, Bcl-6 and Blimp-1 on IgE^{lo} and IgG1^{lo} (filled histogram) *vs* IgE^{hi} and IgG1^{hi} (empty histograms) gated cells, respectively. Data are representative of six experiments.

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 IgE^{lo}CD138^{-} and IgG1^{lo}CD138^{-} cells, but strongly down-regulated in both the IgE^{hi} (IgE^{hi}CD138^{-} and IgE^{hi}CD138^{+}) and IgG1^{hi} (IgG1^{hi}CD138^{-} and IgG1^{hi}CD138^{+}) 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 IgE^{hi}CD138^{+} and IgG1^{hi}CD138^{+} 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 G_0 stage of the cell cycle (Fig. S2). In sum, these observations, consistent with those on the cell surface markers, indicate that IgE^{lo} (IgE^{lo}CD138^{-}) cells have a phenotype with GC B cell-like characteristics, whereas the IgE^{hi} cells represent a later stage of differentiation into a PC-like (IgE^{hi}CD138^{-}) 'plasmablast' phenotype, and only a small proportion appear to be fully differentiated PCs (IgE^{hi}CD138^{+}).

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 naïve 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

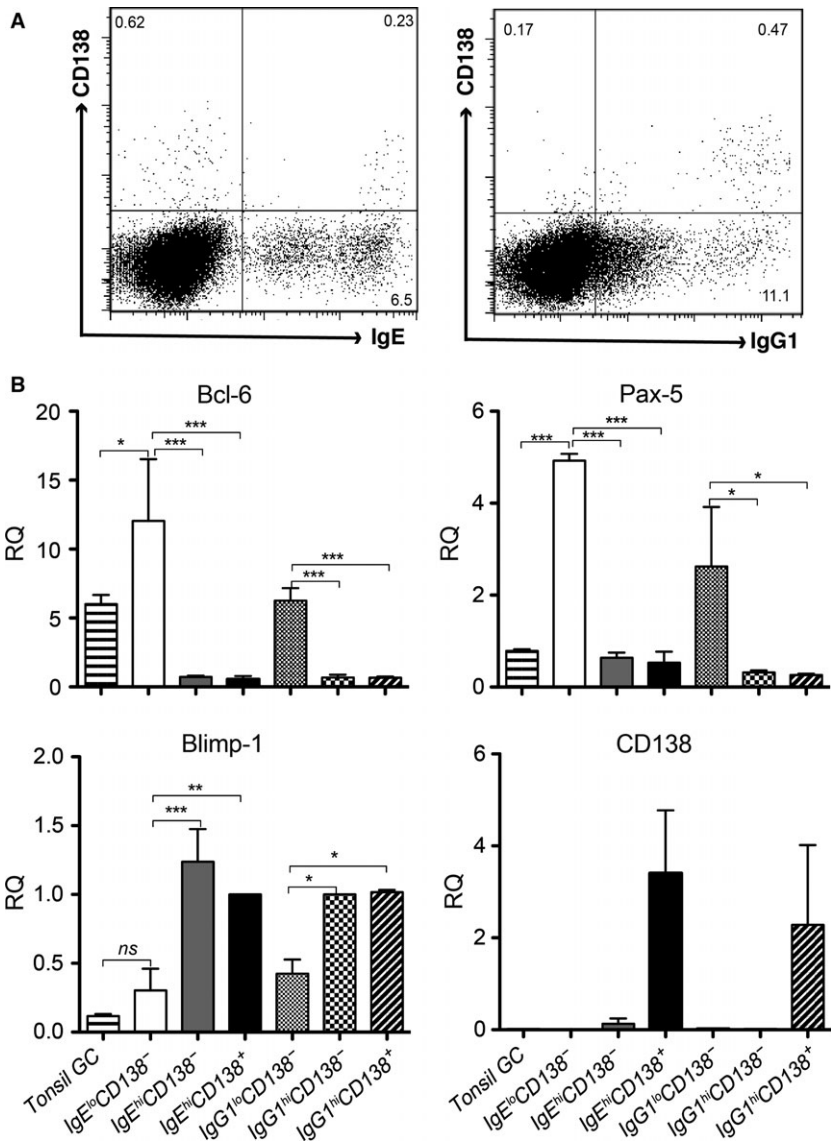

Figure 2 Human IgE^+ and $IgG1^+$ cells have three distinctive stages of differentiation into PCs. (A) On day 10 of total B cultures, the cells were surface-stained for CD138 followed by fixation/permeabilization and intracellular staining for IgE and IgG1. The FACS dot plots show three distinct IgE^+ and $IgG1^+$ cell populations: $IgE^{lo}CD138^-$, $IgE^{hi}CD138^-$and $IgE^{hi}CD138^+$ 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. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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).

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 naïve ($CD27^-CD38^-CD77^-$), 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 naïve and memory B-cell cultures (Fig. 3E).

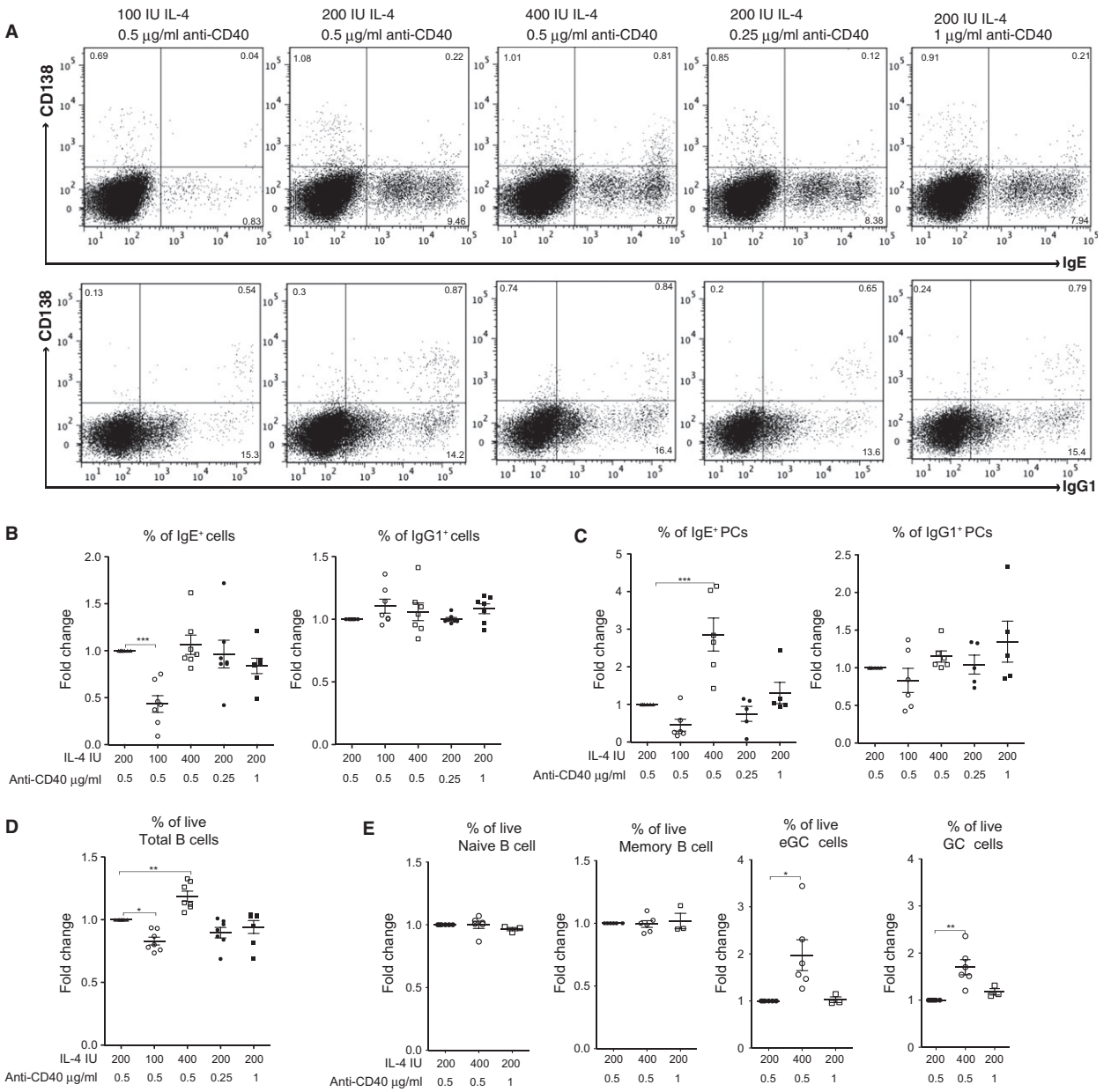

Figure 3 GC-derived B cells and the yields of IgE$^+$ PC are sensitive to changes in IL-4 concentration. To determine the effect of IL-4 and anti-CD40 concentrations on the generation of IgE$^+$ cells, especially IgE$^+$ PCs, tonsil B cells were cultured with varying concentrations of IL-4 and anti-CD40. (A) On day 10 of culture, the cells were stained for IgE and CD138 (top panels) and IgG1 and CD138 (bottom panels). The percentages of total IgE$^+$ and IgG1$^+$ cells (B), IgE$^+$ and IgG1$^+$ PCs (C) and % of live cells in culture (D) were made relative to the 200 IU/ml of IL-4 and 0.5 μg/ml of anti-CD40 concentrations, standard concentrations used to induce CSR to IgE. (E) Percentage of live cells in cultures containing the FACS-sorted naïve, memory, eGC and GC B cells cultured with increased concentrations of IL-4 (400 IU/ml) or anti-CD40 (1 μg/ml). The data show the fold changes in the percentage of live cells relative to the 200 IU/ml of IL-4 and 0.5 μg/ml of anti-CD40 cell culture condition and represent the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (one-way ANOVA, Dunnett's test).

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 unfractionated total B cells as controls, and determined the IgE^{hi}/IgE^{lo} 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^{hi}/IgE^{lo} cell ratios intermediate to that in the naïve and GC B-cell cultures (Fig. 4A). $IgG1^+$ cells, by contrast, despite their similar pattern of differentiation throughout the different B-cell cultures (Fig. 4A), were predominantly $IgG1^{lo}$ (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 and S4) 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 unfractionated 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 $IgG1^+$ 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 $IgG1^+$ PCs were much lower than those of IgE^+ PCs. This is also evident in naïve B-cell cultures, which have no $IgG1^+$ cells at the start of the culture (23), where the yields of IgE^+ PCs are twice those of $IgG1^+$ PCs (Fig. S5). The data demonstrate that IgE^+ B cells have a much higher frequency of PC differentiation than $IgG1^+$ 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 $IgG1^+$ 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 (Iε-Cμ) and IgG to IgE (Iε-Cγ) SCTs were present in $IgE^{lo}CD138^-$, $IgE^{hi}CD138^-$ and $IgE^{hi}CD138^+$ 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 Iε-Cμ and Iε-Cγ SCTs were present in $CD138^-$ cells, but only Iε-Cγ SCTs in the $CD138^+$ cells (Fig. 5B). In contrast, in naïve B-cell cultures, we detected both Iε-Cμ and Iε-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 $IgG1^+$ 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 differentiation pathway of IgE^+ cells

In mouse $IgG1^+$ GC B cells, surface IgG1 is expressed at a 20-fold higher level than in $IgG1^+$ 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 isoforms of human mIgE, $mIgE_L$ and $mIgE_S$, the latter being homologous to the one in the mouse. Stimulated human B cells express a preponderance of the $mIgE_L$ isoform (19–21). To determine the surface expression of the two mIgE 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 mIgE expression was higher in IgE^{hi} than in IgE^{lo} 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^{hi} cells relative to IgE^{lo} cells (Fig. 6B).

Next, we determined the surface expressions of mIgE and mIgG1 at successive stages of IgE^+ and $IgG1^+$ cell differentiation, with the aid of, respectively, anti-IgE omalizumab and a polyclonal anti-IgG1 (Fig. 6C,D). It can be seen that surface mIgG1 is down-regulated, and surface mIgE is up-regulated in the course of PC differentiation in humans, as in mouse (13, 16). Importantly, however, staining with anti-$mIgE_L$, which recognizes only the $mIgE_L$ (32), reveals a reduction in the surface mIgE on IgE^{hi} cells relative to that on IgE^{lo} cells (Fig. 6A). This result was confirmed by the concomitant reduction in surface $mIgE_L$ along the differentiation pathway of IgE^+ cells into PCs (Fig. 6E,F).

In sum, our data provide evidence that the down-regulation of surface $mIgE_L$ is compensated by the up-regulation of

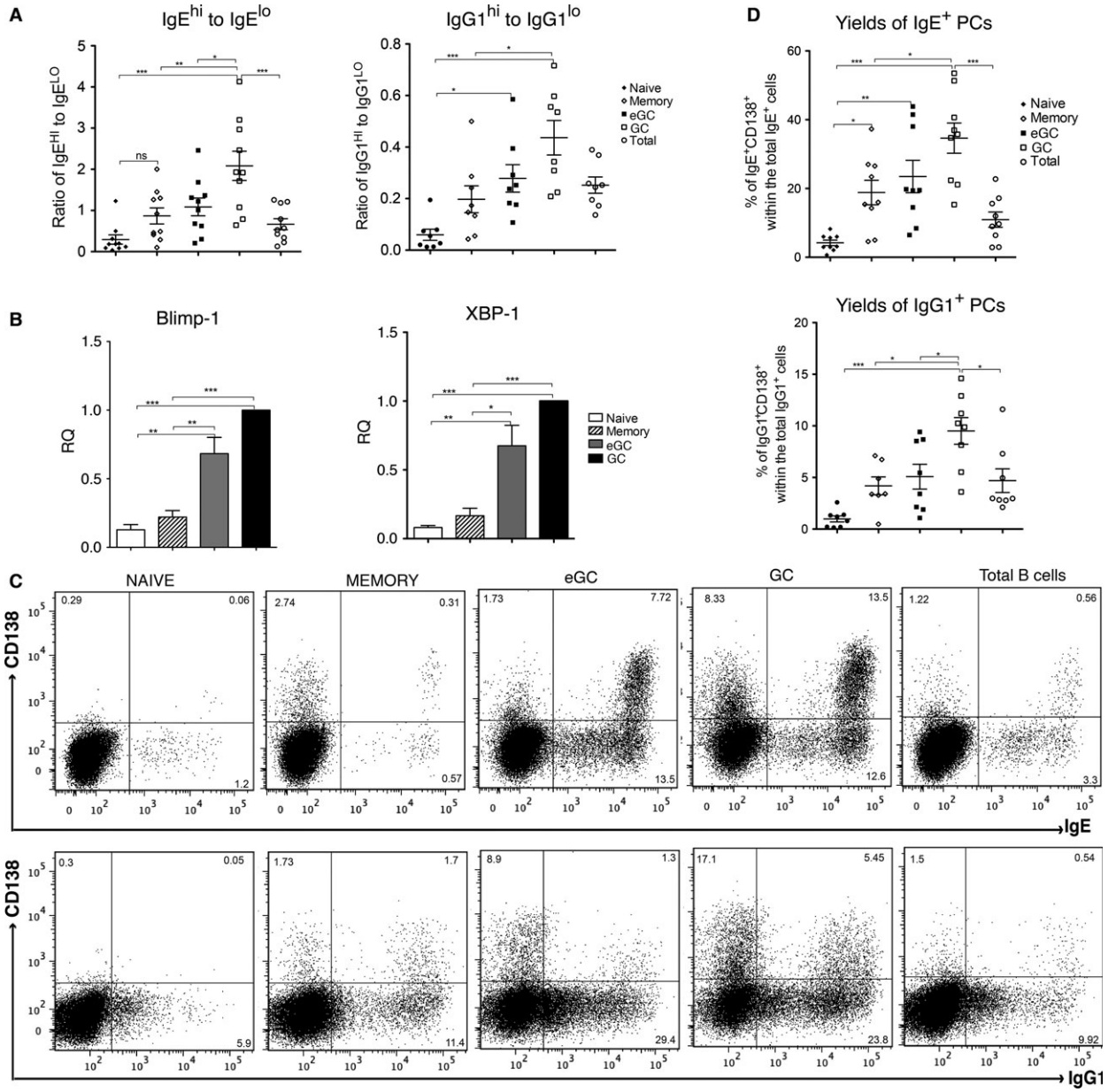

Figure 4 GC B-cell cultures yield the highest percentage of IgE$^+$ PCs. (A) Ratio of IgE^{hi} to IgE^{lo} cells and IgG1^{hi} to IgG1^{lo} cells in IL-4- and anti-CD40-stimulated cultures of naïve, memory, eGC, GC and unfractionated total B cells. (B) Blimp-1 and Xbp-1 expression levels in sorted tonsil B-cell subsets prior to culture with IL-4 and anti-CD40. Data represent the mean ± SD and are derived from 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. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

surface mIgE$_S$, 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 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 (IgE^{lo}CD138$^-$), (ii) IgE$^+$ PC-like "plasmablasts" (IgE^{hi}CD138$^-$) and (iii) IgE$^+$ PCs (IgE^{hi}CD138$^+$). The same developmental sequence was seen to prevail in the

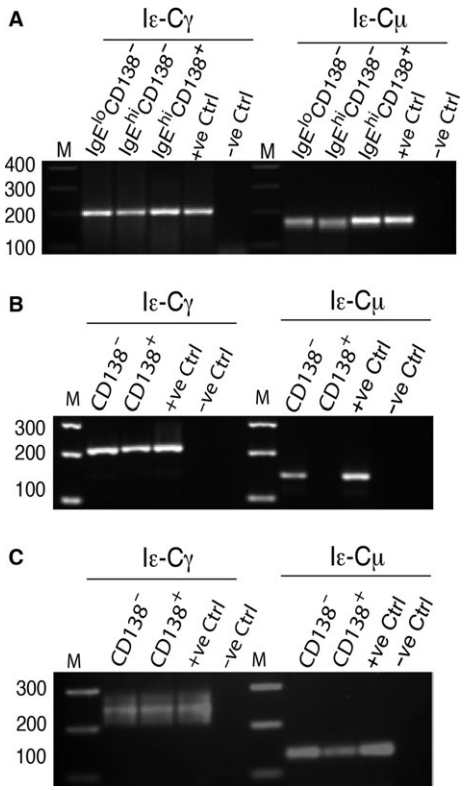

Figure 5 Both direct and sequential switching generate IgE^+ GC B cells and IgE^+ PCs. (A) RNA from the sorted $IgE^{lo}CD138^-$, $IgE^{hi}CD138^-$ and $IgE^{hi}CD138^+$ was used for the detection of Iε-Cμ SCT (167 bp; direct switching) and Iε-Cγ SCT (202 bp; sequential switching) by nested PCR. The 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 (B) and naïve B-cell cultures (C), 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, dH_2O. (M= marker). Data are representative of four different experiments.

human tonsil $IgG1^+$ cells. In contrast, mouse *in vivo* and *ex vivo* studies show the existence of only two distinct IgE^+ ($IgE^{lo}CD138^-$ and $IgE^{hi}CD138^+$) and $IgG1^+$ ($IgG1^{lo}CD138^-$ and $IgG1^{hi}CD138^+$) 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, compared with their $IgG1^+$ counterparts, differentiate 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^{hi} cells are mainly IgE^+ PCs (13, 14), only a minor fraction of IgE^{hi} 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 naïve 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

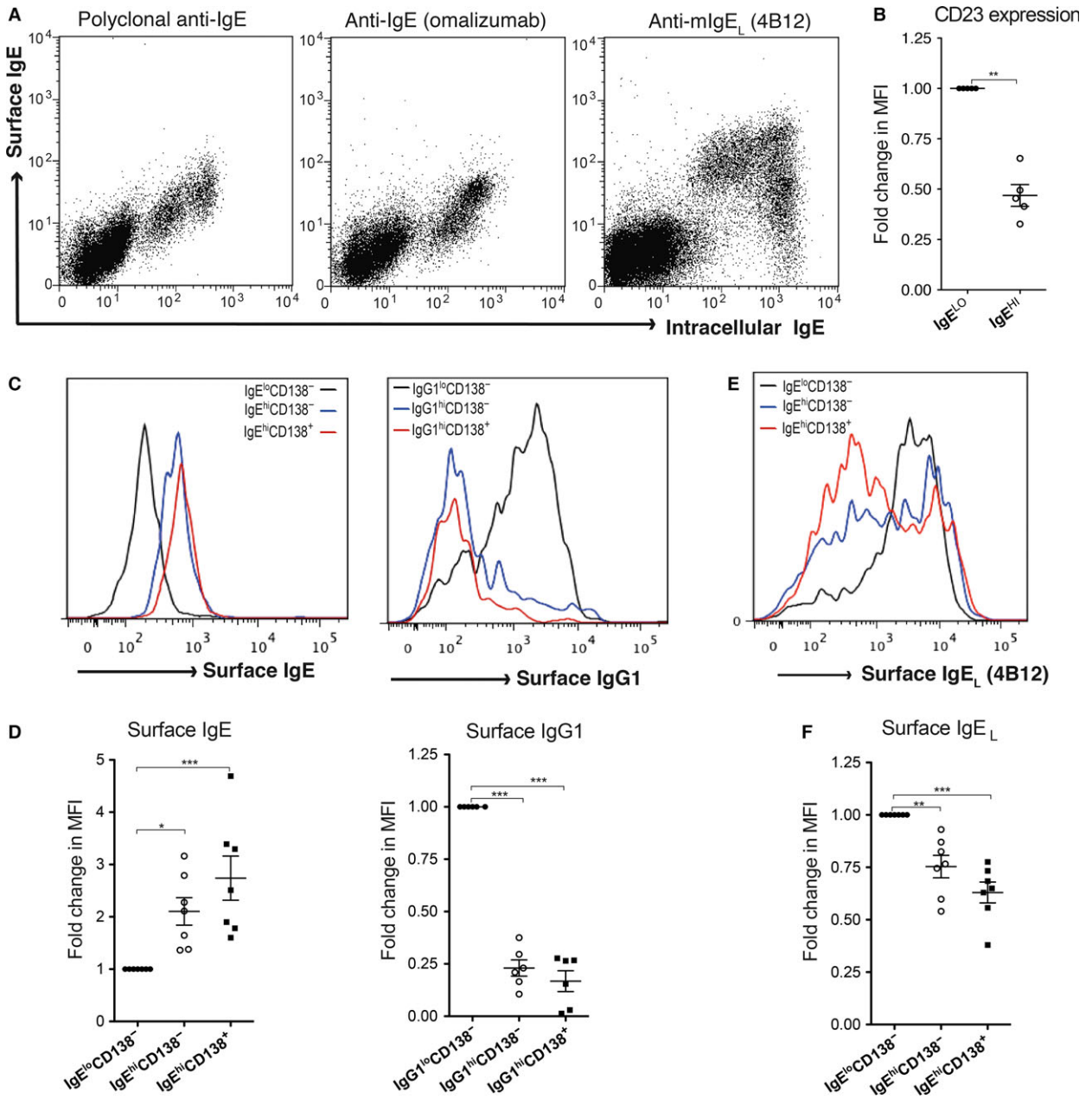

Figure 6 Surface mIgE expression along the differentiation pathway of IgE$^+$ cells. (A) The FACS dot plot shows the day 12 cultured cells surface-stained for IgE with a polyclonal anti-IgE, anti-IgE omalizumab and anti-mIgE$_L$ (4B12), followed by intracellular staining of IgE. Data are representative 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^{hi}CD138$^+$ and IgG1^{lo}CD138$^-$, IgG1^{hi}CD138$^-$, IgG1^{hi}CD138$^+$, respectively. (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 $mIgE_L$ and $mIgE_S$ expression during PC differentiation. Earlier studies have shown that $mIgE_L$ and $mIgE_S$ 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 mIgE 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 $mIgE_L$ 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 $mIgE_L$ 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.

Acknowledgments

This work was in part supported by the Wellcome Trust (Grant: 076343), Asthma UK (Grant: AUK-PG-2013-183) and the Department of Health via the National Institute for Health Research (NIHR) Comprehensive Biomedical Research Centre Award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. We are grateful to Walter Gratzer for helpful comments on the manuscript.

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

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