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BCR Ligation antagonizes the IL-21 enhancement of anti-CD40/IL-4 Plasma cell differentiation and IgE production found in low density human B cell cultures

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Abstract

We sought to discover the mechanisms explaining increased IgE production seen at low cell densities when IL-21 is added to human B cell cultures activated with anti-CD40 and IL-4. When cells were cultured in the absence of BCR ligation, qPCR demonstrated dramatic increases in mRNA for the plasma cell transcription factors BLIMP1 and XBP1. Furthermore, a majority of viable cells expressed high levels of CD38 while losing expression of surface IgD. In contrast, in the presence of BCR stimulation, both the XBP1 mRNA levels and CD38 cell surface expression were markedly reduced, and a large population of cells retained IgD expression, indicating reduced plasma cell differentiation. IgE levels were reduced in the BCR stimulated cultures by 90%, while IgG4 levels remained unchanged. In summary, IL-21 enhances IgE production at low densities through stimulating cell division and plasma cell differentiation and this activity is reduced upon BCR crosslinking.

Keywords

IgE; IL-21; plasma cell; XBP1; BLIMP1; BCR; human B cells

Introduction

IgE, an immunoglobulin isotype produced in low levels in both man and mouse, is involved in atopic diseases such as allergy and asthma and can trigger a lethal reaction known as anaphylaxis. While IgE has potential benefit to helminth infected individuals, such infections are rare in industrialized societies. Certainly, IgE is more problematic than beneficial in that antigen specific IgE against normally innocuous particles triggers unwanted allergic responses. Today, allergies are the 6th leading cause of chronic disease in the US and incidence of both allergic rhinitis and asthma are rising in developed countries [1;2]. The only current treatment to control IgE synthesis is immunotherapy and the effectiveness varies widely with both the respective antigen involved as well as the number of antigens to which the individual reacts [3;4]. There is currently no strategy to decrease overall IgE synthesis; thus, better understanding of both the production and control of IgE synthesis is certainly warranted.

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Isotype switching *in vivo* occurs when antigen-activated IgM⁺ IgD⁺ B cells receive a second activation signal, generally provided by CD40 ligand and a cytokine signal. For IgE switching this cytokine is either IL-4 or IL-13 (reviewed in [5]). *In vitro*, IL-10 added to IL-4/anti-CD40 treated cells has also been shown to enhance IgE production in B cells [6]. Other groups have shown that the addition of IL-4 alone, or in the presence of corticosteroids, can increase IgE production in the human system as well [7]. However, *in vitro* studies using human PBMC or purified B cells have been problematic due to the small amount of IgE produced, usually close to the limits of detection (pg or ng). The recent findings that addition of IL-21 to cell cultures greatly promotes IgE synthesis [8;9–11] will significantly aid these investigations. Our laboratory has previously shown that IgE in the murine system can be optimized at a low cell density [12]. Furthermore, studies by the Hodgkin laboratory [13;14] have indicated that both mouse IgE and IgG1 as well as human IgG synthesis occurs only after multiple rounds of cell division. This same finding was observed with human B cells activated with anti-CD40 and IL-4 in the presence of IL-21 [8]. Other recent studies have supported a role for human IL-21 in enhancing IgE production, as well as IgG1 and IgG3 [8;10]; however, only high cell concentrations were analyzed in those studies. Our previous cell density analyses indicated that IgE as well as total IgG and IgM were all increased at low cell densities [9]. This did not appear to be due to simply media exhaustion, since replenishment of media during culture did not ablate the concentration effect [9]. Because we determined an increased production of IgE in our human *in vitro* culture system in the presence of IL-21, we next wanted to address the mechanism of this increase. The most straightforward explanation for the cell density effect was reduced plasma cell differentiation at high cell densities. Using both cell surface phenotypic analysis and real-time, quantitative PCR (qPCR), we report that the addition of IL-21 promotes plasma cell differentiation of human primary B cells grown at low cell densities. Higher density cultures show reduced proliferation and cell viability post day 7, which potentially explains the decreased differentiation to plasma cells. We also report that anti-IgM stimulation inhibits this plasma cell differentiation which is consistent with other reports claiming IgM crosslinking inhibits the production of IgE [15].

Materials and Methods

Antibodies, reagents and cytokines

rhIL-4 and huIL-21R-Fc chimera were purchased from R&D Systems (Minneapolis, MN). Mouse anti-human IgD-FITC and anti-human IgE-HRP were obtained from Becton-Dickenson Biosciences (San Diego, CA) or Dako Cytomation, Inc. (Ft Collins, CO), respectively. Human IL-21 was prepared as previously described [9]. Mouse anti-human CD40 clone G28-5 (American Type Culture Collection, (ATCC), Manassas, VA) was purified from ascites by salt fractionation and FPLC using MEP-Hypercel chromatography (Pall Life Sciences East Hills, New York). Purified mAb (99%) was eluted with acetate buffer, pH 4.9, and dialyzed against PBS prior to use in culture. Human IgE standards were purified from JW8 IgE myeloma cells (ATTC), grown in Integra CeLLine 1000 flasks (IBS Integra Biosciences, Switzerland). Supernatants were purified by FPLC using MEP-Hypercel (Pall laboratories, East Hills, NY) [8]. IgE elutes at pH 5.5 and was further purified (98%) by gel filtration. Mouse anti-human IgG4-AP, goat anti-human IgG unlabeled, and goat anti-human IgM unlabeled were obtained from SouthernBiotech (Birmingham, Alabama).

Complete culture medium

(CRPMI-10) consisted of RPMI-1640 supplemented with 10% heat inactivated (56 °C, 30 min) fetal bovine serum (Atlanta Biologicals Norcross, GA), 2 mM L-glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, 100 µM non-essential amino acids, 1 mM sodium pyruvate, 50 µg/ml amphotericin B, 50µM 2-mercaptoethanol, and 20 mM HEPES buffer (all from

Invitrogen, Carlsbad, CA). B cells were cultured in complete media supplemented with 10 ng/ml rh IL-4 and 1000 ng/ml α -human CD40 in the presence or absence of IL-21 (10 ng/ml).

Human B cell purification

Human tonsils were obtained from routine tonsillectomies at Henrico Doctors Hospital (Richmond, VA) per approved IRB protocol. Tonsils were placed in RPMI-1640 and mechanically disrupted using a Seward Stomacher 80 Biomaster Lab Blender (Brinkmann, Westbury, NY) at normal speed for 60 seconds. To obtain a single cell suspension, the homogenate was filtered through a 70 micron cell strainer (BD Discovery Labware, Bedford, MA). The resulting product was underlaid with Ficoll-Hypaque (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) (30 ml diluted cells on 13 ml Ficoll-Hypaque solution). The tubes were centrifuged for 20 min at $400 \times g$. The cells were removed under the laminar flow hood, transferred to new tubes and washed in sterile PBS. Assessment for total cell yield was done using Gentian Violet staining and viability using Trypan blue exclusion. To isolate B cells, the tonsillar preparation was incubated with an anti-human IgD conjugated with fluorescein isothiocyanate (FITC) (BD Pharmingen San Diego, CA) at a concentration of 1×10^6 cells/1 μ l antibody (Ab) and 1×10^6 cells/9 μ l buffer for 30 minutes on ice in separation buffer (PBS with 0.25% BSA and 0.19% EDTA). Cells were then washed one time in full volume separation buffer to remove unbound antibody and then re-suspended at a concentration of 1×10^6 cells/1 μ l Ab and 1×10^6 cells/9 μ l buffer with anti-FITC Microbeads (Miltenyi Biotec Auburn, CA) and incubated again on ice for 30 minutes in separation buffer. Post secondary incubation, cells were re-washed in full volume separation buffer and isolated via the Miltenyi MACS cell separation protocol to obtain a >95% pure IgD+ B cell population.

Cell Surface Phenotyping

All cells were tested for cell surface antigen expression by direct immunofluorescence and flow cytometric analysis. The antibodies were anti-human IgD-FITC and anti-CD38-allophycocyanin (APC) (BD Pharmingen San Diego, CA). Briefly, 1×10^6 cells were pelleted in 12mm $\times$ 75mm tubes and washed once in cold FACS buffer (PBS with 0.5% FBS, 0.03% sodium azide). Cells were re-suspended in approximately 80 μ l of FACS buffer and 10 μ l of 1:1 antibody dilutions in PBS were added. Cells were incubated for 30 min at 4°C, washed once in cold FACS buffer, and then re-suspended in 1ml of FACS buffer. Prior to analysis, propidium iodide (PI) stain was added to determine cell viability. Flow cytometric analyses were performed on a Cytomics FC500 Flow Cytometer and data was analyzed using CXP software (Beckman Coulter Fullerton, CA).

Analysis of cell division numbers

Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes Eugene, OR) was prepared according to the manufacturer's recommendations. Resting B cells were washed and re-suspended at 1×10^7 cells/mL with PBS/0.1% BSA. CFSE was then added to final concentration of 0.5–5 μ M and incubated in the dark at 37°C for 10 minutes. Cells were then washed twice and re-suspended in CRPMI-10. CFSE labeled cells were plated at either 1×10^3 cells/well, 1×10^4 cells/well or 1×10^5 cells/well in a 96 well plate and cultured for 8 days with 10 ng/ml IL-4 alone, 1000 ng/ml anti-CD40 alone, or IL-4 and anti-CD40 in the presence or absence of IL-21. Cells were harvested and then analyzed by flow cytometry using an FC500. The percentages of cells in each cell division were determined by setting sequential gates one-half intensity starting with the highest intensity peak, which represents undivided cells.

ELISA protocols

Determination of human IgE levels utilized the mouse monoclonal anti-human IgE antibodies, clones 4.15 and 7.12 (HB-235 and HB-236 from ATCC) as capture antibodies [16]. Antibodies

were plated in combination at 5 µg/mL total in 0.05M borate buffered saline (BBS), pH 8.5. Samples and standards were detected using rabbit anti-human IgE-HRP diluted in PBS/2% FBS. Human IgE standards and samples were serially diluted 1:2 in CRPMI-10. The lower limit of linear detection was consistently ~5 ng/mL. IgG4 concentrations were determined by coating with goat anti-human IgG and detection with mouse anti-human IgG4-AP, used as suggested by manufacturer.

ELISpot Protocol

Flat-bottom Multiscreen-HA 96-well plates (Millipore, Bedford, MA) were pre-coated overnight at 4°C with a monoclonal anti-human IgE antibody (clone 4.15) at 5µg/ml in Dulbecco's PBS (DPBS) (Invitrogen). This antibody was also used in our earlier investigation of IL-21 influence on IgE [9] and shows no reaction to other human Ig isotypes [16]. The plates were then washed with DPBS and blocked using RPMI-1640 with 10% FBS and HEPES for 1 hour. Cells were plated in a serial dilution of cell concentrations identical to the conditions set forth in all other assays. Each condition included three replicates. Following 20-hour culture, cell-containing medium was removed, plates were washed in DPBS with 0.05% Tween-20 (Sigma, St Louis, MO), and incubated with an anti-human IgE-AP antibody (BD Pharmingen) at a 1:1000 dilution at 37°C for 1 hour. Plates were again washed and incubated with substrate solution. Substrate consisted of AMP Substrate (15mg MgCl₂·6H₂O, 10 µl Triton X-405, 25mg NaN₃, 9.6mls AMP (2-amino-2-methyl-1-propanol) in 100mls dH₂O at pH 10.5.) and 1mg BCIP (5-bromo-4-chloro-3-indoyl phosphate) was dissolved in 1ml AMP substrate and filtered. 50µl substrate per well was used. Plates incubated for 1 hour at 37°C until blue spots appeared. Plates were read and analyzed on a CTL 2.01 ELISPOT reader (Cellular Technology Limited Cleveland OH).

RNA Isolation and RT-PCR conditions

RNA was isolated from cells post culture conditions using TRIZOL (Invitrogen) according to manufacturer's protocol. qPCR experiments were performed in the ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, Ca) using the TaqMan® One Step PCR Master Mix Reagents Kit. All the samples were tested in triplicate under the conditions recommended by the fabricant. The cycling conditions were: 48°C/30min; 95°C/10min; and 40 cycles of 95°C/15sec and 60°C/1min. The cycle threshold was determined to provide the optimal standard curve values (0.98 to 1.0). The probes and primers for PAX-5 (#Hs00277134_m1) and BLIMP-1 (#Hs00153357_m1) were synthesized and purchased from ABI. The probes and primers used for XBP1 were designed using the Primer Express® 2.0 version. The forward primer was GCGCCTCAC GCACCTG, the reverse primer was GCTGCTACTCTGTTTTTCAGTTTCC, and the probe was CTCAGCGCCTTCTCCTCGGGG. The probes were labeled at the 5' end with FAM (6-carboxyfluoresceine) and at the 3' end with TAMRA (6-carboxytetramethylrhodamine). Human beta-actin from the Pre-developed TaqMan® Assay Reagents was used as endogenous control. The reactions were performed in the VCU Nucleic Acid Research Facilities.

Data analysis

Data are summarized as mean ± Standard error (SE). The statistical analysis of the results was performed by the student's *t* test. A p-value of <0.05 was considered significant.

Results

ELISPOT analysis of IgE producing cells as a function of initial cell density

Several laboratories [8;10;11] have demonstrated that IL-21 strongly enhances human B cell proliferation and IgE production. Our recent publication in this area demonstrated that the IgE

production induced by CD40L and IL-4 was greatly enhanced at low cell densities [9]. In order to further examine the mechanisms behind the cell density effect we performed IgE ELISPOT analysis. As can be seen in Fig 1, in which the analysis was performed at day 8, a dramatic increase in both spot size and number was seen at the lower cell densities in the presence of IL-21. While ELISPOT values are shown for only a single cell concentration in the absence of IL-21, all cell concentrations exhibited low spot numbers and those seen were small. Thus, it can be concluded that IgE production at the lower cell densities comes from both increasing numbers of IgE producing cells and increased IgE production per cell.

Increased plasma cell differentiation at low cell densities

As suggested by the ELISPOT data, we hypothesize that increased plasma cell differentiation is occurring at low cell densities. This was confirmed by examining message levels for plasma cell specific transcription factors as well as the plasma cell marker CD38 by FACS analysis. Fig 2 shows qPCR data of relative levels of PAX5, BLIMP1 and XBP-1 normalized to human β -actin message. When IL-21 is present in the culture along with anti-CD40 and IL-4, the relative mRNA levels of both BLIMP1 and XBP1 transcription factors are dramatically increased at low cell densities (Fig 2B), with up to a 4-fold increase in relative levels for BLIMP1 and a 3-fold increase for XBP-1 when compared to samples grown in the absence of IL-21 (Fig 2A). In contrast, message levels for PAX5, a transcription factor that is lost upon plasma cell differentiation, decrease at lower cell densities in the presence of IL-21 (Fig 2B). In the absence of IL-21, the increasing levels of XBP1 and BLIMP1 are not observed (Fig 2A). The message levels of PAX5 were quite low in the absence of IL-21, especially at the lowest density culture but decreased in a similar manner to the IL-21 containing cultures. (Fig 2A). The results with XBP-1 and BLIMP1, both of which show progressive increases in message at lower cell concentrations, are consistent with the theory that increased plasma cell differentiation is occurring at the lower cell densities in the presence of IL-21.

Fig 3 shows FACS density plots for plasma cell marker CD38 and naïve cell marker IgD expression 9 days after culture initiation. The top panels show decreasing cell concentrations/well of cells treated with anti-CD40 and IL-4 alone, whereas the bottom panels show the same cell concentrations from cultures that also contain IL-21. In the absence of IL-21, the highest level of CD38 expression (18.6 %) is seen at the highest cell density (Fig 3 top panel). When IL-21 is present at cell densities below 40K/well, CD38+IgD⁻ expression is seen on >75% of the cells (Fig 3 bottom panel). These cells are fully differentiated plasma cells. In the anti-CD40 and IL-4 alone conditions, CD38⁻IgD⁺ expressing cells remain as well as some dual CD38+IgD+ cells. This however is not observed in the populations in which IL-21 is present. Indeed, as shown earlier [9] in the presence of IL-21, proliferation has occurred to a greater extent at all input cell concentrations. This increase in proliferation was seen at least through day 9 (data not shown). Based on this finding, we anticipated that viability of the IL-21 containing cultures would be reduced, and Fig. 4 show the percent viability at the different cell concentrations of a day 9 tonsillar B cell culture and the decreased viability in the presence of IL-21 is shown. Thus, less live cells are recovered at this culture time point. While only day 9 data is shown, this increased differentiation is evident as early as day 4 (data not shown), indicating that the changes are not due to the differences in cell recovery.

We previously demonstrated that IgE production was enhanced especially in low density cultures when IL-21 was present [9]. In order to determine the kinetics of the IgE production, supernatants were collected after 6, 8, 10 and 12 days of culture. In the presence of IL-21, and especially after day 6, the enhancement of IgE production at the low cell densities was clearly evident (data not shown). Given that plasma cell markers BLIMP1 and XBP-1, as identified by qPCR (Fig 2) and cell surface CD38 expression (Fig 3) were both the highest at the examined

low cell densities, this indicates an association between high level IgE production and plasma cell differentiation.

IL-21 does not overcome suppression of plasma cell differentiation by BCR stimulation

Recently, Ettinger *et.al* [8] reported that IL-21 induced plasma cell differentiation and IgG and IgM production were enhanced by co-ligation of CD40 and the BCR; however, in this publication, IgE production was not studied. In addition, this differentiation was suppressed by inclusion of IL-4. Since IL-4 is necessary for IgE production, we examined the effect of the co-ligation as a function of cell concentration on plasma cell differentiation and IgE and IgG4 production. We chose to focus on XBP1 as this transcription factor yielded the most dramatic results when directly comparing the absence or presence of IL-21. Fig 5 shows relative mRNA levels for XBP-1 under co-ligation conditions containing anti-CD40, IL-4 +/- IL-21, +/- anti-IgM. In the presence of IL-21, decreased cell concentrations continue to enhance XBP1 message levels (Fig 5B); however, the induction is significantly decreased in the presence of co-ligation of the BCR. This holds true for samples grown in the absence of IL-21 as well (Fig 5A). Despite the fact the XBP1 levels fail to reach comparable levels in the absence of IL-21, the same marked reduction in XBP1 levels are seen in the presence of BCR co-ligation (Fig 5A).

A similar conclusion is derived from FACS analysis of CD38 vs. IgD expression under co-ligation conditions analyzed on day nine post culture initiation. In the absence of IL-21, the cells show primarily IgD expression with a modest co-expression of CD38 and IgD seen at the highest cell concentrations (Fig 6 top panel). The CD38 expression is reduced in intensity as well as percentage when compared to CD40 ligation alone (Fig 3), examined in a similar time frame. Essentially, no CD38⁺IgD⁻ (mature plasma) cells are seen under co-ligation conditions in the absence of IL-21, and cell recovery was insufficient for analysis below 5k/well initial concentrations. When IL-21 was included in the culture, greatly increased dual expression of IgD and CD38 is observed, and at lower cell concentrations CD38⁺IgD⁻ cells are seen with a maximum level of % at 5k/well (Fig 6 lower panel). Thus, in the presence of IL-4, plasma cell differentiation is still seen at the lower cell densities, albeit at a much lower level than seen in the absence of BCR co-ligation (Fig 3).

In parallel cultures, after 12 days of culture, IgE and IgG4 levels were compared. Minimal IgE was seen in dual ligated cultures without inclusion of IL-21 (data not shown), however, significant levels were observed in the presence of IL-21 (Fig 7A). BCR ligation strongly suppressed IL-21 induced IgE production, and the IgE levels were similar to those seen in cultures that were stimulated with anti-CD40/IL-4 alone. When IgG4 levels were analyzed in the same cultures (Fig 7B), the peak production of IgG4 shifted to a higher starting cell concentration, but quite similar levels of IgG4 were seen at the optimal cell densities for both cultures. Note that the lower cell densities where CD38⁺IgD⁻ cells were seen (Fig 6), this correlates with optimal IgG4 production occurred with CD40 ligation alone.

Dual ligation slows cell proliferation

We had previously shown that IgE production with human B cells required high levels of cell division [9] analogous to findings with mouse IgE production [14] and human IgG production [13]. In order to determine the effect of simultaneous ligation of CD40 and BCR on cell division, CFSE labeled B cells were cultured with anti-CD40 and IL-4 +/- IL-21 in the presence of absence of anti-IgM. We consistently observed fewer divisions for the cultures containing anti-IgM. Fig 8 shows a representative histograms where cell division is compared at day 3 (Fig 8A) and day 6 (Fig 8B). Cultures containing anti-IgM exhibited less division. In a repeat experiment of similar design, we examined cell division at an initial cell input of 2.5, 10 and 75 ($\times 10^4$) cells per well and saw a similar effect on cell division (data not shown) namely that

the cultures containing the anti-IgM showed at least one division less at all concentrations examined. While the difference was small (approximately one less division), the decreased division was consistently seen.

Discussion

Since its cloning and identification in 2000 [17], IL-21 has been studied primarily for its capacity to enhance NK activity and promote tumor immunity as well as promote B cell differentiation, the latter primarily in the human system (reviewed in [18]). This promotion of differentiation is proposed to work primarily by enhancing differentiation to plasma cells, resulting in high level immunoglobulin production. Although initial studies concentrated on IgG, other antibody classes, including IgE, are also enhanced [8;10]. While the mechanism for IL-21 action is not completely clear, the observation that IL-21 induces BLIMP1 and CD38 expression [8], molecules both known to be associated with plasma cells, indicates that a primary mechanism is induction of plasma cell differentiation. Action as a switch factor has also been proposed for human IgG1 and IgG3 [10]. These findings have lead to proposals that promotion of IL-21 production could be used to treat humoral immunity compromised individuals and inhibition of the same to treat autoimmune disease.

As a major interest of this laboratory relates to mechanisms controlling IgE synthesis, the initial findings that IL-21 enhanced human IgE production [11;19], suggested its usefulness in this arena. However, two arguments against such control were the findings in the mouse system that IL-21 deficiency resulted in enhanced IgE synthesis [20;21] and the finding that in the human system, IL-4 inhibited the IL-21 induced plasma cell differentiation [8]. The Hodgkin laboratory had reported that mouse IgE, IgG1 [14], and human IgG [22] production and plasma cell differentiation correlated with cell division, which helps explain the inverse correlation between initial B cell plating concentrations and IgE production that was observed for mouse B cells activated with IL-4 and CD40L [12]. The availability of IL-21 allowed a similar study to be performed with human B cells, and we recently reported a similar inverse correlation with human IgE production, induced by anti-CD40 and IL-4 in the presence of IL-21 [9]. Indeed microgram quantities of IgE were produced with as little as 5000 purified B cell initial input. IL-10 further enhanced IgE production in an additive manner [9].

Given the above mentioned inhibitory effect that IL-4 has on IL-21 induction of plasma cell differentiation [8], we explored the mechanism of the IgE induction further in the present report. Fig 1 demonstrates that plasma cell differentiation, at least as exemplified by ELISPOT assay, is enhanced at the same low cell inputs where maximum IgE production is found. The finding that increased spot count as well as spot size was occurring indicates that both more frequent IgE producing plasma cells as well increased production per cell is induced by IL-21. Both the qPCR and FACS analysis also confirmed plasma cell differentiation was most efficient at low cell densities. Both BLIMP1 and XBP1 message levels increased in the presence of IL-21 at the densities where maximum IgE production occurs. Markers of plasma cell production, such as loss of IgD expression and high level CD38 expression were found at day 9 post culture initiation, the last day where significant levels of live cells were available for FACS analysis. Thus, low cell densities clearly allow IL-21 to promote plasma cell production even in the presence of IL-4 and efficient switching and production of IgE are seen.

Ettinger *et al* [8] reported increased plasma cell differentiation when the BCR was stimulated with anti-IgM in cultures containing anti-CD40 and IL-21, whereas we observed excellent IgE production in the absence of BCR stimulation [9]. Increased proliferation, increased CD38 expression, and increased IgG and IgM production were all seen. These experiments were all done at a single high cell input concentration (10^6 cells/ml) and while switching to IgG subclasses was analyzed, IgE production, indicated as data not shown, was reported to not

change under co-ligation conditions [8]. In order to determine the effect of dual ligation of the BCR and CD40 on IgE production at various cell densities, we added 10 µg/ml anti-IgM to cultures and examined plasma cell differentiation and IgE and IgG4 production. Addition of anti-IgM modulated the low density effect in that less plasma cell differentiation was seen, whether measured by XBP1 levels or CD38 expression (Figs 5 and 6 respectively). The effect on IgE production was substantial in that peak IgE levels were reduced by about 90%. In contrast, when IgG4 production was examined, the peak cell density at which maximum levels of IgG4 were obtained shifted towards a higher density (as also seen with IgE), but similar levels of IgG4 were produced. Final stage plasma cells no longer divide; however, the number of divisions before the plasma cell stage is reached influences the isotype produced. Thus, multiple divisions were found to give increased IgE switching in both mice [14] and humans [8]. We used CFSE to examine cell division numbers in both BCR/anti-CD40 and anti-CD40 alone cultures and found a consistent lag in cell divisions when anti-IgM was present. Since IgG production requires fewer divisions, this may help explain the differential effects on IgE vs. IgG4 production in the cultures (Fig 7). These results are also suggestive in explaining why relatively high polyclonal IgE responses exist such as those seen using *in vitro* stimulation of B cells with CD40L/anti-CD40 and IL-4, when levels of IgE and other isotypes produced are similar [8;12]. In addition, *in vivo* parasite infections also result in a relatively high polyclonal response, while the antigen specific IgE response (where BCR stimulation would occur) to either the parasite or to allergens is generally much lower.

As mentioned in the introduction, the profound influence of IL-21 on the human B cell stimulation and differentiation using *in vitro* models has led to suggestions that IL-21 antagonists could be useful in selective autoimmune diseases and conversely agents that promote IL-21 production could be useful in humoral immune deficiencies. Given the multiplicity of targets for IL-21, it is difficult to predict whether such strategies would indeed be useful. The recent finding that IL-21 promotes IgE production in purified B cell cultures stimulated with anti-CD40 and IL-4, yet inhibits IgE production in PMBC cultures stimulated in a similar manner exemplifies the confounding evidence for clinical use of IL-21. The inhibitory effect was associated with the production of IFN γ [23] which was induced by the activity of IL-21 on T and NK cells. In addition, evidence has been recently reported that NKT cells when stimulated with BCG produce IL-21 and induce apoptosis of IgE switched cells [24]. These findings emphasize the difficulty in predicting activities of a multiple target cytokine (which is true of most cytokines) when examining its activity in purified cell systems. However, plasma cell differentiation primarily occurs in germinal centers, not PMBC, and the environment of the GCs may be much more conducive to IL-21 induced plasma cell differentiation, suggesting that IL-21 production by the T cells found in GCs would have the strongest effect on B cell differentiation.

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Abbreviations used in this paper

CFSE

carboxyfluorescein diacetate succinimidyl ester

Fig 1. ELISPOT analysis of IL-21 containing and control cultures

Purified tonsilar B cells were cultured for 8 days in CRPMI-10 containing IL-4 and anti-CD40 +/- IL-21 (25 ng/ml). IgE positive spots and spot size were determined as indicated in methods. Shown are histograms in which the number of spots at a particular cell concentration (input well cell numbers were 1175 (●), 2350 (○), 4700 (▲) cells per well in the presence of IL-21 and 3.75×10^4 cells (□) per well in the absence of IL-21. All values have been corrected for spot number seen in blank wells. Error bars represent ± 1 SE of triplicate samples and experiment is representative of two of similar design.

Fig 2. Inclusion of IL-21 causes enhancement of BLIMP1 and XBP1 mRNA at low cell densities
 RNA was isolated from day 9 cultures of tonsillar B cells activated with anti-CD40 and IL-4 (10 ng/ml)+/- IL-21 (25 ng/ml). Levels of XBP1 (open), BLIMP1 (hatched), and PAX5 (filled) message were determined by qPCR. Levels are normalized to human Beta actin message. Initial cell input numbers (in thousands) are indicated on the graph. A vs B is the absence or presence of IL-21 respectively. Assay was done in triplicate and error bars represent 1 SD of mean. * represents $p < 0.05$ when lower density samples of each respective transcription factor were compared to samples containing 75K cells/well.

Fig 3. FACS analysis of plasma cell differentiation in the presence or absence of IL-21

Eight days after culture initiation, tonsillar B cells activated with anti-CD40/IL-4 alone (rows 1–2) or with 25 ng/ml IL-21 (rows 3–4) were collected and analyzed for CD38 and IgD surface expression. Percent in respective quadrants is shown in left corner and initial cell input in right corner. Cells were chosen for analysis based upon PI exclusion. Figure representative of four of similar design.

Fig 4. Culture viability on day 9 ± IL-21

Purified B cell cultures were setup from two tonsils in the presence of IL-4 and anti-CD40 alone (●) or the same plus IL-21 (25 ng/ml) (○). On day 9 cells were collected and viability determined by Trypan Blue staining. Results are averages of the two cultures set up in duplicate ±1 SE.

Fig 5. BCR ligation reduces IL-21 enhanced XBP1 transcription

RNA was isolated from day 9 cultures of tonsillar B cells activated with anti-CD40, IL-4, +/- 25 ng/ml IL-21 +/- anti-IgM (10µg/ml). Levels of XBP1 message were determined by qPCR. Levels are normalized to human Beta actin message. Cell concentrations are indicated on the graph. No anti IgM stimulation (filled), anti-IgM (hatched). Panel A shows cells with IL-4 and anti CD40 alone whereas panel B is in the presence of IL-21. Assay was done in triplicate. * represents $p < 0.05$ when no anti-IgM stimulation was compared to samples with BCR ligation

Fig 6. FACS analysis of plasma cell differentiation in the presence or absence of IL-21 in cultures containing both CD40 and BCR stimulation

Cultures are setup and analyzed as in Fig 3 except that anti-IgM (10 μ g/ml) was present. Top two panels depict cell in the absence of IL-21, bottom two panels contain IL-21 (25 ng/ml). Experiment was performed three times. Shown is a representative experiment.

Fig 7. Analysis of the effects of IL-21 on IgE and IgG₄ production after BCR plus CD40 ligation
 Cultures are setup as in Fig 1. In (A), IgE levels were determined on day 12 from cultures containing anti-CD40/IL-4 alone (●) or with 25 ng/ml IL-21 either in the absence (▲) or presence (□) of anti-IgM (10 µg/ml). Figure 7B shows IgG₄ levels after 12 days from cultures containing anti-CD40/IL-4 alone (●) or with IL-21 (▲) or with IL-21 and anti-IgM (□). Figure representative of three of similar design.

Fig 8. Effect of dual ligation of CD40 and BCR on cell division

Tonsilar B cells (10^4 /well) were labeled with CFSE as indicated in Materials and methods and were cultured with CRPMI-10 only (—), with CRPMI-10 containing anti-CD40/IL-4/IL-21 (25 ng/ml) alone (---) or with 10 μ g/ml anti-IgM (····). CFSE fluorescence was determined on day 3 (A) or day 6 (B) post culture initiation. Figure representative of two of similar design.