



IL-21 dependent IgE production in human and mouse in vitro culture systems is cell density and cell division dependent and is augmented by IL-10

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Received 11 August 2005; accepted 3 March 2006

Abstract

IL-21 is known to enhance immunoglobulin production using human in vitro models. Using either PBMC or purified tonsillar B cells both stimulated with anti-CD40, IL-4 ± IL-21, this enhancement was shown to correlate with increased cell division especially for IgE and to a lesser extent for IgM and total IgG. Cell division was monitored by CFSE staining and maximum cell division was found at low initial cell plating densities. A correlation between increased cell division and IL-10-mediated enhancement of IgE production was also seen; however, increased cell division plays a smaller role with IL-10 than IL-21. This is further emphasized in that when IL-10 and IL-21 were added together there was a further synergistic increase in IgE seen, but no accompanying further increase in cell division. The mouse system was also examined for IL-21 effects as a function of cell concentration, and as in humans, IL-21 added to murine cells increased IgE production over IL-4/CD40 stimulated cells at lower cell concentrations; however, IL-21 significantly reduced IgE at higher plated cell concentrations.

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Keywords: IgE; IL-21; IL-4; IL-10; Cell density; In vitro culture

1. Introduction

IgE plays an important role in allergic disease. Evolutionarily, it is thought to be the most recent of the five isotypes that are expressed in humans: IgM, IgG, IgA, IgE, and the B cell surface receptor, IgD. The primary role of IgE is in protecting against parasitic infections, which have been largely eliminated from industrialized societies. However, in the developed world, innocuous particles such as pollen or dust mite particles have become the primary triggers for the IgE response. As much as 20% of the world's population suffers from allergic disease and a higher incidence in developed societies is well known and indeed has increased two to three fold in the last 40 years [1].

For isotype switching to IgE to occur, antigen-activated IgM⁺ IgD⁺ B cells are thought to require two signals [2]; the first being provided by either IL-4 or IL-13 [3,4], the second by CD40L expressed on activated T cells [5]. In vitro, IL-10 added to IL-4/anti-CD40 treated cells has also been shown to enhance IgE production in B cells and PBMC [6]. Human in vitro IgE production studies, while somewhat variable, are usually in the picogram per milliliter to nanogram per milliliter range, requiring highly sensitive ELISAs for detection. This can complicate experiments which seek to examine agents which inhibit IgE production. Generation of an in vitro model in which larger amounts of IgE are produced would greatly assist future studies looking at ways to control IgE synthesis.

Murine studies by several investigators have demonstrated that IgE production, rather than being proportional to B cell concentration, is optimal at very low cell concentrations, in the range of 5×10^2 to 5×10^3 cells/well [7,8]. Studies by Hodgkin et al. have demonstrated that specific

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classes of antibody require multiple B cell divisions before isotype switching can occur, providing a potential explanation for the cell density effect. In the mouse *in vitro* system, a minimum of three divisions are required before IgG is observed; whereas five divisions are required for IgE [9,10]. This may be explained by the observation that mouse B cells display sequential switching first from IgM to IgG₁ and then to IgE [11,12]. Recently, it has been shown that the cell division relationship persists in the human *in vitro* model for IgG [13,14]. However, demonstration of a similar phenomenon for human IgE has been hindered by the low level of IgE that is produced *in vitro*.

In vitro IgE synthesis levels in the murine system easily reach microgram per milliliter (μg/ml) levels suggesting that additional stimuli may be needed for optimal IgE production in the human system. Given its relationship to B cell proliferation, a logical candidate would be IL-21 [15]. In view of this finding, we examined human and mouse IL-21 for its ability to enhance IgE production *in vitro*. While this work was in progress, several reports were published demonstrating that human IL-21 plus IL-4/anti-CD40 could induce class switching to IgG₁ and IgG₃, as well as enhance IgE production [16], and that IL-13 and IL-21 added to IL-4/anti-CD40 stimulated cells increased both proliferation and IgE [4]. However, these studies only examined a single concentration of cells in culture. Furthermore, the dependence of cell division on IL-21 mediated IgE production was not tested, and the IL-13 paper [4] examined B cells purified from PBMC but did not examine cell division and cell density. In this study, both human and mouse IgE production was examined as a function of cell concentration in the presence of combinations of IL-4/anti-CD40 and IL-21. IL-21 stimulation of naïve human tonsillar B cells resulted in increased production of IgE, total IgG, and IgM in a cell density-dependent manner. While mouse IL-21 did enhance B cell proliferation, IgE production was reduced by the inclusion of IL-21 at high cell densities. However, at lower cell densities, IL-21 modestly enhanced IgE production in the mouse system as well. Finally, while IL-10 addition caused an increase in IgE production, increases in cell division were modest. Addition of both IL-10 and 21 gave a synergistic increase in IgE but no further increase in cell division.

2. Materials and methods

2.1. Animals, antibodies, reagents, and cytokines

Six-eight-week-old BALB/c mice were obtained from National Cancer Institute (Frederick, MD) and housed in an accredited animal facility. All animal studies were performed according to an approved IACUC protocol. Baculovirus supernatant containing recombinant murine IL-4 was a gift from Dr. William Paul (National Institutes of Health, Bethesda, MD). Recombinant murine IL-5, recombinant murine interleukin-21, human IL-4, and huIL-21R-Fc chimera were purchased from R&D Systems (Minneapolis, MN).

Recombinant CD40 ligand trimer (CD40LT) and mouse IgG₁ anti-CD40LT clone M15 [17] were obtained from Amgen (Seattle, WA). 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. 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 (CiphaGen, Fremont, CA). Purified mAb (99%) was eluted with acetate buffer, pH 4.9, and dialyzed against PBS prior to use in culture. IL-10 (Sigma, St. Louis, MO) was brought to a working concentration of 12.5 μg/ml. Human IgE standards were purified from JW8 IgE myeloma cells (ATCC), grown in Integra CeLLine 1000 flasks (IBS Integra Biosciences, Switzerland). Collected supernatants were dialyzed three times against PBS and purified by FPLC using MEP-Hypercel. IgE elutes at pH 5.5 and was further purified (98%) by gel filtration. Goat anti-human Ig, goat anti-human and anti-mouse IgM (AP and unlabeled), goat anti-mouse IgG₁-unlabeled, goat anti-mouse IgG₁-AP and mouse anti-human IgM-HRP were obtained from Southern Biotechnologies (Birmingham, Alabama). Goat anti-human IgG-Fc-AP, human polyclonal IgG, and human IgM were obtained from Sigma. Production of biotinylated rat anti-mouse IgE mAb R1E4 and B1E3 has been previously described [7]. [³H]Thymidine (50 Ci/mMole) was purchased from NEN (Boston, MA).

2.2. Human IL-21 preparation

4×10^7 T cells were obtained by MACS purification of disrupted human tonsils and stimulated overnight with PMA (10 ng/ml) and ionomycin (2 μM). RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) and RT-PCR was performed with GTGTCCAAGTCAAG (anti-sense) and GTCTAGCTCTACTGTTGG (sense) primers (Applied Biosystems, Foster City, CA). The IL-21 cDNA was cloned into pCR2.1 (Invitrogen, CA) and confirmed by sequencing. For expression, the cDNA was inserted into pEF4My-cHisA (Invitrogen) using *Bam*HI and *Not*I and used to transfect HEK293T cells (ATCC), using Fugene6 (Roche Diagnostics Corp., Indianapolis, IN) according to manufacturer's directions. Transfected cells were cultured for 72 h and supernatants were collected and pooled. Supernatants were dialyzed against PBS and kept at -70°C prior to use.

2.3. Human B cell purification and culture

Human B cells were purified from PBMC or tonsils. PBMC were obtained from Virginia Blood Services, (Richmond, VA) as a buffy coat preparation, and leukocytes were purified by differential density centrifugation over Ficoll–Hypaque (Amersham Biosciences, Piscataway, NJ). For some experiments, tonsillar B cells were purified as described. Whole tonsils were qualified as an approved

exemption under Institutional Review Board regulations as discarded anonymous tissue. In some experiments CD19⁺ B cells were purified from PBMC. In others IgD⁺ tonsillar B cells were isolated as follows. Tonsil disruption was accomplished using a Collector Tissue Sieve (VWR International, West Chester, PA) using two wire screens, mesh sizes 0.14 mm and 0.0381 mm, respectively. PBMC or disrupted tonsils were diluted 1:2 in RPMI, layered over Ficoll–Hypaque, centrifuged at 200g for 20 min at 4°C, and washed twice with 1% FBS/PBS. The cells were then stained for 30 min at 4°C with mouse anti-human IgD-FITC (isotype I) (Pharmingen). After 2 washes, the cells were incubated for 10 min at 4°C with anti-FITC microbeads (Miltenyi Biotech), washed and re-suspended in PBS containing 1% BSA and 2 mM EDTA. Cells were then placed over a MACS LS separation column with the magnetically retained cells being the IgD⁺ or CD19⁺ fraction. Note that the monoclonal anti-IgD is not stimulatory to B cells, in contrast to polyvalent anti-IgD [18]. For the PBMC purification, CD19⁺ staining was followed by the anti-FITC-microbeads. After removal of the magnet, the retained cells were collected then centrifuged and then resuspended in B cell media 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, 50 µM 2-mercaptoethanol, and 20 mM Hepes buffer (all from Invitrogen Carlsbad, CA). FACS analysis with anti-CD19 indicated B cell purities were above 95% using this protocol. B cells were cultured in 96 well culture plates at the indicated cell densities for 96 h with last 16 h pulsed with [³H]thymidine or 12–14 days for antibody production, respectively.

2.4. Mouse B cell preparation

Resting B cells were purified as previously described [7]. Briefly, B cells were isolated from BALB/c spleens and enriched by complement lysis of T cells and further purified using by Percoll gradient centrifugation. Resting B cells were collected from the 66–70% Percoll layer, washed and placed in culture. To examine the effect of murine IL-21 (mIL-21) on cell proliferation and IgE production, B cells were plated from 0.1×10^3 to 50×10^3 cells/well in 96-well culture plates in the presence of IL-4, IL-5, CD40LT, M15 (standard activation cocktail), and with or without 25 ng/ml mIL-21 in mouse B cell media (RPMI 1640 containing 10% FCS, 50 µM 2-ME, 1 mM pyruvate and $1 \times$ non-essential amino acids) [7] for 3 or 8 days. Standard activation cocktail contained 0.1 µg/ml CD40LT, 5 ng/ml IL-5, and 10,000 U/ml mouse IL-4, and 0.1 µg/ml M15. Proliferation was measured on day three by [³H]thymidine incorporation (1 µCi/well) during the last 8 h of culture and antibody secretion was determined by ELISA after 8 days of culture. Stimulations were conducted in triplicate.

2.5. ELISA protocols

Human IgE ELISA utilized the monoclonal anti-human IgE clones 4.15 and 7.12 (HB 235 and 236 from ATCC) as capture antibodies [19], which were plated in combination at 5 µg/ml in 0.05 M 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 RPMI media containing 10% FBS. The lower limit of linear detection was ~5 ng/ml. Total human IgG was detected by coating plates with 3 µg/ml goat anti-human IgG (H + L) in borate-buffered saline (BBS). Capture antibodies were goat anti-human IgG-Fc-AP, diluted in Tris buffer/0.5% bovine serum. Standard curves were generated from polyclonal human IgG. Human IgM ELISA utilized goat anti-human IgM (Sigma) as capture at 5 µg/ml for coating and samples and standards were detected using mouse anti-human IgM-HRP and detected using HRP substrates (51-2607KC and 51-2606KC BD Pharmingen). Standard curve was generated using polyclonal human IgM.

Mouse IgE, IgG₁, and IgM production was measured by ELISA as described previously [7]. Briefly, mouse IgE ELISA utilized biotinylated rat anti-mouse IgE mab B1E3 and R1E4 [20], while IgG₁ and IgM utilized goat polyclonal reagents from Southern Biotech. Color was generated by addition of AP substrates (S0942-Sigma). Plates were read on a Molecular Devices SpectraMax 250 ELISA plate reader at either 405 nm for alkaline phosphatase (AP), or 450 nm for horseradish peroxidase (HRP) detection, respectively.

ELISA for determining IL-21 content in supernatants were performed using 1 µg/ml rabbit anti-human IL-21 (aa 121–135) in BBS (QED, San Diego, CA) as coat. Blocking used 10% FBS/PBS for 30 min at 37°C. IL-21 (BioSource, Camarillo, CA) was used for standard curve and supernatant dilutions were analyzed. IL-21 was detected using 1:200 chicken anti-human IL-21 (Genway, San Diego, CA). Color was generated using 1:1000 rabbit anti-chicken IgY HRP conjugate (Upstate, Lake Placid, NY) and TMB substrate. 293HEK transfected IL-21 containing supernatants contained 2.5 ng IL-21 per microliter supernatant.

2.6. Analysis of cell division

Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes Eugene, OR) was prepared according to the manufacturer's recommendations. Resting B cells were washed and resuspended at 10^7 cells/ml in PBS/0.1% BSA. CFSE was then added to final concentration of 5 µM (1 µM for mouse studies) and incubated in the dark at 37°C for 10 min. After washing twice, cells were resuspended in B cell media. CFSE-labeled human B cells were plated at the indicated density and cultured for 8 days with 10 ng/ml IL-4 alone, 1000 ng/ml anti-CD40, or IL-4, and anti-CD40. Murine CFSE-labeled cells were plated ± IL-21 at the indicated cell density and cultured for 4 days with IL-4 alone or IL-4, IL-5,

CD40LT, and M15. Post culture cells were analyzed by flow cytometry using a FC500 (Beckman-Coulter, Miami, FL). 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.

2.7. Data analysis

Data are summarized as mean $\pm$ standard deviation (SD). The statistical analysis of the results was performed by the paired student *t* test. A *p* value of <0.05 was considered significant.

3. Results

3.1. Enhancement of B cell proliferation by IL-21

Human IL-21 was cloned by RT-PCR, and supernatants from transfected HEK293T cells were used as the source of IL-21; the amount of IL-21 in the supernatant was deter-

mined by ELISA. As can be seen in Fig. 1A, addition of increasing levels of IL-21 (from transfected supernatant) resulted in increased B cell proliferation, when used in conjunction with anti-CD40 and IL-4. No proliferation was induced by HEK293T control supernatant alone (data not shown), while B cell proliferation was increased 13 fold by the inclusion of IL-21, compared to IL-4/anti-CD40 (Fig. 1B). We determined that the increase was due to IL-21 contained in the supernatant, as the addition of soluble IL-21R-chimera abrogated the effect in a dose dependant manner, and that the supernatant alone had no effect. In addition, the presence of IL-21 caused massive aggregation of the growing B cells in culture (Figs. 1C–E).

3.2. Enhancement of immunoglobulin production is seen at low cell densities

To determine the effect of IL-21 on antibody production in the human system, IgD⁺ tonsillar B cells were purified ($>98\%$) and cultured at increasing cell densities in the

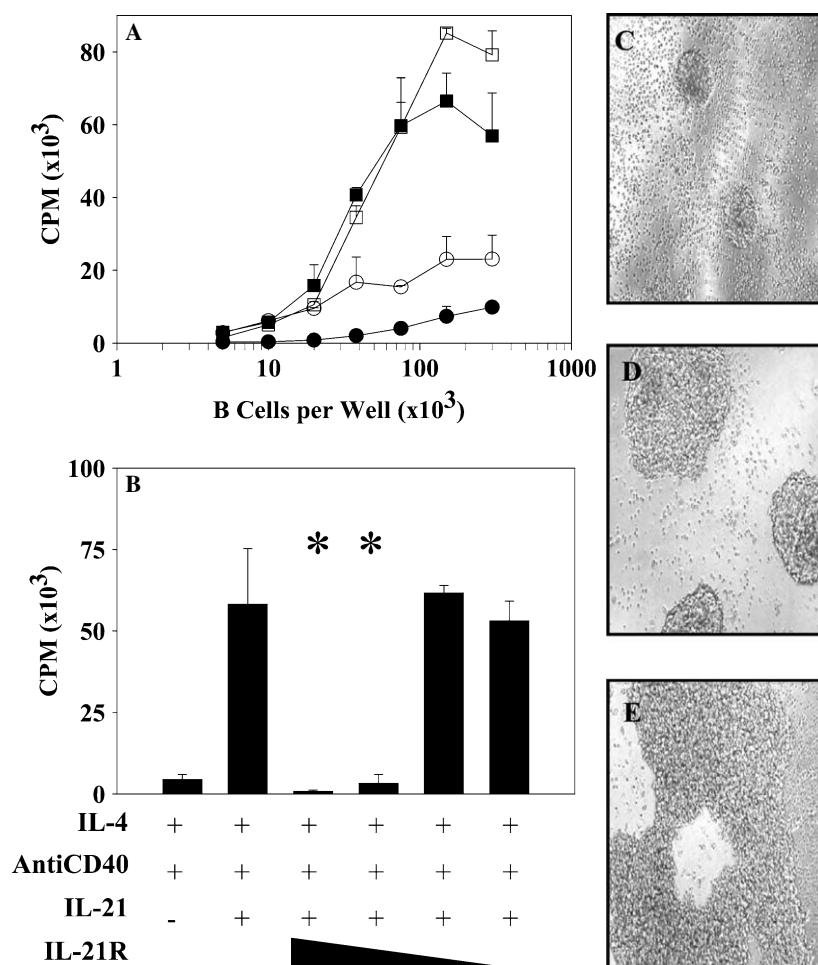


Fig. 1. IL-21 increased human B cell proliferation in a dose-dependent manner. (A) Purified human B cells from PBMC were cultured with IL-4 (10 ng/ml) and anti-CD40 (10³ ng/ml) for 96 h in the presence of IL-21 supernatant (25 ng/ml (□), 2.5 ng/ml (■), 0.25 ng/ml (○), or no IL-21 (●)). Cells were pulsed with 1 μ Ci [³H]thymidine/well for the last 16 h of a 96 h culture. (B) Purified IgD⁺ B cells were cultured and pulsed as in (A). Receptor-Fc chimera was added where indicated at 50, 10, 1, and 0.1 μ g/ml, respectively. Error bars represent one standard deviation (SD) (**p* < 0.02). Experimental results representative of two of similar design. (C–E) Ten times magnification a 5 day culture of IL-4/anti-CD40-activated CD19⁺ B cells purified from PBMC. Cultures were treated with 2.5, 25, and 125 ng/ml IL-21 supernatant, respectively.

presence of anti-CD40 and IL-4 in the absence or presence of increasing concentrations of IL-21. Analysis of IgE levels in supernatants after 14 days of culture revealed that the inclusion of IL-21 resulted in increased IgE production (Fig. 2). Note that the highest level of IgE was obtained at 3000 cells per well in the additional presence of IL-21, one quarter of the cell number that produces maximum IgE when cells are cultured in IL-4/anti-CD40 alone. The inset shows an expanded plot of the IgE production seen in the absence of IL-21. It is shown to emphasize the dependence on cell concentration and the differences when IL-21 is present. The maximum production of IgE occurred at a higher cell density in the absence of IL-21. Controls in which cells were stimulated with IL-4 alone, or IL-4 plus IL-21 only, produced no IgE, indicating that preformed IgE did not contribute to detected IgE levels (data not shown). Note that similar Ig production and proliferation enhancement is seen when B cells are isolated by negative selection (data not shown) confirming that the monoclonal anti-IgD is not influencing B cell activity in the assays.

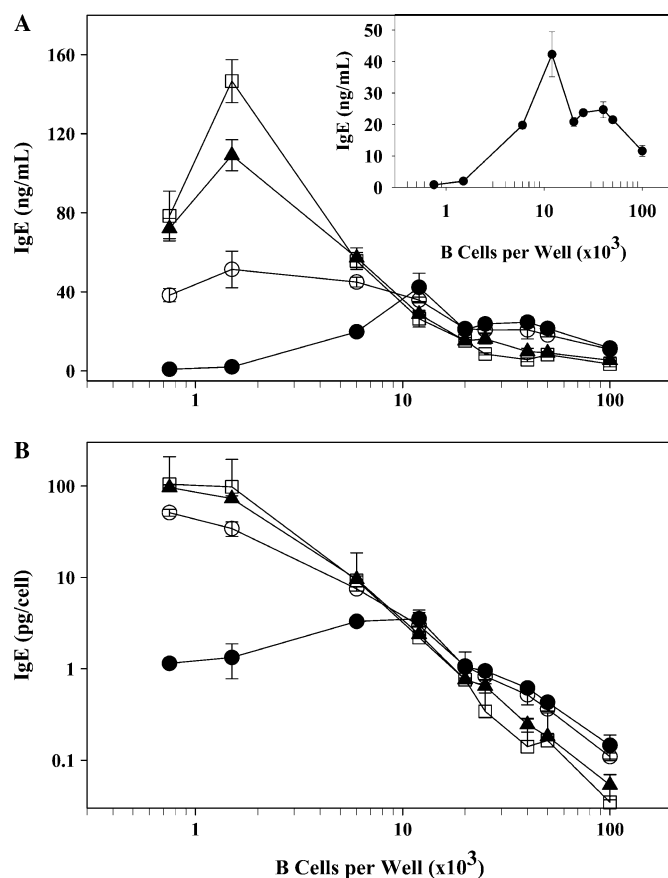


Fig. 2. IL-21 significantly increases IgE production in purified human IgD⁺ B cells. (A) The indicated concentrations/well of IgD⁺ B cells were stimulated with IL-4 and anti-CD40 in the presence of 25 ng/ml (□), 10 ng/ml (▲), 2.5 ng/ml (○), or no IL-21 (●). IgE was measured by ELISA on 12 day supernatants. Data are represented as total IgE production (A) or IgE production/cell (B). (Inset) Enlargement of IL-4/anti-CD40 only conditions, showing optimal IgE in absence of IL-21 is also cell concentration dependent. Error bars represent $\pm$ one SD. Experiment results representative of four of similar design.

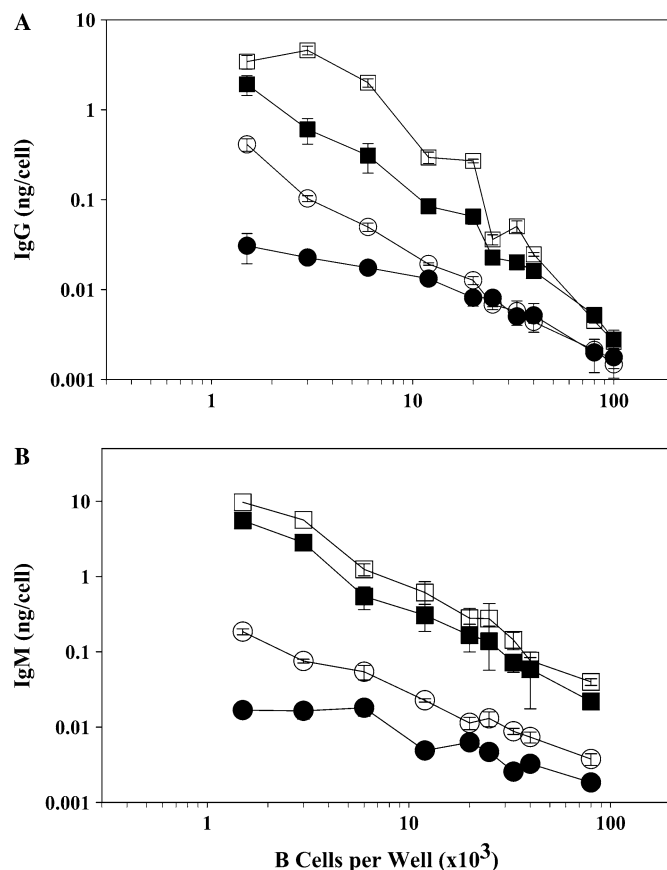


Fig. 3. IL-21 increases IgG and IgM production in human in vitro cultures. IgD⁺ B cells were stimulated with IL-4 and anti-CD40 in the presence of 25 ng/ml (□), 2.5 ng/ml (■), 0.25 ng/ml (○), or no IL-21 (●). Day 12 supernatants were collected and the concentration of secreted IgG (A) or IgM (B) was determined by ELISA and plotted as amount produced/cell. Error bars represent one SD. Experiment representative of three of similar design.

To emphasize the dramatic increase IgE at low cell concentrations, this data is redisplayed as a function IgE produced per input B cell in the presence of IL-21 (Fig. 2B). In addition to IgE, production of other isotypes was also influenced by the inclusion of IL-21 in the culture. Fig. 3A shows production of IgG and Fig. 3B IgM in the presence and absence of IL-21 again, as a function of cell density. The data are plotted as the Ig produced per B cell initially plated, and the increased production of both total IgG and IgM is evident. Thus, the presence of IL-21 resulted in increased immunoglobulin production at low input cell concentrations for all isotypes tested. Thus, in the human in vitro culture system, IgE, as well as IgG and IgM production is cell density dependent and production is enhanced by the presence of IL-21.

3.3. Nutrient depletion does not explain differences in IgE production

As can be seen in Fig. 4A, cells that were treated with IL-4, anti-CD40, and 25 ng/ml IL-21 had the highest level of

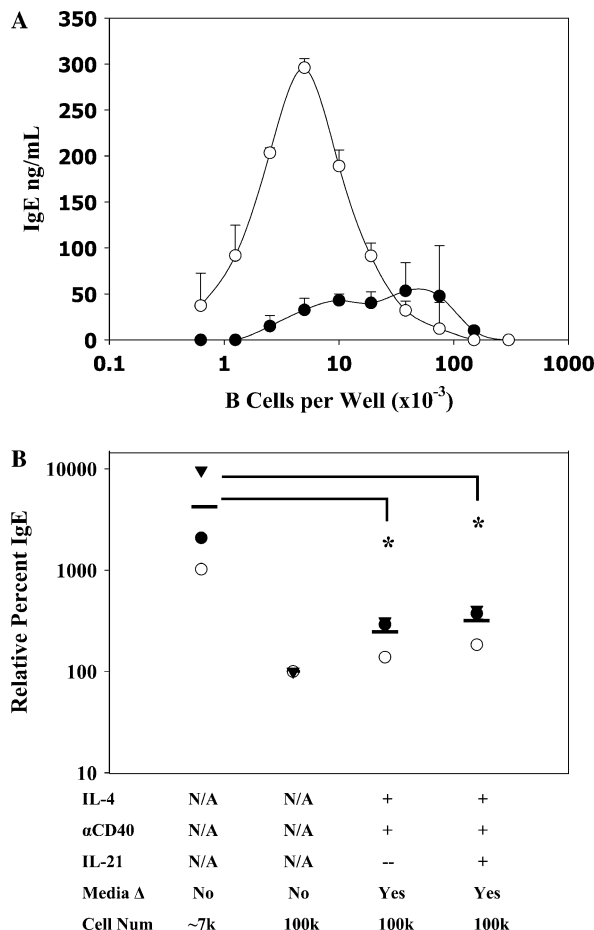


Fig. 4. Media and cytokine replacement does not explain cell density effect. (A) Tonsilar B cells were cultured with IL-4/anti-CD40 alone (●) or the same plus 25 ng/ml IL-21 (○) were plated the indicated cell concentrations and IgE levels determined after day 12. Shown is a representative experiment of 3 performed. (B) Wells plated at 100 K/well with IL-4/anti-CD40/IL-21 were untreated or had one half (100 μ L) media replaced; activation agents were also added as indicated and after day 12 IgE levels determined. Shown represents an average of 3 experiments done in same manner. * $p = 0.002$.

IgE at initial cell plating of 5k/well, while IL-4-anti-CD40 alone had max IgE produced at 75k/well. In view of the increased cell numbers (75k vs. 5k) and accompanying proliferation as seen on day 4 (Fig. 1), we explored whether the Ig differences seen were merely due to nutrient depletion. Since high cell number per well exhibited high levels of proliferation on day 4 (Fig. 1), on day 5 these wells had 100 μ L media replaced either with IL-4/anti-CD40 alone or with IL-21. As can be seen in Fig. 4B, the replacement of media and nutrients did result in a modest increase the levels of IgE produced but the levels remained *ca* 1 log₁₀ less than seen at the optimal cell concentration in the presence of IL-21. In addition, adding increased levels of anti-CD40 and/or IL-4 did not further increase IgE production (data not shown), indicating that these reagents were not limiting. Thus, these results argue against media depletion being exclusively the explanation for the IgE production shift to lower cell densities.

3.4. Increased cell division occurs at low cell inputs in the human in vitro system

Given the correlation between cell division and isotype-specific antibody production, we investigated the contribution of IL-21 to cell division as a function of initial cell concentrations. Cell division was assessed using CFSE-labeled human B cells activated with anti-CD40 and IL-4 in the presence or absence of IL-21 (Fig. 5). Because discrete peaks at each cell division were not clearly evident, estimated cell divisions were determined by dividing the histograms into equally spaced intervals, with the brightest peak (far right) representing cells which had not divided. Increased rounds of cell division, as evidenced by reduced CFSE concentration, were seen when starting cultures had a lower cell input (Compare Figs. 5A–C). These data were used to calculate the proportion of total cells in each round of cell division by partitioning the histogram as described above (Figs. 5B and D). The increase in cell division with IL-21 addition was clearly seen, with dramatically higher cell division evident at lower initial cell inputs (Fig. 5C, solid line). In addition, inclusion of IL-21 resulted in a higher percentage of cells that underwent at least one division, as seen by the lower percentage of cells remaining in an undivided state (Fig. 5D). Culture conditions that exhibited the highest levels of cell division correlated with highest IgE production (Fig. 2), consistent with previous data for IgG and IgE in the mouse [9] and total IgG in the human systems [13].

3.5. IL-21 increases IgE production by IL-10/IL-4/anti-CD40 stimulated PBMC and purified Tonsilar IgD⁺ cells

IL-10 has been shown to increase in vitro IgE production in IL-4 and anti-CD40 stimulated PBMC [21]. We sought to determine whether IL-21 further enhances the amount of IgE produced in cells treated with IL-4/IL-10/anti-CD40 cells. PBMC were treated with IL-4/anti-CD40 plus IL-10. As seen in Fig. 6A, the addition of both IL-10 and IL-21 to IL-4/anti-CD40 treated cells shows higher proliferation than when only IL-21 or IL-10 alone is added. As shown in Figs. 6B and C, IL-10 resulted in an increase in IgE production, with the higher amount of IL-10 (10 ng/ml) (Fig. 6C) giving a 4-fold increase in IgE production, compared to IL-4/anti-CD40 alone. Indeed at this concentration IL-10 was more effective than IL-21 alone in inducing IgE production. The addition of both cytokines further increased IgE production in an additive manner. However, when the lower (1 ng/ml) of IL-10 were used (Fig. 6B), the addition of both cytokines increased IgE production synergistically. To determine whether synergy or addition would be found in B cells alone, purified tonsilar B cells were cultured with IL-4/anti-CD40 in the presence or absence of IL-10 and IL-21. As seen in Fig. 6D, IL-21 had a greater effect on IgE production compared to IL-10. As seen with PBMC, IL-10, and IL-21 added together to IL-4/anti-CD40

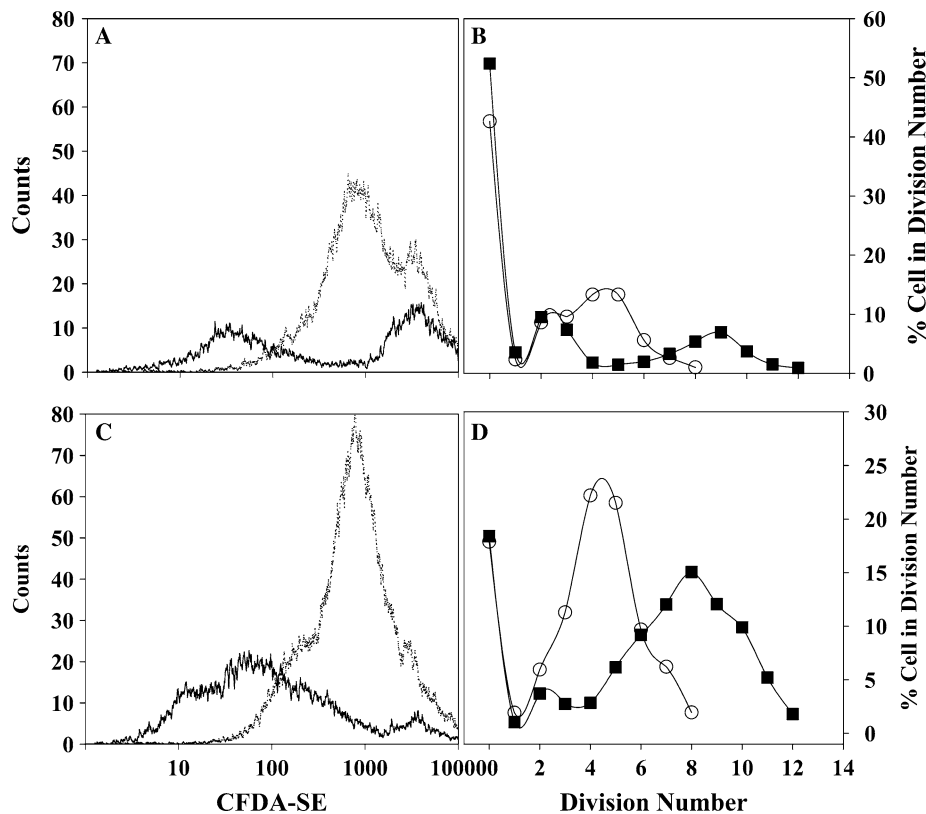


Fig. 5. Cell division is correlated to cell density. Human tonsillar IgD⁺B cells were labeled with CFSE/PBS for 15 min, washed, and cultured for 8 days with IL-4/anti-CD40, without (A and B) or with (C and D) 25 ng/ml IL-21. (A and C) Flow histograms of cells plated at 10^5 (.....) or 10^3 (—) cells/well. (B and D) Show same data plotted as a function of cell division (see Section 2), again with 10^5 (○) and 10^3 (■) cells/well. This experiment is representative of four of similar design.

cells resulted in a synergistic increase in IgE. The levels of IgE produced by IL-4/anti-CD40 alone were 2 ng/ml.

3.6. IL-10 increases IgE production in purified tonsillar B cells and caused a modest increase in cell division

Purified tonsillar B cells were labeled with CFSE and activated with IL-4/anti-CD40 in the presence of IL-10. Similar to the studies with IL-21 (Figs. 7C and D), IL-10 addition also showed IgE production correlated with cell division, albeit to a lesser degree (Figs. 7A and B). When both IL-10 and IL-21 were added together to IL-4/anti-CD40 stimulated cells there was a further increase in total IgE production as seen in Fig. 7E.

To determine the generality of this finding, seven tonsils were collected and IgD⁺ cells were purified and treated with IL-4/anti-CD40 in the presence or absence of IL-10 and 21, added together or separately. IgE production is shown in Fig. 7E. In six of the tonsils there was an increase of IgE over IL-4/anti-CD40 alone when 10 ng/ml IL-10 was added. In one case, IgE production with IL-4/anti-CD40 ± IL-10 was below levels of detection. Interestingly, in that tonsil, the production of IgE was very good of IL-21 alone (145 ng/ml) and IL-10 + IL-21 (340 ng/ml) to IL-4/anti-CD40 stimulated cells. The average IgE production is shown and a significant increase is seen with IL-10/21 as

compared to IL-21 alone; note in general the increase is synergistic rather than additive. With regard to cell division, the highest level of IL-21 resulted in a mean fluorescent intensity (MFI) to around 60; addition of IL-10 did not result in a further decrease in MFI. Thus, the increase in IgE production seen with the double cytokine addition did not correlate with further cell division (Fig. 7F).

3.7. Mouse B cell proliferation is also enhanced by IL-21, but IgE production is inhibited at the peak production values

Current literature indicates that IL-21 is inhibitory to IgE production in the mouse, while stimulatory in humans [4,22], however the relationship of mouse IL-21 and cell density has not been examined. To better compare the human and mouse system, purified mouse B cells were cultured with CD40LT, IL-5, IL-4, and M15 (anti-CD40LT) in the presence or absence of mouse IL-21. As was seen with the human system [15], the addition of mouse IL-21 to activated B cell cultures, also resulted in increased B cell proliferation (data not shown). Analysis of antibody production in response to different initial B cell input indicated that IgE production was influenced in a biphasic manner by the inclusion of IL-21 in the cultures (Fig. 8A). At high cell concentrations, IL-21 inhibited IgE production; however, modest enhancement was seen at low cell inputs. While

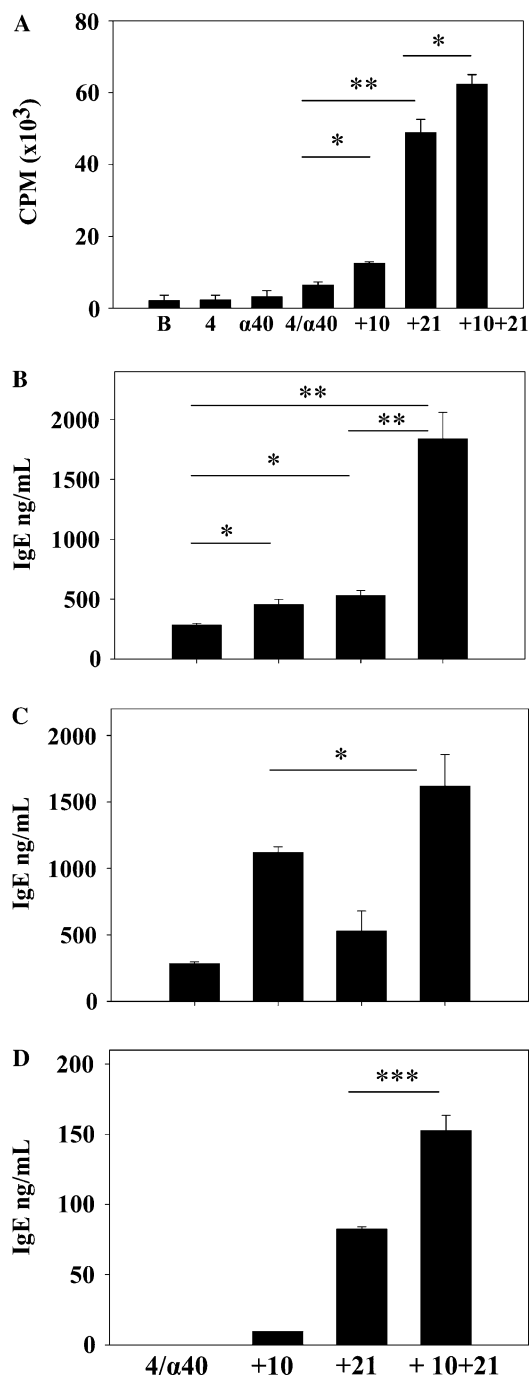


Fig. 6. IL-10 and IL-21 increase proliferation in PBMC, and increase IgE when added alone and synergistically increase IgE when combined with PBMC or IgD⁺ tonsillar B cells. IL-4 and anti-CD40 (4/α40) treated PBMC were plated at 300,000 cells per well and treated with buffer, IL-10, IL-21, or IL-10 and IL-21 combined. Twenty-five nanograms per milliliter IL-21 was used in all conditions. (A) Proliferation was determined by ³H-thymidine incorporation. (B and C) IgE was determined from PBMC activated as in (A); with IL-10 concentrations of (B) 1 ng/ml or (C) 10 ng/ml, respectively, with IL-21 added as indicated. (D) IgE production from 75k cells per well IL-4/anti-CD40 stimulated IgD⁺ tonsillar B cells that were treated with IL-10 (10 ng/ml), IL-21 (25 ng/ml), or IL-10, and IL-21 combined was determined. In (B–D), IgE levels were determined by ELISA from day 14 supernatants. Cultures were plated in duplicate, collected on day 14, pooled and determined by ELISA. These two results are representative of seven experiments. Statistics were paired Student's *t* test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

mouse IgG₁ and IgM production were not inhibited by the addition of IL-21 at any concentration, the bulk of IgG₁ production was shifted towards lower cell density (Fig. 8B). Mouse IL-21 had minimal effect on the cell concentration dependence for IgM production (Fig. 8C).

To determine whether changes in cell division explained the inhibitory effects of IL-21, CFSE-labeled mouse B cells were incubated with CD40LT, IL-4, and IL-5 ± IL-21. Since mouse cell activation occurs more rapidly than human, after 4 days of culture, cell division numbers were examined by flow cytometric analysis. As with the human system, addition of IL-21 resulted in increased cell division as indicated by the peak at decreased CFSE intensity. Thus, at least at these low cell inputs (500 cells/well) mouse IL-21 addition caused increased cell division, analogous to what was seen in the human B cell cultures Fig. 9.

To directly compare the mouse and human in vitro systems, we analyzed the inhibition or enhancement of IgE production as a function of the B cells per well due to the addition of IL-21 relative to IL-4/anti-CD40 conditions alone. This analysis is shown in Fig. 10 and this figure clearly shows that both the human and mouse systems exhibit a similar activity in the presence of IL-21. To show the variability that is seen, two experiments are plotted in this manner. Note that the primary difference is that the curve is shifted towards lower cell concentrations in the mouse system, while both systems exhibit enhancement of IgE at low and inhibition at high cell densities, respectively. The mean cell number of the mouse system where an the maximum increase of IgE production due to IL-21 was in the hundreds of cells per well (mean = 625 cells per well, 2 experiments) The human system also displayed a similar trend of IgE production as was found in the mouse system, but the cell density where that was found was considerably higher. The average of six experiments was 47k cells per well ± 25k. Thus, while there is more donor variability in the human system, perhaps related to the condition of the tonsillar cells, almost 2 log₁₀ higher cell numbers give maximum IgE compared to the mouse system.

4. Discussion

One strategy to reduce allergic symptoms is to block the IgE from binding to the FcεRI by using anti-IgE antibodies [23,24]. This has been found to be clinically effective and is currently an available treatment. Since the anti-IgE therapy does not reduce IgE synthesis [24], there must be near total blockage of the IgE binding, since granule release requires cross linking of as few as ten basophil- or mast cell-bound IgE molecules. Another approach is to try to limit IgE synthesis and this necessitates a good understanding of the mechanisms involved in the isotype switch to this antibody class.

In vitro IgE studies in the human system have been hampered by the low production levels. In the mouse in vitro system IgE production is in the microgram per milliliter, whereas in the human, IgE levels are usually in the upper picogram to nanogram levels per ml. Therefore we sought to determine a set of conditions that could be used to induce

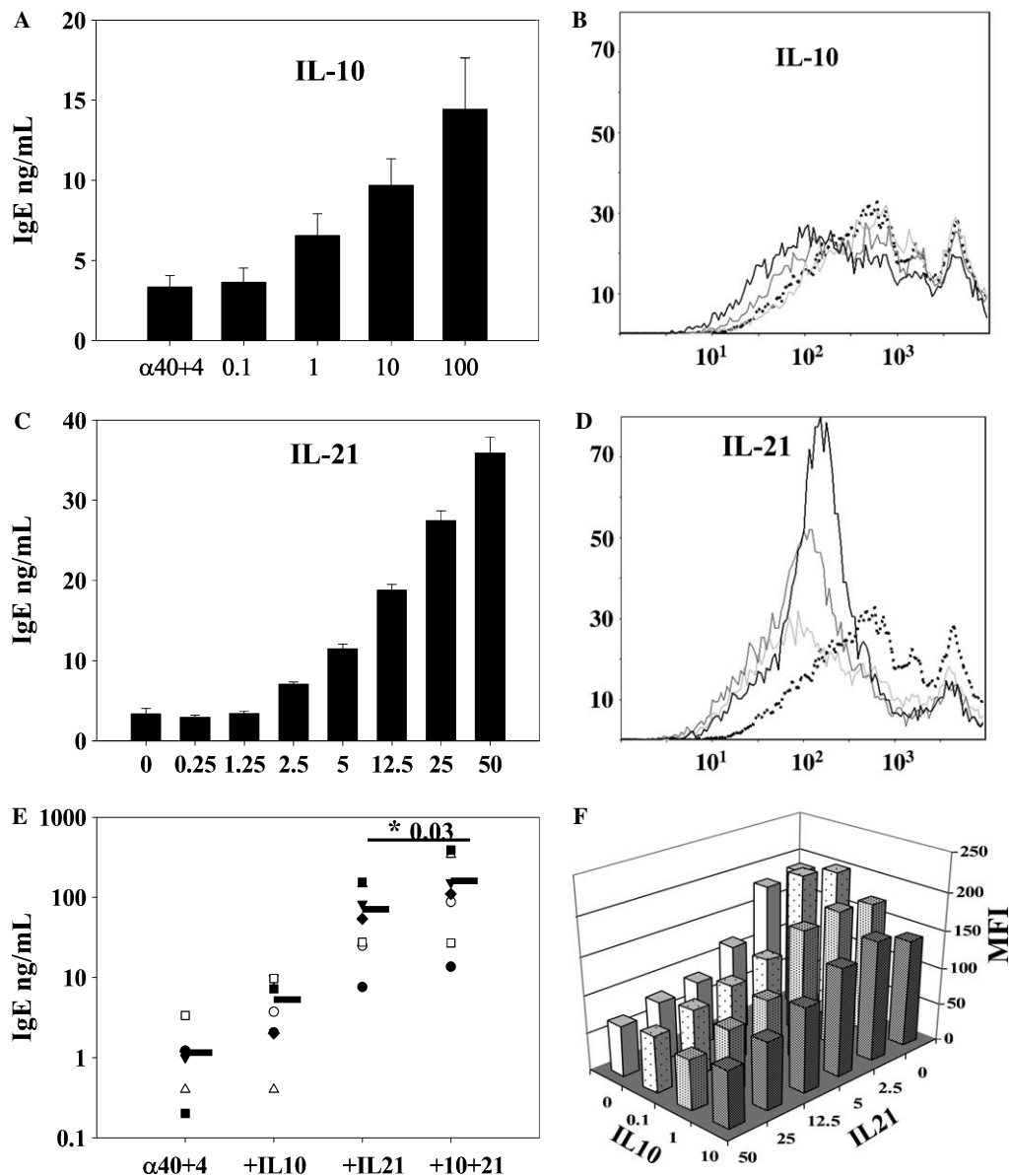


Fig. 7. IL-10 increases IgE production in IL-21/IL-4/anti-CD40 stimulated tonsillar B cells and correlates with increased cell division: IgD⁺ B cells were treated with IL-4/anti-CD40 and the indicated concentrations of IL-10 and either (A) IgE (day 12) or (B) cell division (day 8) was determined. The dotted line, light grey, med grey, and black lines represent 4/40, or 4/40 plus 0.1, 1, 10 ng/ml IL-10, respectively. (C and D) Represents similar conditions as in (A and B) except IL-21 were used. In (D) the dotted line, light grey, med grey, and black lines represent 4/40, or 4/40 plus 5, 25, 50 ng/ml IL-21, respectively. (E) Mean IgE values from seven IgD⁺ B cell tonsil preparations are shown; conditions shown are 4/40 or 4/40 plus IL-10 (10 ng/ml), IL-21 (50 ng/ml) or same levels of both IL-10 and IL-21. IgE levels were determined by ELISA on day 14 (**p* < 0.05). (F) Relative cell division represented by decreasing mean fluorescent intensity (MFI) values from anti-CD40-/IL-4-activated CFSE-labeled B cell cultures containing the indicated levels of IL-10 or IL-21 (both are ng/ml) are shown. Flow analysis was done on day 8 and values are representative of two experiments.

maximal IgE production in the human in vitro model. Cho et al. [7] found that the level of mouse IgE produced in vivo was cell density dependent, and further examination of the cell density phenomenon indicated that the number of cell divisions was the most likely explanation for the increased IgE production [8]. A series of studies, primarily by the Hodgkin laboratory [9,10,13,14] have demonstrated that increased murine cell division correlates with higher levels of IgE and to some extent, IgG production after appropriate in vitro stimulation. One of these studies examined the relationship between cell division number and IgG production

using human B cells [13]. However, IgE was not examined, presumably due to the low amounts of IgE produced.

One of the newest members of the hematopoietic cytokine family is IL-21 and indeed this is the most recent cytokine that uses the common γ_c chain as part of its receptor. Although the initial report of IL-21 demonstrated its ability to co-stimulate human B cell proliferation [15], the major focus of research into IL-21 functions has revolved around NK and CTL activation, especially involving tumor progression [25–27]. As mentioned in the introduction, recent reports have shown that IgE production is enhanced using

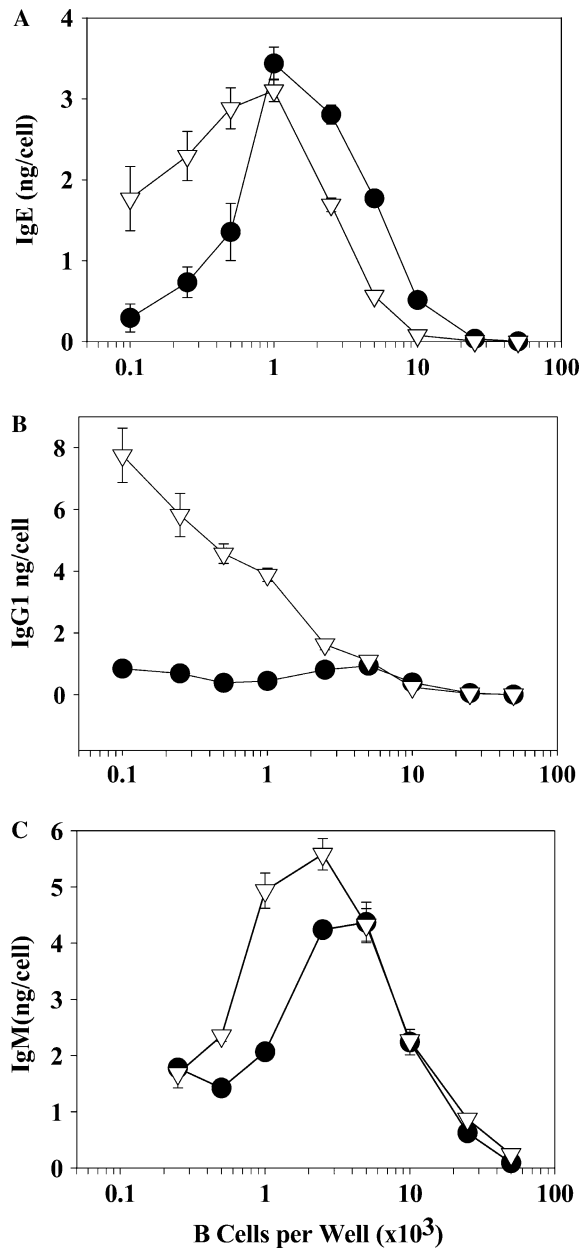


Fig. 8. mIL-21 has a biphasic effect on IgE and IgG₁. (A) IgE levels, (B) IgG₁ levels, or (C) IgM levels were measured from resting B cells plated at 1×10^2 – 5×10^4 cells/well and incubated for 8 days with the cocktail alone (CD40LT/IL-4/IL-5/M15) (●) or cocktail and 25 ng/ml mIL-21 (▽). Supernatants from the cells were collected on day 8 and assayed for the respective immunoglobulin by ELISA. Error bars represent one SD. This experiment is representative of three of similar design.

the human in vitro culture model [4,16]. Several studies in mice, however, have indicated that IL-21 may be beneficial in inhibiting Type I allergic disease. IL-21R $\alpha^{-/-}$ mice exhibit normal lymphoid development, but have elevated serum IgE levels [28]. In addition, treatment of BALB/c mice with IL-21 inhibited antigen-specific IgE production as well as eosinophil recruitment into airways [22]. In addition in this same study, IL-21 was shown to inhibit in vitro IgE production induced by LPS and IL-4, albeit at a single cell concentration.

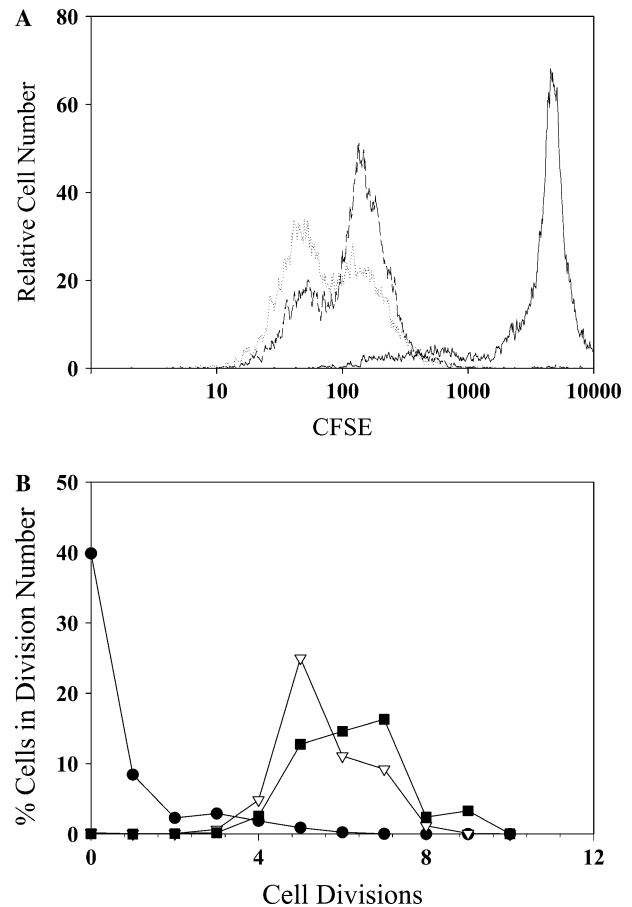


Fig. 9. Increased cell division is evident at low cell densities in mouse IL-21 containing cultures. Splenic B cells were labeled with CFSE and cultured at 500 cells/well for 4 days then analyzed by flow cytometry. (A) Histogram of cells cultured with IL-4 only (—), stimulation cocktail (CD40LT/IL-4/IL-5/M15) (---) or stimulation cocktail plus 5 ng/ml mIL-21 (.....). (B) Analysis of division numbers from flow data IL-4 only (●); activation cocktail plus IL-21 (■), or without IL-21 (▽), respectively. Experiment is representative of three of similar design.

A cell density-dependent relationship to human IgE production is also seen when IL-21 is used in conjunction with IL-4 and anti-CD40 (Fig. 2). Interestingly, IgG and IgM production levels are also cell density dependent when both IL-4 and IL-21 are added to anti-CD40 stimulated B cells (Fig. 3). Confirmation that increased cell division is occurring is shown in Fig. 5 with CFSE-labeled B cells, seen both with lower cell numbers compared to higher cell numbers, and with the addition of IL-21 to the IL-4/anti-CD40 cocktail providing even greater divisions. Our lab has shown previously that the higher number cell divisions at the lower cell densities relative to higher cell densities cannot be explained due to depleted nutrients [8]. Also, studies here support that the lower IgE production in IL-4/anti-CD40/IL-21 stimulated cells at higher cell densities is not due to nutrient or stimulation depletion. The human IgM results were somewhat surprising, since in the murine system, IgM production is directly proportional to cell numbers; the more cells added the greater IgM yield [8]. This indicates that in the human in vitro system, increasing B cell densities

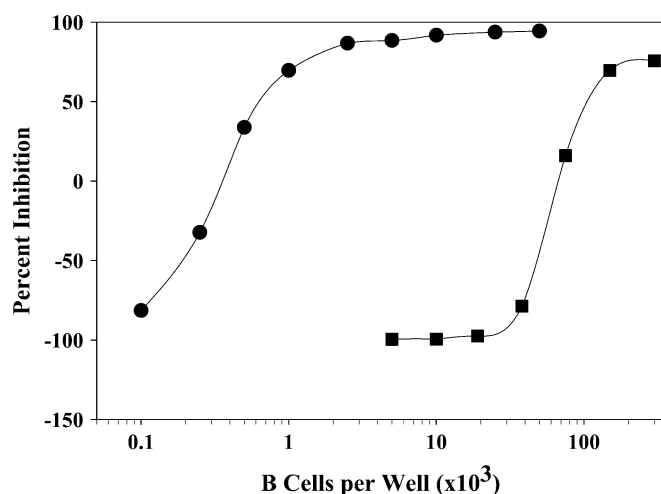


Fig. 10. Comparison of the effect of cell density on human and mouse IgE production. (A) The influence of IL-21 on the amount of mouse (●) or human (■) IgE produced is shown as a function of cell density to illustrate that IL-21 causes both enhancement and inhibition of IgE synthesis, depending on the initial cell input. Mouse B cells were activated with CD40LT/IL-4/IL-5/M15 plus 25 ng/ml mIL-21, while the tonsil B cells were activated with IL-4/anti-CD40 plus 25 ng/ml human IL-21 supernatant. The formula used was IgE level at: $((\text{no IL-21} - \text{IL-21}) / \text{no IL-21}) \times 100$. Two experimental results for mice are shown. Two experimental results for human cells are shown, representative of seven experiments of similar design.

beyond a relatively low (5×10^3 cells/well) cell concentrations, results in decreased antibody production per cell, compared to maximal antibody production seen at lower cell densities. This even holds true in the absence of isotype switching, as exemplified by IgM.

In the previous study, correlating cell division with human IgG production [13], the highest level of IgG production was seen when IL-4 was used in conjunction with IL-10. IL-10 had not previously been tested in conjunction with IL-21, with regard to cell division. We observed that the addition of IL-10 to IL-4/anti-CD40 stimulated cells increased both IgE production (Fig. 7A) and cellular division (Fig. 7C). And as the addition of IL-10 to IL-21/IL-4/anti-CD40 treated cells also increased IgE, we anticipated that there would be a corresponding increase in cellular division, when both cytokines were used. However, no further increase in cellular division was seen (Fig. 7F). Interestingly, cell division, as exemplified by reduced CFSE MFI, reaches the same limit seen after the addition of the highest dose of IL-21 (Fig. 7F). In the case of the two cytokines used, 50 ng/ml IL-21 was able to reach this division limit while the highest dose of IL-10 (100 ng/ml) was not. Additionally, the MFI at all IL-21 concentrations could not be further lowered even with the additional stimuli of IL-10 added. One possible explanation is that the increase in IgE is due to an increase of IgE per cell, induced by IL-10, without increased cell division. Another possibility is there could be additional divisions and IgE production occurring after day 8 when the CFSE analysis occurred and day 14 when the supernatants were collected and analyzed for IgE. However, it has been argued that IL-21 can drive B cells

into terminally differentiated non-dividing Ig-secreting plasma cells [29]. This could explain that increase in Ig production without the further increase in cellular division.

Since IL-10 has previously been used in the in vitro human IgE synthesis models, we wished to determine the relative efficacy of IL-10 vs. IL-21 and whether the combination was synergistic. As can be seen in Figs. 6 and 7, synergy was seen with PBMC and with purified B cells. Note that the production of IgE due to IL-21 was consistently higher than IgE from IL-10. Correspondingly, IL-21 also caused a greater increase in cell division than was seen with IL-10 alone, both with PBMC and purified IgD⁺ tonsillar B cells. When the two cytokines were combined and added to IL-4/anti-CD40 stimulated cells, a further increase in IgE was seen; the increase was synergistic rather than additive. As discussed above, this synergistic increase in IgE occurs without additional cell division. This possibility can be explained due to IL-21 inducing the differentiation of both cell cycling and terminally differentiated cells [29]. As IL-21 can drive cells to fully differentiated plasma cells, it is likely that the increase of IgE seen in the IL-10/IL-21 treated cells is from the increase in plasma cell Ig secreting cell numbers.

Studies in the murine system have shown that IL-21 has an inhibitory effect on IgE production, both in vivo and in vitro [22,28]. Since the published studies in vitro used a single, relatively high, cell density, the effect of IL-21 on mouse B cells was re-examined as a function of cell concentration. Consistent with other reports [15], we confirmed that IL-21 enhanced the proliferation of mouse B cells stimulated with CD40LT and IL-4, and an optimal amount (based on proliferation) of IL-21 was added to increasing concentrations of mouse B cells. As shown in Fig. 8A, addition of IL-21 shifted the peak of maximal mouse IgE production to lower cell densities. Mouse IgG₁ production was also marginally increased at lower cell densities (Fig. 8B); IgM levels (Fig. 8C) were minimally influenced by IL-21 addition, and remained proportional to cell density, as reported for IgM with CD40LT and IL-4 stimulated cells [8]. The lowest cell densities analyzed in the mouse model are of particular interest; as IgE levels were enhanced at these cell densities. Indeed, when IL-21-mediated influence on IgE production is plotted against their respective cell densities, both the human and mouse in vitro systems give a quite similar pattern (Fig. 10). Thus, a primary difference in the two systems is that optimal IgE production is seen in the mouse at lower cell densities. Depending upon the cell density being examined, IL-21 can cause both enhancement and inhibition of IgE synthesis in both the human and mouse in vitro systems.

Overall, the data suggest that IL-21 is driving the IL-4/anti-CD40 treated cell populations to have increased divisions and increased IgE production, and in the additional presence of IL-10 to further increase in IgE without the accompanying increase in cellular division, especially at the lower cell concentrations. Recent data argues that the presence of IL-4 inhibits the plasma cell differentiation induced by IL-21. However, this is primarily seen with anti-IgM/anti-CD40 stimulation [29], not CD40 stimulation alone.

IgE production in human in vitro culture models requires the presence of IL-4 (or the related IL-13), regardless of the stimulus, and IL-21 plus anti-CD40 did not lead to detectable IgE production (data not shown and [16,29]). Our data demonstrates that IL-21 and IL-4 drives increases in IgE production by CD40 stimulated B cells and that this is linked to increased cellular division and likely cellular differentiation. Finally, the synergy of IL-10 plus IL-21 on IL-4 and anti-CD40 stimulated human B cells provides an interesting avenue for examining the relationships between the two cytokines and the cell signaling pathways. This data provides an important and novel system in which to investigate the mechanism of IgE production and regulation in both model systems.

Acknowledgments

The authors acknowledge Dr. Natalie McCloskey and Jamie Sturgill for help in obtaining optimal media for human B cell culture. Dr. Suzanne Barbour, and Jamie Sturgill are thanked for comments on the manuscript. Thanks to Dr. Linda Macgovern and her staff from Henrico Doctor's Hospital, Richmond, VA for willing and considerable assistance with acquiring tonsils.

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