

IL-21 effects on human IgE production in response to IL-4 or IL-13

Nancy Wood^a, Karen Bourque^a, Debra D. Donaldson^a, Mary Collins^a, Donata Vercelli^b, Samuel J. Goldman^a, Marion T. Kasaian^{a,*}

^a Wyeth Research, Cambridge, MA, USA

^b Department of Cell Biology, Arizona Respiratory Center, University of Arizona, Tucson, AZ, USA

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Abstract

In human atopic disease, IgE sensitizes the allergic response, while IgG4 is protective. Because IL-4 and IL-13 trigger switch recombination to both IgE and IgG4, additional agents must regulate the balance between these isotypes to influence susceptibility or tolerance to atopy. In this report, we define in vitro conditions leading to activation or inhibition of human IgE and IgG4 production by IL-21. IL-21 reduced IL-4-driven IgE synthesis by mitogen-stimulated human PBMC. IL-21 inhibition of human IgE production was not a direct effect on B cells, was not seen following B cell activation with IL-13, and was overcome by CD40 ligation. Neither IFN- γ , IL-10, IL-12, CD40L expression, nor apoptosis was responsible for the inhibitory effect. In contrast, IL-21-stimulated secretion of IgG4 from PBMC. Our findings indicate that IL-21 may influence the production of both human IgE and IgG4, and thus contribute to the regulation of atopic reactions.

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1. Introduction

IgE generated in response to allergen challenge triggers potent effector mechanisms associated with atopic disease [1,2]. When bound to high affinity receptors on mast cells and basophils, IgE can be cross-linked by allergen, leading to degranulation and the release of histamine, leukotrienes, cytokines, and chemokines. These agents directly mediate the symptoms of wheezing and bronchoconstriction in asthma associated with early and late phase allergic reactions and late phase inflammatory responses [1,2]. The central role of IgE in these responses is supported not only by the detection of allergen-specific IgE in atopic subjects but not healthy controls, but also by the demonstration that neutralization of IgE is

an effective therapeutic strategy for the treatment of atopic disease [3].

While generation of allergen-specific IgE is central to development of atopy, allergen-specific IgG4 is thought to be non-pathogenic [4] or even protect against atopic reactions [5–7]. Levels of IgG4 specific for allergen can be higher in non-atopic than atopic subjects, and increase in allergics following successful therapy [5–7]. Interestingly, both IgE and IgG4 are produced in response to the same signals. IL-4 or IL-13 binding to receptors utilizing IL-4R α triggers phosphorylation of STAT6, which translocates to the nucleus and interacts with STAT6-responsive elements in the C ϵ and C γ 4 promoter regions [8,9]. If IgG4 is protective, while IgE is a potent trigger of atopic reactions, then it becomes important to elucidate the factors influencing production of these isotypes. Several cytokines, including TGF- β [10–12], IFN- α [12–14], and IFN- γ [13,14] suppress IgE and IgG4 production. In contrast, IL-6 [15], IL-7 [16],

* Corresponding author. Fax: +1 617 665 5584.

E-mail address: mksaiaian@wyeth.com (M.T. Kasaian).

and IL-9 [16,17] enhance both IgE and IgG4 production. Other agents, including IL-12 [18,19], IL-12-inducing immunostimulatory DNA [20], and IL-10 [21] have been reported to inhibit IgE while enhancing IgG4.

IL-21 is a T cell-derived cytokine that triggers T cell and NK cell activation [22,23], and has several reported effects on B cells, including proliferation [22,24] and apoptosis [25]. The observation that IL-21R-deficient mice have high levels of circulating IgE [23,26] led to the suggestion that this cytokine may influence IgE switch recombination. This was confirmed in murine systems by the demonstration that IL-21 inhibits IL-4-driven IgE production by B cells in vitro and in vivo [26,27]. Interestingly, IL-21R-deficient mice have low levels of serum IgG1, analogous to human IgG4 [23,26,27]. The finding of low serum IgG1 in association with high IgE indicates that IL-21 may differentially influence IgE and IgG1 switch recombination. This was supported by experiments in which IL-21 administered to mice throughout the course of OVA immunization reduced IgE production, while increasing OVA-specific IgG1 [27], and by the observation that treatment of murine B cells with IL-21 enhanced IgG1 production in vitro [26,27]. That IL-21 could also affect IgE production in humans was indicated by a polymorphism in the human IL-21R gene locus, which is associated with high serum IgE [28]. Direct effects of IL-21 on human IgE and IgG4 production have not been thoroughly evaluated, however.

In this report, we investigate the influence of IL-21 on in vitro IgE production by human B cells, and describe its role in IL-4 vs. IL-13-directed immunoglobulin synthesis. In contrast to its effects in murine systems [26,27], IL-21 enhanced IgE production from purified human B cells stimulated with anti-CD40 mAb and either IL-4 or IL-13. An inhibitory effect was seen only in unfractionated PBMC, and only in response to IL-4 and not IL-13. Moreover, IL-21 itself directly induced IgG4 secretion from unfractionated PBMC. These findings are discussed in terms of the immunoregulatory role of IL-21.

2. Materials and methods

2.1. PBMC isolation and culture

Peripheral blood from healthy human donors was drawn into heparinized Vacutainer tubes (Becton–Dickinson, Mountain View, CA). Mononuclear cells were isolated by centrifugation over Histopaque-1077 (Sigma, St. Louis, MO). For culture of whole PBMC, cells were plated at 2×10^6 /ml in 96-well round-bottomed plates in RPMI containing 10% heat-inactivated FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine. For PHA activation, PBMC were plated with 2 µg/ml PHA-P (Sigma). After 2 days, fresh media were added lacking PHA but containing 25 ng/ml recombinant

human IL-4 or 50 ng/ml recombinant human IL-13, ± 20 ng/ml recombinant human IL-21 (R&D Systems, Minneapolis, MN). These levels were determined by dose titration for each cytokine. For anti-CD40 mAb activation, PBMC were plated with 1 µg/ml anti-human CD40 mAb (BD Pharmingen) in the presence of cytokines. For both PHA- and anti-CD40 mAb-activated cultures, media containing fresh cytokines was added every 5 days. On days 14–21 of PHA cultures, or days 6–12 of anti-CD40 mAb cultures, media were harvested for determination of antibody levels. These time points reflect the more rapid time course for IgE production under conditions of anti-CD40 treatment vs. PHA stimulation. Cells were harvested early (days 3–5) or later (days 10–14) in the course of culture for RNA isolation.

2.2. B cell enrichment

PBMC isolated as described above were incubated with B cell enrichment cocktail (RosetteSep; StemCell Technologies, Vancouver, British Columbia, Canada), and B cells isolated according to the manufacturer's instructions. The resulting population was $>88\%$ CD20+ B cells. B cells were plated at 2×10^5 /ml in media containing 1×10^6 /ml irradiated (1500 RAD) autologous PBMC as feeders, and treated with anti-CD40 mAb or cytokines as described above. On days 6–12 of culture, media were harvested for determination of antibody levels.

2.3. ELISA for human Ig isotypes

ELISA plates (EIA/RIA plates; Corning Costar, Acton, MA) were coated with 1 µg/ml goat anti-human IgE (KPL, Gaithersburg, MD) or 3 µg/ml mouse anti-human IgG4 (Southern Biotechnology Associates, Birmingham, AL) in 0.1 M sodium carbonate, 0.1 M sodium bicarbonate buffer, pH 9.6, overnight at 4°C. Plates were blocked for 1 h with 0.5% gelatin and 1% polyvinylpyrrolidone (Sigma, St. Louis, MO) in PBS. Plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween), then incubated with culture supernatant or human IgE (Biodesign Int, Kennebunk, ME) or IgG4 (Sigma) isotype standards for 4 h at room temperature. After washing with PBS-Tween, plates were incubated for 2 h at room temperature with biotinylated antibody directed against human IgE (KPL) or IgG4 (Southern Biotechnology Associates). Plates were washed and incubated with HRP-labeled streptavidin (Southern Biotechnology Associates) for 1 h at room temperature. Plates were washed and incubated with the peroxidase substrate Sure Blue (KPL). The reaction was stopped by adding 0.1 N HCL, and absorbance at 450 nm was read in a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). To demonstrate isotype specificity, purified human IgM, IgG isotypes, or IgA (BD Biosci-

ences Pharmingen, San Diego, CA) were run in the IgE and IgG4 ELISAs and produced no signal (data not shown). The limit of sensitivity of the IgE ELISA was 0.3 ng/ml. The limit of sensitivity of the IgG4 ELISA was 4 ng/ml.

2.4. Cytokine analysis

Cytokine levels in culture supernatants were determined using assay kits for IL-10 (Biosource International, Camarillo, CA; sensitivity <0.2 pg/ml), IL-12 (Biosource International; sensitivity <2 pg/ml), IFN- γ (R&D Systems; sensitivity <8 pg/ml) or TGF- β 1 (R&D Systems, sensitivity <7 pg/ml).

2.5. Proliferation assays

Enriched human B cells were cultured in RPMI containing 10% FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 2 mM L-glutamine at 2×10^5 /well in 96-well round-bottomed plates. Anti-CD40 mAb and cytokines were added as described above. On day 3, cultures were pulsed with 0.5 μ Ci/well [^{3} H]thymidine (Perkin-Elmer NEN, Boston, MA), and harvested 5 h later onto glass fiber filter mats. [^{3} H]Thymidine incorporation was determined by liquid scintillation counting.

2.6. Apoptosis assay

Apoptosis was measured by flow cytometry using a Annexin V-FITC Apoptosis Detection Kit (Calbiochem, La Jolla, CA). PBMC were cultured as described above, and apoptosis measured at 24 and 48 h following addition of cytokines. Cells were incubated with annexin V-FITC and APC-conjugated anti-human CD19 (BD Pharmingen) for 15 min at room temperature and washed. Propidium iodide was added and fluorescence was analyzed using a BD FACSCalibur cytometer and CellQuest software (BD Biosciences).

2.7. RNA isolation

On day 5 of PBMC or B cell cultures, cells were harvested from microtiter wells, pooled, washed with PBS, lysed with RLT buffer (Qiagen, Valencia, CA), and prepared with QiaShredder. RNA was prepared using the RNA Mini Kit (Qiagen) according to manufacturer's instructions.

2.8. Reverse-transcription and PCR analysis of germline transcripts

RNA prepared as described above was transcribed to cDNA using the Promega Reverse Transcription kit (Promega, Madison, WI). PCR was performed using the Clontech Advantage PCR kit (BD Biosciences Clontech,

Palo Alto, CA) and the following primer sequences and conditions. GAPDH was amplified in 25 cycles of 1 min each at 94, 65, and 72 °C using the primers GAPDH forward: 5' CACAGTCCATGCCATCACTG 3' and GAPDH reverse: 5' TACTCCTTGGAGGCCATGTG 3' [29]. I ϵ germline transcript was amplified in 38 cycles of 1 min each at 94, 65, and 74 °C using the primers I ϵ germline forward: 5' GACGGGCCACACCATCCACAGGCACCAAATGGACGAC 3' and I ϵ germline reverse: 5' CAGGACGACTGTAAGATCTTCACG 3' [30]. I γ 4 germline transcript was amplified in 38 cycles of 1 min each at 94, 65, and 76 °C using the primers I γ 4 germline forward: 5' TTGTCCAGGCCGCGCAGCATCACCAGA 3' and I γ 4 germline reverse: 5' ATGGGCA TGGGGGACCAATTTGGA [31]. Mature IgE transcripts were amplified in 38 cycles of 1 min each at 94, 69, and 74 °C using a JH consensus forward primer: 5' ACCCTGGTCACCGTCTCCTCA 3' [32] combined with the I ϵ reverse primer. CD40L transcripts were amplified in 32–33 cycles of 1 min at 95 °C, 1 min at 66 °C, and 3 min at 72 °C, using forward: 5' CACCTTC TCTGCCAGAAGATAACCATTTCAA 3' and reverse: 5' TTATGAAGACTCCAGCGTCACGTCCACCA 3' primers spanning the entire CD40L coding region [33]. All of the above primers were prepared by Eurogentec (San Diego, CA). PCR for human IFN- γ was performed using the gene specific relative RT-PCR kit from Ambion (Austin, TX). Amplified products were run on 1.2% agarose gels containing ethidium bromide.

2.9. Real time RT-PCR

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Oligonucleotides were designed to human GAPDH, IL-12p35, IL-10, CD3, and IL-12R β 2 using Primer Express software (Applied Biosystems Division of Perkin-Elmer, Foster City, CA) and synthesized by Eurogentec. Forward and reverse primer sequences were as follows: GAPDH forward 5' TCCAT GACAACCTTGGTATCGT; GAPDH reverse 5' GTC TTCTGGGTGGCAGTGA; IL-12p35 forward 5' CCA CTCCAGACCCAGGAATG; IL-12p35 reverse 5' GACGGCCCTCAGCAGGT; IL10 forward 5' GCCAGG GCACCCAGTCT; IL10 reverse 5' TCGAAGCATGT TAGGCAGGTT; IL-12R β 2 forward 5' AGACATTT ATTGTGACTATTTGGACTTTG; IL-12R β 2 reverse 5' AGTAACCTTGGCTGTGAAATTGG; CD3 forward 5' CATCCCAAAGTATTCATCTACTTTTC; CD3 reverse 5' CCCAGTCCATCCCCAGAGA; CD40L forward 5' GCTTTGAAATGCAAAAAGGT GAT; and CD40L reverse 5' TTGTTTACTGCTGG CCTCACTT. Probes were labeled on the 5' end with the reporter dye, 6-carboxyfluorescein (FAM) and on the 3' end with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA). Probe sequences were as follows: GAPDH probe 5' AGGACTCATGACCACAGTCCA

TGCC; IL-12p35 probe 5' TCCCATGCCTTCACCAC TCCCAA; IL10 probe 5' AGAACAGCTGCACCC ACTTCCCAGG; IL-12R β 2 probe 5' AATCAACCTC ACCCCTGAATCACCTGAA; CD3 probe 5' TCGCC GTCCCCTTTTGACAGC; CD40L probe 5' AGAAT CCTCAAATTGCGGCACATGTCAT. Reactions were set up using a reverse transcriptase q-PCR MasterMix (Eurogentec) and 50 ng of template RNA per reaction. Samples were run in duplicate on the Prism 7000 Sequence Detection System (Applied Biosystems) using the following RT-PCR program: [1] 30 s cycle at 48 °C, [50] 10 s cycles at 95 °C, [1] 15 min cycle at 95 °C, and [1] 1 s cycle at 60 °C. Data were analyzed using Prism 7000 software. Each result was fit to a standard curve generated from a positive control source of RNA and expression values were normalized to GAPDH.

2.10. Statistical analysis

All observations were reproduced in 2–6 separate experiments. Data between treatment groups were compared using Student's *t* test. For analysis cytokine effects on IgE production in microcultures, the IgE level in each microwell with a given treatment was taken as a separate determination, with $n = 24$ –36 per treatment. For analysis of cytokine effects on IgE or cytokine production in bulk cultures, replicate cultures were established per treatment. *p* values of <0.05 were considered significant.

3. Results

3.1. IL-21 enhances IL-4- and IL-13-driven IgE synthesis in human B cells

IgE switch recombination can be triggered by exposure of B cells to a CD40 cross-linking agent in the presence of IL-4 or IL-13. To investigate the effects of IL-21 on this process, B cells were enriched from human PBMC to $>88\%$ purity, and stimulated with anti-CD40 mAb in the presence of IL-4 or IL-13. CD3 $^{+}$ cells were undetectable. Individual cultures were established in 24–36 microtiter wells per treatment. In the absence of IL-4 or IL-13, none of the wells contained IgE, consistent with a lack of detectable IgE-producing cells. When IL-4 or IL-13 was added, most of the microcultures contained IgE-producing cells (Fig. 1A), with detectable IgE in the supernatant (Fig. 1B). Although limiting dilution analysis was not performed to calculate the exact frequency, an increase in the number of IgE-positive microcultures was taken to indicate an increased frequency of IgE-producing B cells. Addition of IL-21 to IL-4 or IL-13 consistently increased IgE production over levels seen with IL-4 or IL-13 alone (Figs. 1A and B). The percentage of IgE-producing wells was virtually 100% with IL-4 or IL-13+IL-21, and increased from 61% with IL-13

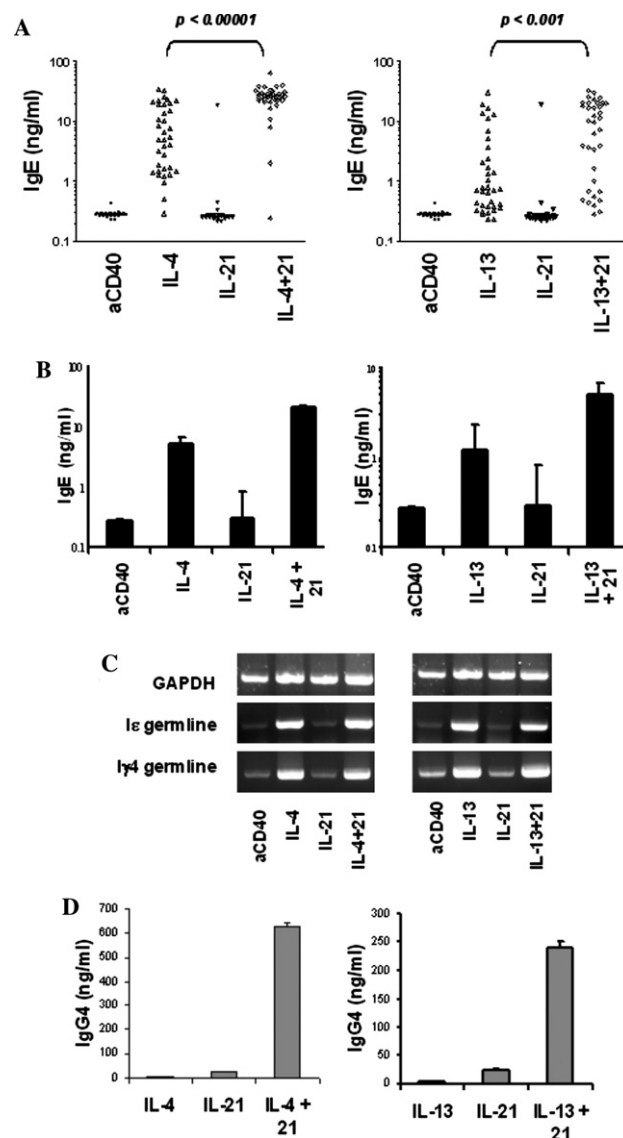


Fig. 1. IL-21 potentiates IgE and IgG4 release from purified B cells. B cells were isolated from human PBMC by magnetic bead separation. Cells were treated with anti-CD40 mAb plus the indicated cytokines as described in Section 2. On day 14, cells and supernatants were harvested. (A) IgE levels in supernatants of individual microwells. (B) Mean $\pm$ SEM of IgE levels in wells treated with the indicated cytokine, assayed on day 14 of culture. (C) PCR for expression of GAPDH, Iε germline transcript, and Iγ4 germline transcript. (D) IgG4 levels in pooled wells treated with the indicated cytokine. No IgG4 was detectable in cells treated with anti-CD40 mAb alone (not shown).

alone to 78% with IL-13+IL-21. IL-4 and IL-13 also induced production of the Iε germline transcript (Fig. 1C), which is associated with de novo Ig switch recombination to the Cε locus.

IL-4 and IL-13 also induced generation of the Iγ4 germline transcript (Fig. 1C), but in our culture system, IL-4 or IL-13 alone was not sufficient to support IgG4 production and release from the cells (Fig. 1D). In contrast, IL-21 alone generated only background levels of Iγ4 germline transcript (Fig. 1C), but did stimulate low levels

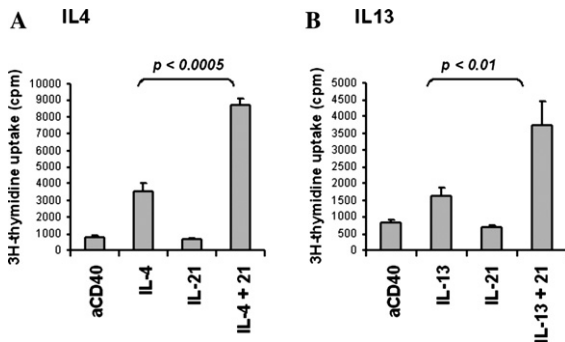


Fig. 2. IL-21 synergizes with IL-4 or IL-13 to drive B cell proliferation. B cells were isolated from human PBMC by magnetic bead separation. Cells were treated for 48 h with anti-CD40 mAb plus the indicated cytokines. [³H]Thymidine was added for the final 24 h, and incorporation determined by liquid scintillation counting.

of IgG4 release into the supernatant of anti-CD40 mAb-treated B cells. Addition of IL-21 to IL-4 or IL-13 strongly enhanced IgG4 production over levels seen with IL-4 or IL-13 alone (Fig. 1D). In fact, very little IgG4 was released from the cells unless IL-21 was added to the cultures.

IL-21 stimulates proliferation of human B cells that have been treated with anti-CD40 mAb [22], and the proportion of cells undergoing isotype switch recombination increases with cell division [34]. To determine if increased B cell proliferation could help to account for the enhanced levels of IgE and IgG4 seen in the presence of IL-21, we evaluated [³H]thymidine incorporation by purified B cells under the culture conditions used above. Results show that IL-21 enhanced B cell proliferation above levels seen with IL-4 or IL-13 alone (Fig. 2).

3.2. IL-21 enhances IgE synthesis in unfractionated PBMC stimulated with anti-CD40 mAb and IL-4 or IL-13

In addition to its reported effects on B cells, IL-21 has potent effects on human T cells. It induces T cell proliferation [22,23,35], and potentiates cytokine production in the presence of TCR cross-linking agents and appropriate costimulation [23,36]. Therefore, it was of interest to investigate IL-21 effects on IgE production under conditions in which T cells were also present and could respond to the cytokine.

Unfractionated PBMC were treated with anti-CD40 mAb in combination with IL-4 or IL-13 to drive IgE production. IgE was measured in the supernatants 7–14 days later. In combination with IL-4 or IL-13, IL-21 produced a modest increase in levels of IgE and IgG4 protein (Figs. 3A, B, and D). The percentage of IgE-producing wells increased from 86% with IL-4 alone to 100% with IL-4 + IL-21, and increased from 19% with IL-13 alone to 56% with IL-13 + IL-21. Consistent with this, IL-4- and IL-13-induced I ϵ germline transcript, J κ -C ϵ mature transcript, and I γ 4 germline transcript all were maintained in the presence of IL-21 (Fig. 3C).

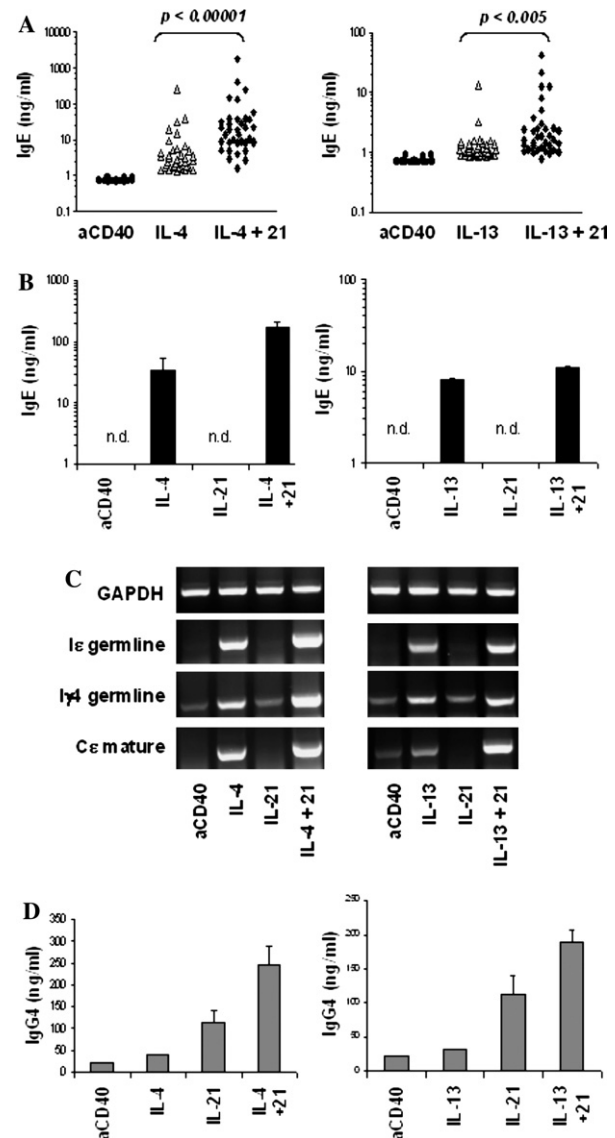


Fig. 3. IL-21 potentiates IgE and IgG4 release from PBMC stimulated with anti-CD40 mAb. Unfractionated human PBMC were treated with anti-CD40 mAb plus the indicated cytokines, as described in Section 2. (A) IgE levels in supernatants of individual microwells, assayed on day 7 of culture. There was no detectable IgE in wells treated with IL-21 alone (not shown). (B) IgE levels in pooled wells treated with the indicated cytokine, assayed on day 21 of culture. (C) PCR for I ϵ and I γ 4 germline transcripts was performed using cells isolated on day 3 of culture. PCR for C ϵ mature transcript was performed using cells isolated on day 10. (D) IgG4 levels in pooled wells treated with the indicated cytokine, assayed on day 9 of culture. Levels of IgG4 produced in response to IL-4 + IL-21 or IL-13 + IL-21 were not significantly greater than those produced by IL-21 alone ($p > 0.05$).

3.3. IL-21 blocks IgE synthesis in unfractionated PBMC stimulated with PHA and IL-4

Activated T cells are the only known source of IL-21. In the next series of experiments, effects of IL-21 were investigated under conditions in which Ig class switch recombination was dependent on T cell activation.

Unfractionated PBMC were treated with the T cell mitogen, PHA, to induce CD40L expression [51]. Upon addition of IL-4 or IL-13, IgE was released into the supernatant within 14–21 days. In this T cell-dependent system, IL-4-driven IgE production was blocked by IL-21, which greatly reduced the levels of IgE released into the supernatant (Figs. 4A and B). The percentage of IgE-producing wells decreased from 47% with IL-4 alone to 6% with IL-4 + IL-21. Interestingly, this effect was not seen when IgE synthesis was initiated with IL-13. Cells treated with IL-13 + IL-21 produced more IgE than

those treated with IL-13 alone (Figs. 4A and B), as had been seen with purified B cells (Fig. 1A). The percentage of IgE-producing wells increased from 31% with IL-13 alone to 68% with IL-13 + IL-21. Taking into account that T cells respond to IL-4 but not to IL-13, these observations point to a T cell-dependent mechanism for the inhibitory activity of IL-21 on IL-4-driven IgE production in this system.

To further investigate this inhibitory activity, I ϵ germline transcription was examined. With PHA stimulation, I ϵ germline transcript was detectable early after addition of IL-4 or IL-13 (days 3–5 of culture). Although IL-21 blocked IgE production in IL-4-treated cultures, it did not prevent this initial induction of I ϵ germline transcript by either IL-4 or IL-13 (Fig. 4C).

3.4. IL-21 increases IgG4 production in unfractionated PBMC stimulated with PHA

In PHA-stimulated PBMC cultures, treatment with IL-4 or IL-13 induced high levels of I γ 4 germline transcript. PBMC treated with IL-21 or with no added cytokine also showed detectable transcript (Fig. 4C). By days 14 and 15, much higher levels of IgG4 were found in cultures that had been treated with IL-21 than in those treated with IL-4 or IL-13 alone (Fig. 4D). Addition of IL-4 was inhibitory for IL-21-induced IgG4 production, whereas addition of IL-13 was not (Fig. 4D).

3.5. CD40L expression is maintained in the presence of IL-21

An inhibitory effect of IL-21 on IgE and IgG4 production was seen when PHA was used to induce co-stimulatory signals for IL-4-driven IgE production (Fig. 4). In contrast, when anti-CD40 was used to directly cross-link CD40 in PBMC cultures, IL-21 did not block IgE production in response to IL-4 (Fig. 3). Thus, we considered the possibility that IL-21 reduced CD40L expression by PBMC stimulated with PHA and IL-4. CD40L mRNA is labile, and expression is thought to be transcriptionally regulated [37,38]. Using real time-PCR, we examined CD40L transcript levels in PHA-stimulated PBMC cultures early after addition of cytokine (day 4), or at a later time point (day 14), at which IgE was measurable in the cell supernatants. At both time points, cells treated with IL-4 or IL-4 + IL-21 showed strong CD40L mRNA expression, while transcript levels with IL-21 alone were not elevated over those seen with PHA (Fig. 5A). These findings were supported by PCR amplification using primers spanning the entire CD40L coding region (Fig. 5B). This result clearly demonstrates that the presence of IL-21 does not block CD40L transcription, indicating that CD40L expression is not reduced under conditions in which IgE production is inhibited.

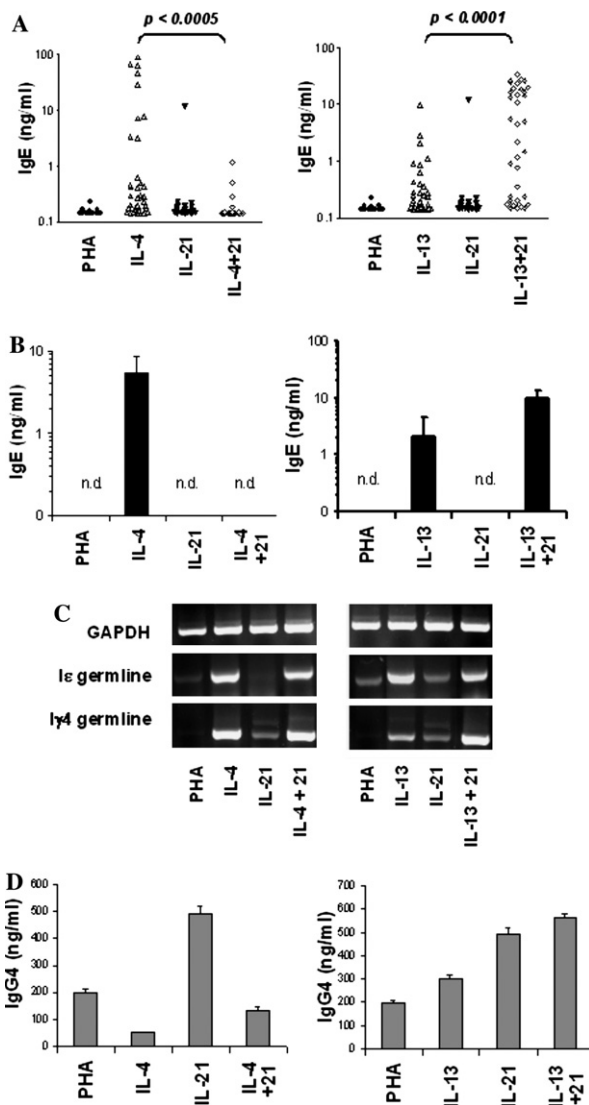


Fig. 4. IL-21 inhibits IgE production but not IgG4 release in PBMC stimulated with PHA. Unfractionated human PBMC were treated with PHA and cytokines as described in Section 2. (A) IgE levels in supernatants of individual microwells, assayed on day 21 of culture. (B) IgE levels in pooled wells treated with the indicated cytokine, assayed on day 21 of culture. (C) PCR for I ϵ and I γ 4 germline transcripts, using cells isolated on day 3 of culture. (D) IgG4 levels in pooled wells treated with the indicated cytokine, assayed on day 21 of culture. Addition of IL-4 but not IL-13 significantly decreased in IgG4 levels in response to IL-21 ($p < 0.0005$).

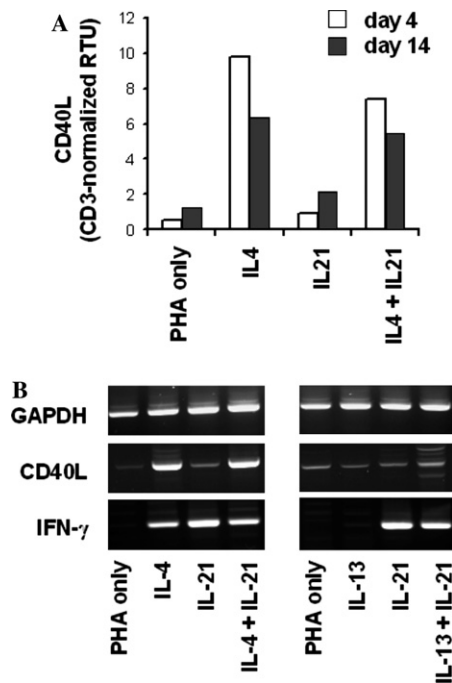


Fig. 5. CD40L gene transcription is maintained in the presence of IL-21, and IFN- γ gene transcription is induced by IL-21. Unfractionated human PBMC were treated with PHA and cytokines as described in Section 2. (A) Real time-PCR was used to quantitate the level of CD40L mRNA relative to CD3 mRNA on day 4 (open bars) or day 14 (filled bars) of culture. Data are expressed as relative Taqman units (RTU). (B) On day 14 of culture, cells were harvested and analyzed for CD40L or IFN- γ gene expression by RT-PCR.

3.6. IL-21 induces expression of IFN- γ

Several experiments were done to address whether treatment of PHA-stimulated, IL-4-activated PBMC with IL-21 resulted in generation of cytokines that block IgE production. The ability of IFN- γ to antagonize IgE synthesis has been well-characterized [10,13,14,39], and IL-21 is known to stimulate IFN- γ gene transcription in human T and NK cells [36,40]. Therefore, the expression of IFN- γ transcript was examined in PHA-stimulated PBMC treated with IL-4, IL-13, or IL-21. Early in the cultures, when IgE germline transcript was detectable, IFN- γ gene expression was seen under all treatment conditions (data not shown). By day 14 of culture, when IgE could be assayed from the supernatant, IFN- γ gene expression was seen only in cultures treated with IL-4 or those treated with IL-21 (Fig. 5). Thus, IFN- γ transcripts were found both in cultures treated with IL-4 + IL-21, in which IgE production was reduced, and in those treated with IL-13 + IL-21, in which IgE production was maintained.

3.7. IL-21 induces IL-10 production by PBMC, but does not affect production of IL-12 or expression of IL-12R β

IL-10 is a multi-potent cytokine that has been reported to stimulate [41] or inhibit [21] B cell IgE

synthesis, depending on the presence of other cytokines or co-stimulatory signals. We asked whether IL-10 was produced in IL-21-treated PBMC cultures and could help to explain the inhibition of IgE production that was seen in the presence of IL-4. IL-21 was found to boost IL-10 production by PBMC, both in PHA-stimulated cultures (Fig. 6A), where IgE production was inhibited (Fig. 4), and in anti-CD40 mAb-stimulated cultures (Fig. 6B), where IgE production was not inhibited (Fig. 3). Furthermore, comparable IL-10 levels were seen in the presence of IL-4 or IL-13 (Figs. 6A and B). Real time-PCR analysis confirmed that IL-21 increased IL-10 production, but the increase was seen whether or not IgE was released (data not shown). To address the role of IL-10 more directly, neutralizing antibody to IL-10 was added to the PHA-stimulated cultures, and did not overcome the inhibitory effect of IL-21 on IgE production (data not shown).

Several other cytokines have been reported to block IgE production, including IL-12 [19], and TGF- β [10]. Both IL-12 (Fig. 6C) and TGF- β (data not shown) could be detected in PBMC cultures, but levels were similar in cells treated with IL-4 or IL-13, in the presence or absence of IL-21. IL-21 also had no effect on IL-12R β gene expression induced by PHA, IL-4, or IL-13 (Fig. 6D). Thus, neither IFN- γ , IL-10, IL-12, nor TGF- β could satisfactorily account for the inhibitory effect of IL-21 on IL-4-driven IgE production.

3.8. IL-21 does not drive B cell apoptosis in PHA-stimulated PBMC cultures

IL-21 has been shown to induce apoptosis of primary murine B cells [25]. Thus, it is possible that B cells of PHA-stimulated PBMC cultures treated with IL-21 were driven to apoptosis, accounting for the decrease in IgE production. To address this issue, PHA-stimulated PBMC were stained with anti-CD19 to identify B cells, and assayed for binding of PI and FITC-annexin by flow cytometry. Late apoptotic cells (PI⁺/FITC-annexin⁺) could be distinguished from early apoptotic (PI^{neg}/FITC-annexin⁺) or viable (PI^{neg}/FITC-annexin^{neg}) B cells. Results show that addition of IL-21 resulted in a minor increase in the percentage of apoptotic CD19⁺ cells in IL-4-treated cultures, but that the level of apoptosis was not different than that seen in cultures treated with IL-13 or IL-13 + IL-21 (Fig. 7). Thus, induction of B cell apoptosis does not account for the inhibitory effect of IL-21 on IgE production.

3.9. Adding back IL-13 does not restore IgE production in PBMC treated with IL-4 and IL-21

Because IL-21 inhibited IgE production from PHA-stimulated PBMC in response to IL-4 but not IL-13 (Fig. 4), we asked whether the presence of all three

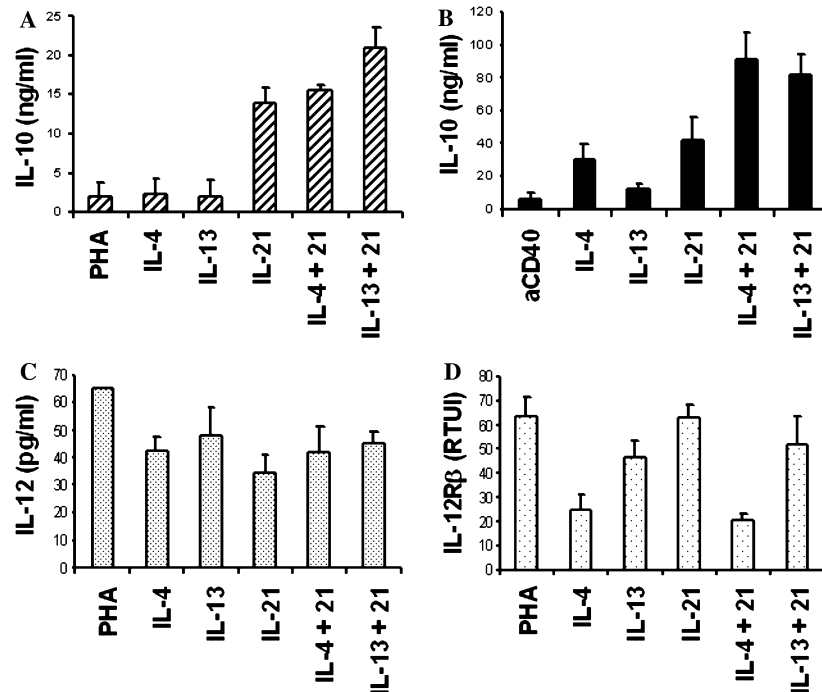


Fig. 6. Cytokine levels in PBMC cultures. (A) Unfractionated PBMC were treated with PHA and cytokines as described in Section 2. IL-10 levels were measured in pooled supernatants collected on day 7 of culture. (B) Unfractionated human PBMC were treated for 48 h with anti-CD40 mAb plus the indicated cytokines. On day 2 and every 4 days thereafter, media were changed and fresh cytokines added. IL-10 levels were measured in pooled supernatants collected on day 7 of culture. (C) PHA-stimulated PBMC were treated with the indicated cytokines. IL-12 levels were measured in pooled supernatants collected on day 6 of culture. (D) PHA-stimulated PBMC were treated with the indicated cytokines. IL-12R β transcripts were quantitated by real time-PCR in cells collected on day 6 of culture. Data are expressed as relative Taqman units (RTU).

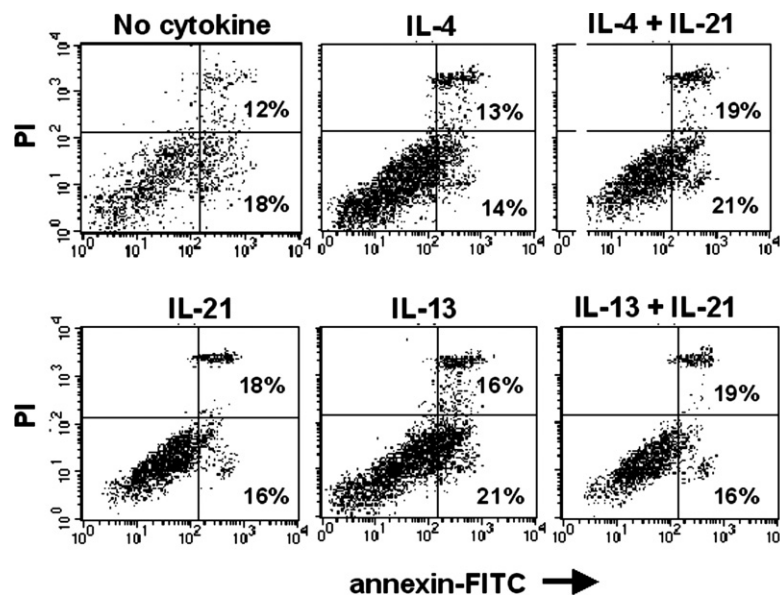


Fig. 7. IL-21 does not induce high levels of B cell apoptosis. Unfractionated human PBMC were treated with PHA on day 0. Cytokines were added on day 2. On day 4, cells were stained with APC-conjugated anti-human CD19, PI, and FITC-annexin. Gated B cells were analyzed for percentages of late apoptotic cells (PI⁺/annexin⁺; upper right quadrant) and early apoptotic cells (PI^{neg}/annexin⁺; lower right quadrant).

cytokines would have a net activating or inhibitory effect. Results showed that the combination of IL-4 and IL-13 was inhibitory for IgE production. Thus, IL-13 did not rescue IgE production from PHA-stimulated PBMC

treated with IL-4 and IL-21 (Fig. 8A), whereas addition of IL-4 reduced the IgE production that was normally seen in PHA-stimulated PBMC treated with IL-13 and IL-21 (Fig. 8B).

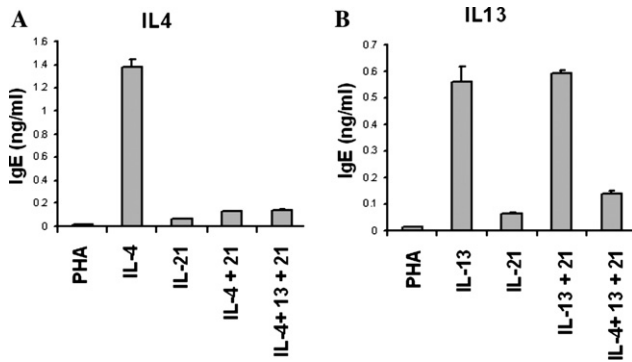


Fig. 8. IL-13 does not rescue IgE production from PHA-stimulated PBMC treated with IL-4 and IL-21. Unfractionated human PBMC were treated with PHA and cytokines as described in Section 2. On day 14, IgE levels were determined in pooled supernatants. (A) Effects of IL-13 and IL-21 on IgE production in response to IL-4. (B) Effects of IL-4 and IL-21 on IgE production in response to IL-13.

3.10. CD40 ligation overcomes the inhibitory effect of IL-21 on IgE production

We have observed that in human PBMC stimulated with anti-CD40 and IL-4, the addition of IL-21 boosts IgE production (Fig. 3). In contrast, in PBMC stimulated by PHA and IL-4, the addition of IL-21 blocks IgE production (Fig. 4). To help reconcile these observations, PHA-activated PBMC were treated with anti-CD40 in combination with IL-4 in the presence or absence IL-21. Under these conditions, IL-21 did not inhibit IgE production, but rather boosted levels of IgE above those seen with IL-4 alone (Fig. 9). Thus, anti-CD40 was able to overcome the inhibitory effect of IL-21 on IgE production by mitogen-activated PBMC.

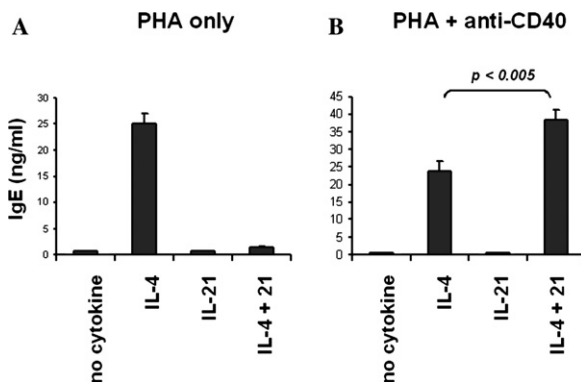


Fig. 9. Anti-CD40 rescues IgE production in PHA-stimulated PBMC cultures treated with IL-4 and IL-21. Unfractionated human PBMC were treated with PHA on day 0. Cytokines were added on days 2, 5, and 10. On day 15, IgE levels were determined in pooled supernatants. (A) Cells treated with PHA and cytokines only; (B) anti-CD40 added on day 2 of culture.

4. Discussion

IgE switch recombination in vitro requires two distinct signals: (i) the cytokines IL-4 or IL-13 to drive generation of the I ϵ germline transcript; and (ii) engagement of the B cell surface CD40 antigen to promote deletional switch recombination [42]. Cytokines provide important regulation of this process. IL-21 has been shown to inhibit IgE production in murine systems [26,27], but its effects on human IgE production have not been explored in detail. We have examined the effects of IL-21 on human IgE production under three different models of activation and found that, depending on the conditions, IL-21 can be stimulatory or inhibitory.

IL-21 is a pleiotropic cytokine produced by activated T cells, that has effects on many immune cell types [22,23]. Under appropriate conditions, it induces B cell proliferation [22] or B cell apoptosis [25]. In murine systems, IL-21 blocks IgE production both in response to IL-4 and mitogen stimulation in vitro, and specific immunization in vivo [26,27]. Accordingly, IL-21R-deficient mice have increased resting levels of serum IgE compared to wild-type mice [23], and produce higher levels of IgE upon immunization or infection [26]. In isolated murine B cells, IL-21 directly antagonizes IL-4 and LPS-induced I ϵ switch recombination [27]. Because IL-21 does not alter proliferation or STAT6 phosphorylation of murine B cells in response to IL-4 and LPS [27], the mechanism of its inhibitory activity has not been defined.

We now report that IL-21 enhances IL-4- or IL-13-mediated IgE production by isolated human B cells. IL-21 potentiated IgE synthesis not only by purified B cells but also by IL-4- or IL-13-treated PBMC in which B cell activation was achieved with anti-CD40 mAb. Resting human peripheral blood B cells express IL-21 receptor, and IL-21 can potentiate anti-CD40-induced B cell proliferation [22]. Consistent with this, we observed increased [^{3} H]thymidine incorporation by IL-4 or IL-13 treated B cells in the presence of IL-21. Thus, the enhancement of IgE production seen in the presence of IL-21 may be a consequence, at least in part, of IL-21-mediated B cell expansion.

In contrast, an inhibitory effect of IL-21 was observed when PHA-activated T cells were used as the source of costimulatory signals for IgE production. Under these conditions, IL-21 blocked IgE synthesis driven by IL-4 but not IL-13. Although not conclusive, these observations point to a T cell-dependent mechanism, as PHA is a T cell mitogen and T cells respond to IL-4 but not IL-13. Because anti-CD40 antibody could overcome the inhibition, CD40L function or expression is implicated. Moreover, we observed I ϵ germline transcript in the absence of IgE synthesis, which is characteristic of defects in CD40L expression [43,44] or CD40 signalling [45]. Nevertheless, CD40L transcripts, which are labile

and limiting for protein expression [37,38] were not decreased by IL-21. Thus, we speculate that IL-21 may elicit additional cell surface signals that block T cell–B cell interaction in this system, or reduce the strength of the CD40L signal.

Several cytokines have been described to antagonize IgE production, including TGF- β [10,46,47], IFN- γ [10,13,14,46], IL-10 [21,48], and IL-12 [18]. We compared levels of these cytokines in cultures in which IgE was produced or inhibited. TGF- β was detected in all cultures, but showed no association with IgE levels (data not shown). IFN- γ transcription was elicited by IL-21 in PHA-stimulated PBMC cultures, in agreement with previous reports [36,40], but was not associated with loss of IgE synthesis. It was maintained with either IL-4 + IL-21 treatment, in which IgE production was blocked, or IL-13 + IL-21 treatment, in which there was no inhibition.

IL-10 blocks IgE production in a monocyte-dependent manner, such that it has no inhibitory activity on purified B cells [49], similar to the current findings with IL-21. Although IL-10 was found in PBMC cultures, it was not associated with inhibition of IgE production. Equivalent levels were seen in cultures stimulated with anti-CD40 mAb or PHA, although IgE was only inhibited with PHA. In PHA-treated PBMC, comparable levels of IL-10 were produced with IL-4 + IL-21, which was inhibitory for IgE production, and with IL-13 + IL-21, which was stimulatory. Neutralizing antibody to IL-10 did not reverse the inhibitory effect of IL-21 (data not shown). These observations indicate that IL-10 is not responsible for the effects of IL-21 seen in this system.

IL-12 has also been reported to reduce IL-4-driven IgE production by unfractionated PBMC, but not by purified B cells [18], similar to the current observations with IL-21. Furthermore, IL-21 may influence lymphocyte responses to IL-12. IL-21 up-regulates transcription of IL-12R β 2 in a human NK cell line and in primary human T cells [40], greatly enhances IL-12-mediated IFN- γ secretion by mouse NK cells [23], and promotes IL-12-mediated STAT4 binding to the IFN- γ -activated sequence of the IL2R α gene [40]. Nevertheless, we found no differences in levels of IL-12 protein, IL-12 mRNA (data not shown), or IL-12R β 2 transcript between cultures in which IgE production was inhibited and those with high levels of IgE. Thus, we were unable to define a cytokine responsible for the inhibitory effect of IL-21 on IgE production by PHA-stimulated PBMC.

IL-21 drives apoptosis of murine B cells, even those that have been stimulated with LPS [25,50]. IL-4 cannot rescue IL-21-treated B cells from apoptosis, but pre-activation with anti-CD40 mAb is protective [25]. Thus, unfractionated PBMC stimulated with IL-4 and PHA may have reduced IgE production because the B cells had undergone apoptosis, whereas those stimulated with anti-CD40 mAb were protected. We found apoptosis of B cells in PBMC cultures, which was marginally

increased with IL-21, but addition of IL-4 produced no more apoptosis than IL-21 alone or IL-13 + IL-21. Thus, conditions leading to reduced IgE production were not associated with enhanced B cell apoptosis.

In murine systems, no clear mechanism has been reported for the inhibitory effect of IL-21 on IgE production [26,27]. Similarly, in our studies with human cells, the mechanism for the inhibitory effect of IL-21 on IL-4-dependent, T cell-driven IgE production remains undefined. It is noteworthy in this regard that like IL-21, other cytokines, including IFN- γ [39], IL-10 [49], and IL-12 [18] all have been reported to inhibit IgE production by human PBMC but not by anti CD40-stimulated purified B cells. These observations suggest that important regulatory mechanisms for human B cell IgE production remain to be elucidated.

The differential effects of IL-21 on mitogen-activated PBMC stimulated with IL-4 vs. IL-13 are intriguing. Although these cytokines share common functions, mice lacking either IL-4 or IL-13 do not generate wild-type levels of IgE [51,52], suggesting that one cytokine alone cannot fully compensate for loss of the other. Indeed, IL-13 may be the major driver for atopic responses, as selective neutralization [53] or deletion [54] of IL-13 protected mice from development of asthma pathology despite the presence of IL-4. Recently, Hajoui et al. [55] have shown that IL-13 production by B cells themselves is required for generation of IgE in response to IL-4, and propose that B cell production of IL-13 is necessary for IL-4-induced IgE synthesis. Consistent with this, we observed IL-13 production in PHA-stimulated PBMC treated with IL-4, which was reduced by half with added IL-21 (data not shown). Furthermore, our findings that IL-21 antagonized IL-4-induced IgE generation in PHA-stimulated PBMC, while the IL-13 response remained robust, could signal a role for IL-21 in regulation of this autocrine pathway.

Whereas IgE directed against allergens is necessary and sufficient for development of atopic disease [56], IgG4 has long been thought to be protective [4–7]. We found that treatment of mitogen-activated PBMC with IL-21 alone stimulated IgG4 release. No IgE was produced under these conditions, suggesting that IL-21 may be capable of shifting the balance between IgG4 and IgE under appropriate circumstances. In contrast, although IL-4 or IL-13 generated high levels of I γ 4 germline transcript, we and others [57] found that these cytokines alone did not result in detectable IgG4 protein release from human peripheral B cells. Ig gene rearrangement and antibody secretion are differentially regulated events [58]. IL-4 induces IgG4 switch recombination in naïve B cells, but may repress secretion of mature protein in those cells that have already switched to IgG4 [59]. The opposite was seen with IL-21 alone, which did not induce I γ 4 germline transcript above unstimulated levels, but strongly enhanced secretion of IgG4 protein.

IL-21 induces secretion of all human IgG isotypes, while promoting de novo switch recombination specifically only to IgG1 and IgG3 [57]. Release of protein without de novo transcription suggests that IL-21 promoted the activation or expansion of B cell clones that had been committed in vivo to generation of IgG4, a process previously shown to account for the IL-4/IL-13-independent generation of IgG4 in vitro [60].

Taken together, these studies show that IL-21 stimulates or inhibits IgE and IgG4 production by human B cells depending on activation conditions. IL-21 has similarly contradictory responses in other systems. Under appropriate conditions, it can induce NK cell activation and/or apoptosis, stimulate or limit T cell expansion, and induce or inhibit T cell IFN γ production [61]. In the murine system, IL-21 triggers apoptosis of B cells treated with LPS, but co-stimulates proliferation of B cells treated with anti-CD40 or anti-IgM [25,50,62]. It has been proposed that IL-21 acts as a checkpoint for productive immune responses, driving activation and proliferation under permissive conditions, while promoting apoptosis of lymphocytes activated inappropriately or in an unfavorable environment [50,61,62]. In the context of the current study, IL-21 appears to exert a regulatory influence over human IgE production, either boosting levels or ensuring against over-production of this critical effector molecule.

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