

IFN- γ -Mediated Inhibition of Human IgE Synthesis by IL-21 Is Associated with a Polymorphism in the *IL-21R* Gene¹

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IL-21 is a cytokine produced by CD4⁺ T cells that has been reported to regulate human, as well as, mouse T and NK cell function and to inhibit Ag-induced IgE production by mouse B cells. In the present study, we show that human rIL-21 strongly enhances IgE production by both CD19⁺CD27[−] naive, and CD19⁺CD27⁺ memory B cells, stimulated with anti-CD40 mAb and rIL-4 and that it promotes the proliferative responses of these cells. However, rIL-21 does not significantly affect anti-CD40 mAb and rIL-4-induced C ϵ promoter activation in a gene reporter assay, nor germline C ϵ mRNA expression in purified human spleen or peripheral blood B cells. In contrast, rIL-21 inhibits rIL-4-induced IgE production in cultures of PBMC or total splenocytes by an IFN- γ -dependent mechanism. The presence of a polymorphism (T-83C), in donors heterozygous for this mutation was found to be associated not only with lower rIL-21-induced IFN- γ production levels, but also with a lower sensitivity to the inhibitory effects of IL-21 on the production of IgE, compared with those in donors expressing the wild-type IL-21R. Taken together, these results show that IL-21 differentially regulates IL-4-induced human IgE production, via its growth- and differentiation-promoting capacities on isotype-, including IgE-, committed B cells, as well as via its ability to induce IFN- γ production, most likely by T and NK cells, whereas the outcome of these IL-21-mediated effects is dependent on the presence of a polymorphism in the *IL-21R*. *The Journal of Immunology*, 2006, 177: 5006–5013.

Immunoglobulin E plays an important role in the pathogenesis of allergic disorders, such as allergic asthma, allergic rhinitis, and atopic dermatitis, which is due to its ability to bind to the high-affinity IgE receptor, Fc ϵ R1, on mast cells and basophils, thereby triggering immediate-hypersensitivity reactions that result eventually in the release of pharmacological mediators including histamine, leukotrienes, cytokines, and chemokines. In addition, following its interaction with CD23, IgE facilitates capture and uptake of allergens by B cells, leading to perpetuation and worsening of allergic conditions, characterized and accompanied by increased IgE serum concentrations (reviewed in Ref. 1). IgE serum levels are also elevated in parasitic diseases, in which it has a role in host immune defense mechanisms, as well as in some rare genetic disorders of the immune system, including Wiskott-Aldrich syndrome (2), Hyper-IgE syndrome (3) and immune dysfunction polyendocrinopathy enteropathy X-linked syndrome (4). Under nonpathological conditions, however, serum IgE levels are much lower than those of other isotypes, indicating that synthesis of IgE is tightly regulated to avoid allergic immune reactions.

The molecular mechanisms underlying the production of IgE are similar to those for other isotypes and are controlled by specific signals from cytokines, as well as the TNFR superfamily member CD40 (reviewed in Ref. 5). They involve a two-step process of

DNA excision and ligation, followed by a deletional class switch recombination (CSR)³ event (reviewed in Ref. 6). CSR results in the deletion of DNA between switch (S) regions, which are located upstream to every C region gene of the Ig H chain, except for δ . A prerequisite for CSR to the production of IgE is the activation of the promoter of the I ϵ exon, located immediately upstream of the S ϵ region, which leads to the transcription of an unarranged, germline, ϵ gene. As the magnitude of germline ϵ transcription is quantitatively correlated with CSR efficiency (6), its modulation directly affects the capacity of a B cells to produce IgE (7). The Th2 cytokines IL-4 and IL-13 are potent inducers of C ϵ germline transcription in human B cells (8, 9), functioning through the activation of STAT6 (review in Ref. 10). Among the human cytokines that are known to inhibit IgE synthesis, only TGF- β has been shown to directly repress C ϵ germline transcription, whereas IFN- γ and IFN- α act on other stages of the regulatory process, resulting in IgE production (7).

Results from experimental mouse models have indicated an inhibitory role for IL-21, a typical four-helix-bundle cytokine, the receptor of which shares the common γ -chain with the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (11), in the production of IgE. Mice deficient in the IL-21R have higher serum IgE levels, compared with those of wild-type animals, and respond with increased IgE production to infection with *Toxoplasma gondii*, a parasite that, in wild-type mice, induces a strong Th type 1 cell-dependent IFN- γ response (12). Moreover, in a different study, injection of IL-21 into mice was found to inhibit Ag-specific IgE, but not IgG2a, responses and this effect was reportedly due to the inhibition of IL-4-induced germline C ϵ transcription by IL-21 (13). Recently, an association between a single nucleotide polymorphism (T-83C) in the *IL-21R* and the presence of elevated IgE serum levels has been reported (14), suggesting a negative regulatory function of IL-21 in the production of this isotype in humans as well.

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³ Abbreviations used in this paper: CSR, class switch recombination; EFl α , elongation factor 1 α .

Therefore, in the present study, we have analyzed the effects of IL-21 on IL-4-induced human IgE synthesis *in vitro* and have investigated whether these effects are associated with a polymorphism in the *IL-21R* gene.

Materials and Methods

Donors and cells

All human PBMC and spleen cells used in this study were obtained in accordance with the guidelines of the ethical committee of the Montpellier University Hospitals. Human PBMC were isolated from 15 healthy donors and 17 atopic patients (Service des Maladies Respiratoires, Centre Hospitalier Universitaire (CHU) Arnaud de Villeneuve) by centrifugation over Ficoll-Hypaque (Amersham Biosciences). Atopy was defined on the basis of elevated, allergen-specific serum IgE levels, a positive skin prick test, and a positive clinical score, as a result of exposure to common aeroallergens. Highly purified (purity, >98%) CD19⁺ spleen B cells (Service de Chirurgie Digestive, CHU St. Eloi) were obtained from human spleen fragments of healthy organ donors by positive selection using specific mAb-coated magnetic beads (Miltenyi Biotec) and a preparative magnetic cell sorter (Miltenyi Biotec), according to the manufacturer's recommendations. Purified CD19⁺ B cells (purity, >98%) were also isolated from PBMC (Etablissement Français du Sang) using the Rosettesep procedure (StemCell Technologies), according to the manufacturer's recommendations. Naive B cells were obtained following staining of CD19⁺ B cells with a FITC-conjugated anti-CD27 mAb (BD Pharmingen) and sorting of CD19⁺CD27[−] B cells, using a FACS Vantage (BD Biosciences), according to the procedure described by Scheffold et al. (15). Reanalysis of sorted cells showed >99% purity. The EBV-negative Burkitt lymphoma cell line BL-2 clone 20, containing all regulatory elements of the human IgE germline promoter, has been described (16).

Culture conditions

Stimulation of B cells for proliferative responses or induction of Ig production was conducted as follows: naive or memory CD19⁺ human B lymphocytes (10⁶/ml) were cultured with 1 μ g/ml anti-CD40 mAb 89 (17), in the presence or absence of combinations of rIL-4 (gifts from Dr. F. Brière, Schering-Plough, Dardilly, France) and rIL-21 (a gift from Dr. D. Foster, Zymogenetics, Seattle, WA) in flat-bottom 96-well culture plates (Nunc) in Yssel's medium (18), supplemented with 10% FCS in sextuplicate in a final volume of 200 μ l. PBMC (10⁶/ml) were cultured with the same combinations of cytokines, in the presence or the absence of the anti-CD40 mAb. Where indicated, a neutralizing polyclonal goat anti-human IFN- γ receptor 1 (anti-IFN- γ R1) Ab (R&D Systems), an anti-IFN- γ R1 mouse mAb (BD Biosciences), or a normal purified mouse IgG1 (Southern Technologies) was added at a concentration of 2 μ g/ml at the initiation of the cultures and subsequently at days 2 and 4, at the same concentration. After 12 days of incubation at 37°C and 5% CO₂, culture supernatants were collected and IgE content was measured. The production of IFN- γ was determined on the same culture supernatants collected at day 12 in parallel cultures. Proliferative responses were measured after 48 h of culture. For analysis of C ϵ transcripts, purified CD19⁺ B cells were cultured under the same experimental conditions and cells were harvested for mRNA isolation after 5 days of culture. rTGF- β (PeproTech) was used as a positive control in the C ϵ promoter gene reporter assay.

Proliferation assay

Proliferative responses were measured by thymidine incorporation of stimulated B cells. After 48 h of culture, 37 kBq (1 μ Ci) of tritiated thymidine ([³H]TdR; Amersham Biosciences) was added to the cultures, and after another 18 h of culture, the cells were harvested onto glass fiber sheets, using an automated cell harvester (Tomtec). Radioactivity was measured, using a scintillation counter (Wallac). Results are expressed as mean and SD of triplicate cultures.

Measurement of IgE and IFN- γ production

IgE and IFN- γ content of culture supernatants was determined by isotype- and cytokine-specific ELISA, respectively, as described previously (19, 20).

cDNA synthesis, RT-PCR analysis, and Northern blot analysis

RNA extraction, reverse transcription, and amplification of cDNA (corresponding to 1 μ g of RNA per PCR sample) was conducted as described (21). The nucleotide sequences of PCR primers were as follows: C ϵ , sense I ϵ , GAC GGG CCA CAC CAT CC; antisense C ϵ , CGG AAG GTG GCA TTG GAG G; β -actin, sense, GCT GCT GAC CGA GGC CCC CCT GAA C; and antisense, CTC CTT AAT GTC ACG CAC GAT TTC.

Germline ϵ and β -actin mRNA transcripts were analyzed by Northern blotting using cRNA probes, complementary to C ϵ and β -actin mRNA, as described (8). The presence of transcripts for the IL-21R in BL-2 clone 20 cells was determined by RT-PCR, using the following primer sequences: sense, TGCCCCGACCTCGTCTGCT; antisense, TGGCCTCGTCCTTCAGCT.

Analysis of IgE germline RNA transcripts by real-time PCR, using elongation factor 1 α (EF1 α) levels as an internal control, was conducted using the following PCR primer sequences (21): upstream PCR primer, located in I ϵ exon in X56797 position 591–617: GGG AGC TGT CCA GGA ACC CGA CAG AGC; downstream PCR primer, located in C ϵ exon (X95746): GGG GTG AAG TCC CTG GAG C; upstream PCR primer for detection of elongation factor EF1 α : TTT GAG ACC AGC AAG TAC TAT GTG ACT; downstream PCR primer for detection of elongation factor EF1 α : TCA GCC TGA GAT GTC CCT GTA A.

Analysis of C ϵ promoter gene activity

BL2-clone 20 cells were seeded at 10⁶ cells/ml in a 96-well flat-bottom tissue culture plate (Nunc) in Yssel's medium, supplemented with 10% FCS and incubated with 10 ng/ml rIL-4, 1 μ g/ml anti-CD40 mAb, and 1 μ g/ml goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) to cross-link the anti-CD40 mAb. The cells were then lysed and luciferase activity was determined, according to the manufacturer's instructions, using the dual-luciferase reporter assay system (Promega) on a LuminoscanRS luminometer (Labsystems). In parallel cultures, cell viability was determined by trypan blue exclusion. Results from preliminary kinetics experiments showed that IL-4/anti-CD40 mAb-mediated induction of the C ϵ promoter increased with the time up to 96 h with significant induction occurring after 24 h of incubation, which was consistent with that of germline RNA induction (8). Based on this result, an incubation time of 48 h was chosen for subsequent experiments conducted in the presence of rIL-21 or rTGF- β .

Genotyping of the IL-21R gene

Genomic DNA was isolated from peripheral blood by standard phenol-chloroform extraction, followed by ethanol precipitation (22). A 521-bp PCR fragment including the −83T>C polymorphism was amplified using the primers: 5'-GCC TGC TGC ATC TAG TGT C-3' (upstream) and 5'-CCG TGC TTC ATG AGA AAG A-3' (downstream). PCR were conducted in a total volume of 50 μ l containing 100 ng of DNA, 0.5 μ M of each primer, 0.2 mM each dNTP, 1.5 mM MgCl₂, 5 μ l of 10 $\times$ buffer, and 1 U of TaqDNA polymerase (Invitrogen Life Technologies). Samples were denatured at 94°C for 10 min followed by 32 cycles at 94°C for 45 s, 55°C for 30 s, 72°C for 60 s, and a final 10-min extension at 72°C. The fragments were sequenced using the ABI Prism Big Dye Terminator DNA Sequencing kit (Applied Biosystems) according to the manufacturer's instructions, and analyzed on an ABI Prism 310 Genetic Analyser (PerkinElmer). The IL-21R sequencing primer was as follows: 5'-CGT GCT TCA TGA GAA AGA TG-3'.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U* test for unpaired, intergroup, comparison and the Wilcoxon test for intragroup comparison. Probability values of $p \leq 0.05$ were accepted as significant.

Results

IL-21 enhances IgE production, as well as proliferative responses by human CD19⁺ B cells stimulated with anti-CD40 mAb in the presence of IL-4

The capacity of rIL-21 to modulate the production of rIL-4-induced IgE production was investigated using purified human CD19⁺ peripheral blood- or spleen-derived B cells, obtained from healthy nonatopic donors, that were stimulated with anti-CD40 mAb in the presence of rIL-4 and increasing concentrations of rIL-21. As expected, addition of rIL-4 to cultures of these cells induced the production of IgE (Fig. 1, A and B). rIL-21 alone did not induce IgE synthesis but strongly enhanced IL-4-induced IgE production, in a dose-dependent fashion, irrespective of the origin of the B cells. Similar results were obtained for purified CD19⁺CD27⁺ memory B cells (results not shown). rIL-21 also enhanced rIL-4-induced IgE production in purified splenic CD19⁺CD27[−] naive B cells (Fig. 1C). Optimal IgE production-enhancing capacity of rIL-21 was observed at concentrations between 1 and 10 ng/ml cytokine.

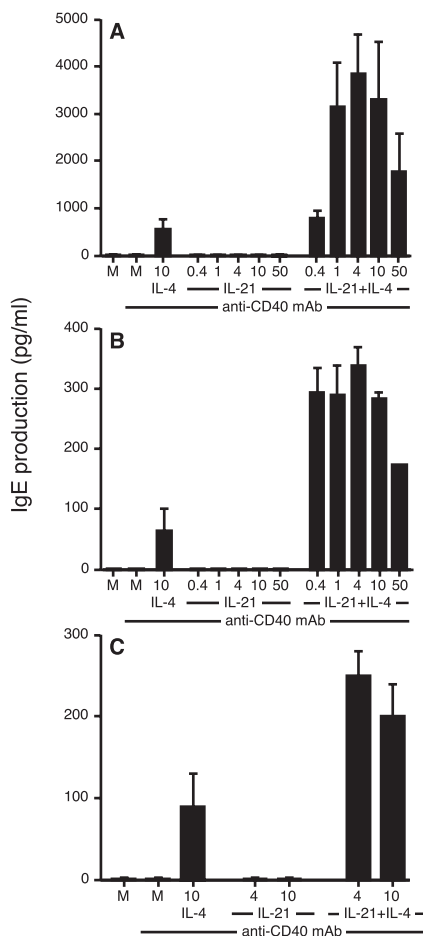


FIGURE 1. IL-21 enhances IgE production by CD19⁺ B cells stimulated with anti-CD40 mAb and IL-4. Human CD19⁺ B cells, purified from spleen (A) or peripheral blood (B) and CD19⁺CD27⁻ naive splenic B cells (10⁶/ml) (C), were cultured in medium alone or stimulated with anti-CD40 mAb (1 μ g/ml) in the absence or presence of rIL-4 (10 ng/ml) and various concentrations of rIL-21. After 12 days of culture, supernatants were harvested and analyzed for IgE content by isotype-specific ELISA. Results represent mean $\pm$ SD from three independent experiments. M, medium only.

Moreover, rIL-21 strongly enhanced rIL-4 and anti-CD40 mAb-induced proliferative responses of both purified CD19⁺CD27⁻ naive, as well as CD19⁺CD27⁺ memory B cells to a similar extent (Fig. 2).

IL-21 affects neither C ϵ promoter activity nor expression of germline C ϵ transcripts induced by anti-CD40 mAb and IL-4

The activation of the C ϵ promoter by IL-4, resulting in the expression of C ϵ -specific germline transcripts, is considered to be an obligatory event, preceding successful isotype switching of human B cells to the production of IgE. Therefore, the effect of IL-21 was tested in a germline C ϵ promoter gene reporter assay, using the BL-2 clone 20 transfectant, containing all regulatory elements of the human germline C ϵ promoter. BL-2 cells stimulated with anti-CD40 mAb and rIL-4 expressed transcripts for the IL-21R (results not shown), indicating that these cells are likely to be responsive to IL-21. Stimulation of BL-2 clone 20 cells with rIL-4 and anti-CD40 mAb induced a strong increase in the reporter gene expression, which was totally inhibited by the addition of rTGF- β , used as a positive control (Fig. 3). The addition of rIL-21 did not significantly enhance the IL-4/anti-CD40 mAb-induced C ϵ promoter activity. To further investigate whether the enhancement of IgE

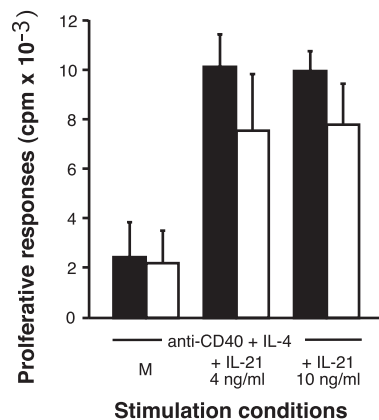


FIGURE 2. IL-21 enhances anti-CD40 mAb and IL-4-induced proliferation of CD19⁺ human B cells. Purified human splenic CD19⁺CD27⁻ naive (black histograms) and CD19⁺CD27⁺ memory (open histograms) B cells were stimulated with anti-CD40 mAb (1 μ g/ml) and rIL-4 (10 ng/ml) in the absence (M) or presence of rIL-21. After 48 days of culture, proliferative responses were measured by [³H]TdR incorporation. Results are expressed as the mean $\pm$ SD of two independent experiments. Proliferative responses of all cultures in the absence of rIL-4 are <500 cpm.

synthesis by rIL-21 was reflected in an increase in IL-4-induced isotype switching, the expression of germline C ϵ RNA by CD19⁺ B cells, stimulated with anti-CD40 mAb, rIL-4, and rIL-21, was determined by Northern blot analysis. Stimulation of purified human spleen- (Fig. 4A) or peripheral blood (Fig. 4B)-derived B cells with the combination of anti-CD40 mAb and rIL-4 resulted in a strong induction of the expression of germline C ϵ transcripts, whereas stimulation via CD40 alone did not have any effect. Expression of these transcripts was not observed in cells stimulated with rIL-21 and anti-CD40 mAb. Addition of rIL-21 did not affect germline C ϵ transcript expression, induced by IL-4 and anti-CD40 mAb, neither in human spleen- nor in peripheral blood-derived B cells (Fig. 4, A and B), which was confirmed by results from quantitative PCR analysis showing that the addition of rIL-21 to purified CD19⁺ peripheral blood B cells, stimulated with rIL-4 and anti-CD40 mAb, did not increase the expression of C ϵ transcripts levels, compared with those induced with rIL-4 alone (Fig. 4C). Taken together, these results indicate that the enhancement of IL-4-induced IgE synthesis by IL-21 is not associated with an enhancing effect of the latter cytokine on germline C ϵ RNA synthesis and C ϵ promoter activity, but is likely to be the consequence of its capacity to promote expansion of activated B cells.

IL-21 indirectly inhibits IL-4-induced IgE production via the induction of IFN- γ production by T and/or NK cells

IL-21 has been shown to enhance IFN- γ production by human T and NK cells (23). Furthermore, IFN- γ is known to strongly inhibit IL-4-induced IgE production by B cells (24), suggesting that IL-21 might have an inhibitory role on IgE synthesis through the enhancement of production of this cytokine. This possibility was investigated by comparing the effect of rIL-21 on rIL-4-induced IgE production by populations of purified CD19⁺ B cells with that obtained by unfractionated PBLs, containing T and NK cells. Addition of rIL-4 to cultures of PBMC, in which the latter lymphocytes were present, resulted in the production of low, but reproducible, levels of IgE (Fig. 5). In contrast to its enhancing effect on purified CD19⁺ peripheral B cells (Fig. 1), rIL-21 inhibited the production of IgE in cultures of PBMC, at concentrations as low as 3 ng/ml, in 10 of 15 healthy donors tested (Fig. 5A). However, at

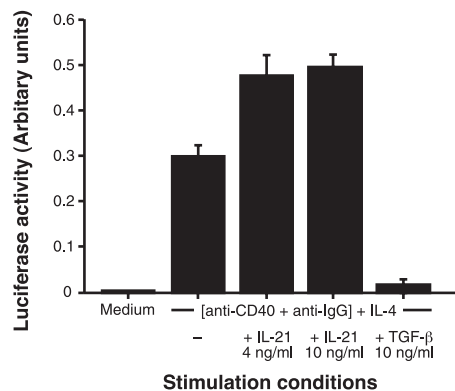


FIGURE 3. Effect of IL-21 germline C ϵ promoter activity, induced by anti-CD40 mAb and IL-4. The Burkitt lymphoma cell line BL-2 clone 20 was cultured in medium alone or stimulated with anti-CD40 mAb (1 μ g/ml) and cross-linked with goat-anti-mouse IgG (1 μ g/ml) and rIL-4 (10 ng/ml), in the absence (–) or presence of rIL-21 or rTGF- β , respectively, for 48 h, and germline C ϵ promoter activity was determined by luciferase assay. Results of two independent experiments are shown.

a lower concentration of rIL-21 (1 ng/ml), IL-4-induced IgE production by B cells of these donors was significantly and reproducibly enhanced. In the five other donors, the inhibitory effects of rIL-21 on IgE synthesis were only detected at concentrations equal to or higher than 30 ng/ml, whereas rIL-21 enhanced IL-4-induced IgE production at concentrations ranging between 1 and 10 ng/ml (Fig. 5B). These results suggest the existence of a differential sensitivity of these two groups of donors to respond to the inhibitory effects of IL-21.

To determine whether the IL-21-induced inhibitory effects could be explained by its capacity to induce the synthesis of IFN- γ , production levels of the latter cytokine were measured in cultures of PBMC stimulated with rIL-4 and rIL-21. Levels of IFN- γ detected in these cultures were found to inversely correlate with those of rIL-4-induced IgE production, both in the sensitive (Fig. 6, A and B) and the less sensitive (C and D) group of donors. It is of note that addition of rIL-21 to cultures of PBMC, stimulated with rIL-4 in the presence of an anti-CD40 mAb, resulted in a strong enhancement of IgE production (Fig. 6, E and F), reminiscent of that observed in cultures of purified B cells. To directly demonstrate that IL-21 inhibits the production of IgE, via the induction of IFN- γ production by non-B cells, either a neutralizing goat-anti-IFN- γ R1 Ab or a mouse-anti-IFN- γ R1 mAb was added, at various time points, to cultures of PBMC stimulated with both rIL-4 and various amounts of rIL-21. The addition of either anti-IFN- γ R1 Ab at the onset of the cultures alone did not affect the IL-21-mediated inhibition of IgE production (Fig. 7). However, their repeated addition during the first 4 days of culture resulted in a complete neutralization of IFN- γ production, thereby restoring the production of IgE to levels comparable, or even superior, to those observed with rIL-4 alone. The addition of a nonrelevant isotype-matched control Ab had no effect (Fig. 7). At a concentration of 90 ng/ml, IL-21 completely inhibited IL-4-induced IgE synthesis, irrespective of the presence of the polyclonal goat-anti-IFN- γ R1 Ab, indicating that under these conditions the amount of IFN- γ induced by IL-21 could no longer be neutralized.

The magnitude of inhibition of IL-4-induced IgE synthesis by IL-21 is determined by a genetic polymorphism of the IL-21R

It has been reported that the presence of a polymorphism (T-83C) in the gene encoding the human IL-21R is associated with elevated serum levels of IgE, in both healthy and, to a greater extent, atopic individuals (14). Because of the differential sensitivity of PBMC to

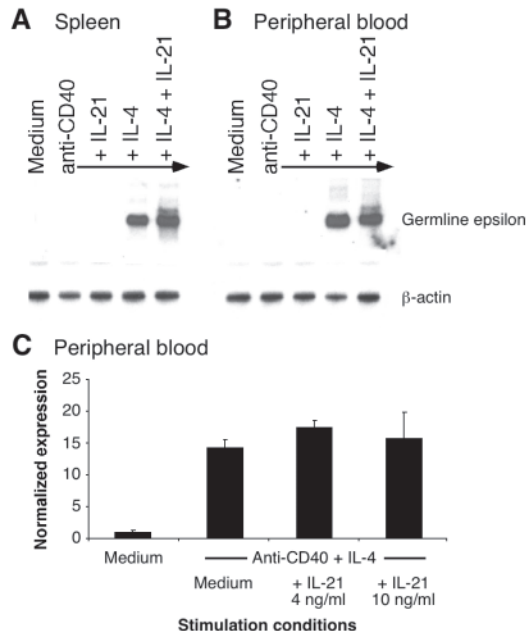


FIGURE 4. IL-21 does not affect expression of germline C ϵ transcripts, induced by anti-CD40 mAb and IL-4 in human B cells. Human CD19⁺ B cells, isolated from spleen (A) or peripheral blood (B), were cultured in medium alone or stimulated with anti-CD40 mAb (1 μ g/ml) in the absence or presence of combinations of rIL-4 (10 ng/ml) and rIL-21 (10 ng/ml). After 5 days of culture, expression of mRNA specific for germline C ϵ and β -actin, transcripts were evaluated by Northern blot analysis. C, Purified human CD19⁺ peripheral blood B cells were cultured under the same experimental conditions and levels of germline C ϵ mRNA expression were determined by real-time PCR, using EF1 α mRNA expression levels as an internal control. Results of two independent experiments are shown.

respond to the effects of rIL-21, as described above, we therefore determined, in a sizeable cohort of donors consisting of both healthy and atopic individuals, whether their clinical status, as well as the presence of this polymorphism, might affect the capacity of IL-21 to modulate IL-4-induced IgE synthesis in vitro. In a first series of experiments, we determined whether PBMC of all healthy and allergic donors included in the study differed in their capacity to respond to the IgE production-inducing effects of IL-4. As shown in Fig. 8A, levels of rIL-4-induced IgE production in vitro did not differ significantly ($p = 0.56$, Mann-Whitney U test) between the healthy and allergic donors. Next, the capacity of rIL-21,

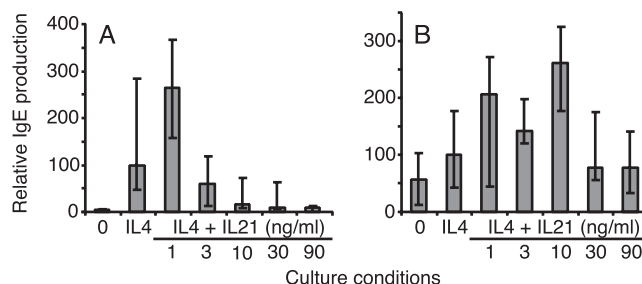


FIGURE 5. The magnitude of inhibition of IL-4-induced IgE production by IL-21 is donor dependent. Human PBMC (10^6 /ml), isolated from healthy donors ($n = 15$), were stimulated with combinations of rIL-4 (10 ng/ml) and increasing concentrations of rIL-21. After 12 days of culture, supernatants were harvested and analyzed for IgE content by isotype-specific ELISA. Data represent median $\pm$ 75% percentiles of IgE production, obtained for each culture condition, thereby distinguishing a group of donors sensitive (A; $n = 10$) or less sensitive (B; $n = 5$) to the effects of rIL-21.

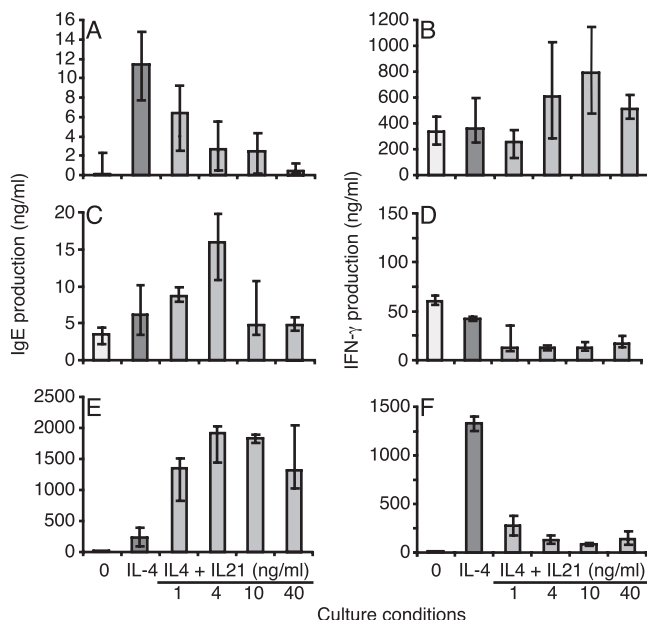


FIGURE 6. The modulatory effects of IL-21 on IL-4-induced IgE synthesis by B cells and IFN- γ production by T and/or NK cells are inversely correlated. Human PBMC from a group of donors classified as sensitive ($n = 6$) (A and B) or less sensitive ($n = 3$) (C and D) to the effects of IL-21 were stimulated, as described in Fig. 5, or PBMC of the same donors, irrespective of their classification (E and F) ($n = 9$) were stimulated under the same conditions in the presence of 1 μ g/ml anti-CD40 mAb. After 12 days of incubation, the culture supernatants were analyzed for IgE (A, C, and E) and IFN- γ (B, D, and F) content by specific ELISA. Data represent the median $\pm$ 75% percentile of IgE or IFN- γ production obtained for each culture condition.

at a concentration of 10 ng/ml, to inhibit or to enhance rIL-4-induced IgE synthesis in vitro, was expressed as the percentage of variation of response, compared with that obtained in rIL-4 alone. Similarly, the rIL-21-induced modulating effects on rIL-4-induced IgE production did not differ in a statistically significant manner between the two groups (Fig. 8B), indicating that the latter effects are not linked to the presence of atopy.

Finally, we determined whether the observed differential sensitivity to the IL-21 modulating effects on IL-4-induced IgE synthesis is associated with a genetic polymorphism in the *IL-21R* gene. For this analysis, we used PBMC from a total of 7 healthy and 17

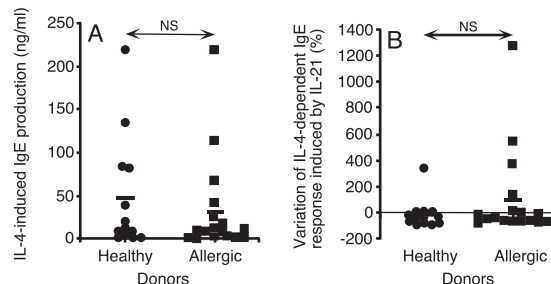


FIGURE 8. Effects of IL-4 and IL-21 on IgE production by PBMC in vitro do not differ between healthy and allergic donors. Human PBMC (10^6 /ml), isolated from 13 healthy and 17 atopic donors, were stimulated with rIL-4 (A) or with a combination of rIL-4 and rIL-21 (B) (both at 10 ng/ml). After 12 days of incubation, culture supernatants were analyzed by IgE-specific ELISA, and comparative statistical analysis was conducted using the Mann-Whitney *U* test.

atopic donors, genotyped for the presence or absence of the T-83C mutant *IL-21R* gene. Results from sequencing analysis showed that 13 of 24 donors were homozygous for the wild-type allele of the *IL-21R* gene, whereas 11 of 24 donors were heterozygous, expressing one copy of the mutated gene (Table I). None of the donors, carrying this polymorphism, expressed two copies of the mutant allele. Again, no statistically significant differences could be observed between the two groups of donors with respect to the capacity of IL-4 to promote IgE synthesis in vitro (Fig. 9A). However, as shown in Fig. 9B, the variation of rIL-4-induced IgE production, mediated by rIL-21, was significantly different between the donors expressing the wild-type alleles and those carrying a mutant copy of the *IL-21R* gene ($p = 0.0054$, Mann-Whitney *U* test). Hence, in vitro IgE synthesis by PBMC of 12 of the 13 donors (92%), homozygous for the wild-type allele, was already inhibited by 10 ng/ml rIL-21, whereas this concentration of rIL-21 did not inhibit, but rather enhanced rIL-4-dependent IgE production in vitro by 7 of the 11 individuals (64%) who carried one copy of polymorphic gene. Finally, rIL-21 induced higher levels of IFN- γ production by PBMC from wild-type individuals, compared with those carrying the *IL-21R* polymorphic variant (Fig. 9C; $p = 0.018$, Mann-Whitney *U* test). Taken together, these results suggest that the presence of at least one copy of the T-83C mutation of the *IL-21R* gene is associated with a decreased capacity of IL-21 to induce the production of IFN- γ , resulting in a

FIGURE 7. IL-21-induced inhibition of IgE production is mediated by an IFN- γ -dependent mechanism. Human PBMC (10^6 /ml), isolated from three healthy donors, were stimulated as described in Fig. 5. Either a neutralizing polyclonal goat anti-IFN- γ R1 Ab (A), a mouse anti-IFN- γ R1 mAb (B and D), or a normal mouse IgG1 (C and E) was added at a concentration of 2 μ g/ml at the initiation of the cultures or at a 2-day interval, as indicated in the figure. After 12 days of incubation, culture supernatants were analyzed for IgE content. Data represent the median $\pm$ 75% percentile of IgE obtained for each culture condition.

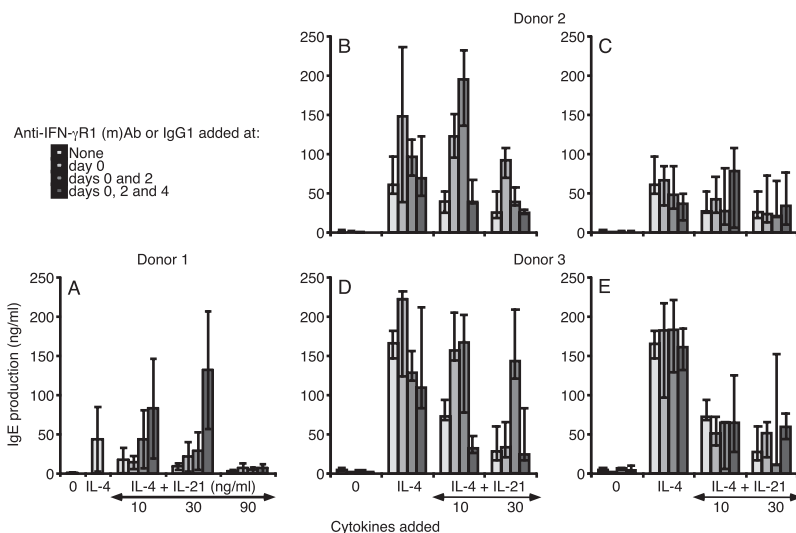


Table I. Characterization of the donors^a

Donors	Age	Sex	Atopy ^b	<i>IL-21R</i> Gene ^c
Healthy				
1	51	M	None	WT
2	40	M	None	WT
3	27	M	None	WT
4	60	M	None	WT
5	50	M	None	Variant
6	24	F	None	WT
7	32	F	None	WT
Atopic				
1	65	F	Latex, Dp, Gr	WT
2	36	F	Peni, DHE	WT
3	44	F	Peni, Dp	WT
4	51	F	Peni, Dp, latex	WT
5	46	M	Gr	WT
6	29	M	Gr, AINS	WT
7	35	F	Peni, Dp	WT
8	57	M	Latex, Dp, Gr	Variant
9	35	F	Latex, Dp, Gr, Ckrh	Variant
10	45	M	Dp	Variant
11	59	M	Peni	Variant
12	44	F	Peni	Variant
13	58	F	Latex, Dp, Gr	Variant
14	26	F	Latex, peni, Dp, Gr	Variant
15	42	F	Latex, Dp, Ckrh	Variant
16	28	M	Gr	Variant
17	54	F	Latex, Gr, quinol	Variant

^a Genomic DNA was isolated from peripheral blood of 7 healthy nonallergic donors and 17 atopic patients, and the *IL-21R* gene was analyzed by sequencing as indicated in *Materials and Methods*.

^b Dp, *Dermatophagoides pteronyssinus*; Gr, grass pollen; Ckrh, cockroach; Peni, penicillin; quinol, quinolone; DHE, dihydroergotamine; AINS, anti-inflammatory nonsteroid drug.

^c WT, Donors carrying both wild-type alleles of the *IL-21R* gene; variant, donors carrying one copy of the polymorph T-83C allele.

reduced sensitivity of the individuals carrying this allele to the inhibitory effect of IL-21 on the production of IgE.

Discussion

The production of IgE, following CSR by B cells, is regulated by molecular events that result from interactions between CD40, expressed on B cells, and CD154, expressed on activated T cells, as well between IL-4 and IL-13 with their respective receptors. Because of the potent, potentially harmful and life-threatening biological effects of allergen-specific IgE, plasma levels of this isotype are much lower compared with those of plasma IgG, even in highly atopic individuals, indicating that its production must be tightly regulated. In addition to various cell surface receptors and transcription factors (reviewed in Ref. 1), several cytokines have

been identified that exert negative regulatory effects on the production of IgE by B cells, among which IFN- γ , IFN- α , TGF- β , and IL-10 are the most prominent. In the present study, we provide evidence that IL-21, a four-helix-bundle cytokine, has differential, inhibitory, as well as stimulatory, effects on human IgE synthesis.

An inhibitory role for IL-21 on the production of IgE has already been established in experimental mouse models. Mice deficient in the IL-21R have enhanced IgE levels (12), whereas injection of rIL-21 into BALB/C mice was found to prevent Ag-specific IgE, but not IgG2a, responses (13). However, in contrast to the latter study in which it was reported that IL-21 directly inhibited IgE production of splenic mouse B cells stimulated with LPS and rIL-4, our data point to an enhancing effect of human IL-21 on IL-4-driven IgE production by anti-CD40 mAb stimulated human splenic or peripheral B cells that we show to be associated with its strong proliferative effects on both naive and memory B cells in agreement with a recently published study (25) and that confirms the growth-promoting effects of IL-21 on activated B cells, irrespective of their isotype commitment (11). Indeed, it has been reported by Tangye et al. (26) that successful isotype switching requires entry of B cells into the proliferation phase and therefore IL-21 might affect both naive B cells as well as already isotype (but not necessarily IgE)-committed B cells. The latter conclusion is supported by a recent report by Ettinger et al. (25) showing that IL-21 induces not only the proliferation, but also the differentiation of anti-CD40 mAb-activated human naive and memory B cells into IgG and IgA Ab-secreting plasma cells. However, IL-21 does not induce IgE synthesis by anti-CD40-activated memory B cells in the absence of IL-4 (this study and Ref. 25), indicating that the differentiation of already IgE-committed memory B cells into IgE-secreting plasma cells requires other signals, most likely IL-4 or IL-13.

Whereas mouse rIL-21 reportedly inhibits IL-4-induced germline C ϵ transcription in activated B cells (13), we demonstrate that human rIL-21 does not affect germline C ϵ transcription, neither in splenic, nor in peripheral blood B cells, as detected by Northern blotting, as well as by the more sensitive real-time PCR analysis. Additional support for the latter notion comes from the observation that IL-21 does not inhibit C ϵ promoter activation induced by IL-4 and anti-CD40 mAb. Induction of germline C ϵ transcription is a required step in the process leading to IL-4- and IL-13-mediated IgE synthesis, and the common denominator of the action of cytokines that inhibit IgE production is often their capacity to regulate germline C ϵ transcription (6). The lack of inhibition of germline C ϵ transcription by human rIL-21 is therefore in agreement with its enhancing, rather than inhibiting, effects on the production of IgE by purified B cells. It should be stressed that any effect of

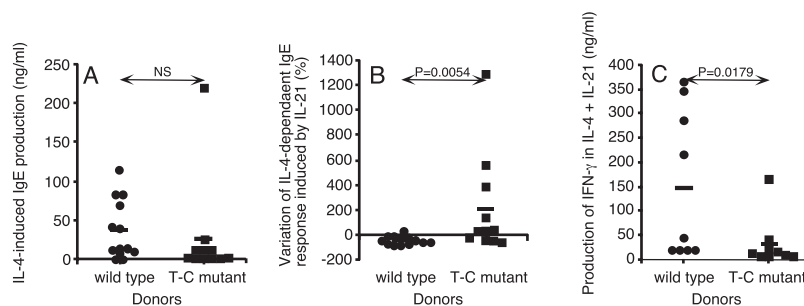


FIGURE 9. The magnitude of variation of IL-4-induced IgE production by IL-21 is associated with a polymorphism in the *IL-21R* gene. Human PBMC (10^6 /ml), isolated from 7 healthy and 17 atopic donors, genotyped for the presence or absence of the T-83C mutant *IL-21R* gene, were stimulated with rIL-4 (A) or with a combination of rIL-4 and rIL-21 (both at 10 ng/ml) (B and C). After 12 days of culture, supernatants were analyzed by ELISA for IL-4-induced IgE production (A), percentage of variation of IL-4-induced IgE production in the presence of rIL-21 (B), and rIL-4- and rIL-21-induced IFN- γ content (C). Comparative statistical analysis was performed between wild-type and T-83C mutant *IL-21R* gene carrying donors, using the Mann-Whitney *U* test.

IL-21 on IL-4-induced germline C ϵ transcription can only be attributed to its effect on naive cells, or on IgG-producing memory cells, that undergo CSR to the production of IgE. Although we have not used purified CD19⁺CD27⁻ naive B cells for Northern blot analysis, splenic and peripheral blood CD19⁺ B cells populations contain both naive and memory B cells, and their use therefore is appropriate to determine whether or not inhibition of germline C ϵ transcription is the mechanism whereby IL-21 affects IL-4-induced IgE production.

The reason for the discrepancy between the results obtained with mouse and human B cells is not clear. It could be argued that the use of the Northern blotting technique, conducted in the present study, is a more reliable method, compared with single RT-PCR analysis, used by Suto et al. (13). In contrast, our results confirm those from a very recently published study, obtained with RT-PCR analysis, showing that human rIL-21 does not inhibit IL-4-induced germline C ϵ transcription in purified B cells (27). It is of note that similar differences between human and mouse have been described with respect to the molecular mechanism of action of IFN- γ , another cytokine with strong inhibitory effects on IgE synthesis (24). In particular, results from Northern blot analysis have shown that IFN- γ does not suppress IL-4-induced germline C ϵ transcripts in anti-CD40 mAb-activated human peripheral blood and splenic B cells (7). In contrast, using the same technique, the inhibitory effects of IFN- γ on the production of IgE by mouse B cells were found to correlate with its capacity to repress IL-4-induced germline C ϵ transcription in vitro, which was furthermore confirmed by its inhibitory activity in a germline ϵ promoter assay (28). These results seem therefore to be part of a large number of immunological differences between mouse and human, shaped by differences in natural selection of both species, as has been reviewed recently (29).

However, as in the mouse, IL-21 was found to exert inhibitory effects on IgE synthesis, because it inhibits IL-4-driven human IgE synthesis in vitro in cultures of PBMC. This effect was dependent on the presence of IFN- γ , as demonstrated by the dose-dependent reversal of rIL-21-mediated inhibition using neutralizing anti-IFN- γ R1 (m)Abs. These results are supported by previous studies in the literature showing that IL-21 induces transcripts for IFN- γ in human T and NK cells (23) and that it strongly enhances, in synergy with IL-15, the production of IFN- γ by these cells (30). Moreover, also in cultures of PBMC stimulated with rIL-4 and rIL-21, and therefore containing IFN- γ , no inhibition of germline C ϵ transcription could be detected (results not shown), which confirms the results from a previous study (7), suggesting that IFN- γ acts at other stages of the regulatory process leading to IgE production. Very recently, it was reported that human IL-21 inhibits IgE synthesis in PBMC, stimulated with PHA and IL-4 (27). Based on an equal presence of IFN- γ transcripts in these cultures containing IL-4 or IL-4 and IL-21, it was concluded by the authors that the inhibitory effect of IL-21 was not mediated via the production of IFN- γ , thereby suggesting the presence of other, yet to be determined, regulatory mechanisms (27). However, this conclusion is questionable because the reversal of IL-21-mediated inhibition by the neutralization of IFN- γ in the cultures, as well as the inverse correlation between production levels of IgE and IFN- γ , clearly demonstrate the implication of the latter cytokine in the regulation of IgE synthesis by IL-21. In conclusion, we would like to argue that the capacity of IL-21 to inhibit IgE synthesis via the induction of IFN- γ production in the mouse has not been formally addressed, and despite immunological differences between human and mouse, as mentioned above, the latter possibility cannot be excluded at present. Finally, although IL-21 induces apoptosis of primary murine B cells (31, 32), this mechanism is unlikely to underlie the decrease in human IgE production observed in the present study. As reported by Wood et al. (27), apoptosis of B cells in PHA-

stimulated cultures of human PBMC treated with IL-21 did not differ significantly from those cultured in the absence of the latter cytokine. Moreover, because stimulation of PBMC with this mitogen induces high levels of IFN- γ production, these results indicate therefore that neither a direct, nor an indirect, effect of IL-21 is observed on human B cell death.

The modulating action of IL-21 on the synthesis of human IgE is therefore the outcome of a balance between its ability to directly promote the growth of both CD19⁺CD27⁺ memory B cells and CD19⁺CD27⁻ naive B cells, undergoing CSR to the production of IgE, as well as to inhibit IL-4-induced IgE synthesis, in an indirect manner, via the induction of IFN- γ production by T and NK cells. This differential effect of IL-21 is somewhat reminiscent of that of IL-10, previously reported to augment IgE production by isotype-committed memory B cells (20), despite its capacity to inhibit IL-4-induced germline C ϵ transcription (33). The drawback of in vitro culture of PBMC, in the absence of polyclonal B or T cell-activating agents, is the production of only very low levels of IgE. However, similarly to what was described for IL-10 (33), the inhibitory effects of rIL-21 on rIL-4-induced IgE synthesis in PBMC were abolished when an anti-CD40 mAb was added to cultures of these cells. These results indicate that polyclonal stimulation of B cells through CD40, although of physiologic relevance, delivers a strong polyclonal activation signal that, because of its intensity, is likely to mask the potential inhibitory effects of IL-21 that, in ongoing immune responses in vivo, are limited to Ag-reactive T and B cells. To circumvent the use of mitogens aimed to induce CD40L expression on T cells and to improve CD40-mediated activation of B cells, we have tried to analyze the effect of rIL-21 in an in vitro allergen-driven culture system. However, similar to Jeannin et al. (33), we were not able to obtain allergen-specific IgE responses in PBMC of atopic donors in the absence of exogenous IL-4 (results not shown).

The results presented in this study indicate that healthy, as well as atopic, individuals respond with a differential sensitivity to the modulatory effects of IL-21 on the synthesis of IgE. Moreover, this differential sensitivity was found to be associated with the presence of a polymorphism in the gene encoding the IL-21R, which is the consequence of a nucleotide substitution in the 5' flanking region of exon 1B (T-83C) (14). We focused on this polymorphism, because among the four *IL-21R* gene variants that were reported in the literature, only the presence of the T-83C variant was found to be associated with enhanced IgE serum levels in healthy and, to a greater extent, in atopic individuals (14). As presented here, T or NK cells from individuals who carry one allele of this *IL-21R* gene variant produce less IFN- γ following stimulation with IL-21, compared with those from individuals carrying the wild-type gene. Accordingly, B cells from the former individuals require higher amounts of IL-21 to achieve a comparable degree of IFN- γ -mediated inhibition of IgE production. Moreover, IgE production levels, at lower concentrations of IL-21, seem to be more prominent in the *IL-21R* gene variant group. It is therefore possible that, due to its strong growth-promoting effects on Ig-committed B cells (8), IL-21 also differentially affects the proliferation of B cells in relation to the polymorphism of its receptor expressed on the latter cells. However, the latter possibility, implying that the interaction between the IL-21R and IL-21 might be altered in B cells expressing the variant IL-21R, remains to be explored.

Because in both groups of donors IL-21 enhanced IgE production already at the lowest concentrations used, it seems that relatively higher concentrations of IL-21 are necessary to stimulate IFN- γ production by T or NK cells, compared with those required to stimulate IL-4-induced IgE production by B cells. An explanation for this finding could be that the levels of the IL-121R differ

among the cell lineages on which it is expressed. Support for this notion comes from a recent study in the mouse in which it was shown that expression levels of the IL-21R are consistently higher on activated B cells, compared with activated T and NK cells (32). Whether this is also true with respect to the expression of the human IL-21R remains to be determined. Likewise, the decrease in IL-21-mediated production of IFN- γ from individuals carrying the T-83C variant of the IL-21R could also be due to lower expression levels of this receptor by T or NK cells compared with individuals homozygous for the wild-type *IL-21R* gene.

According to the original study describing the existence of an *IL-21R* gene polymorphism, individuals with either one or two variant *IL-21R* alleles can be identified (14). Moreover, it was reported that individuals homozygous for the T-83C variant gene had higher serum levels of IgE, compared with heterozygous individuals, suggesting a gene dose effect. Unfortunately, none of the subjects studied here was homozygous for the T-83C variant gene and the possibility that the presence of both variant genes would result in a more pronounced effect of IL-21, compared with the presence of one variant allele only, could not be investigated. In contrast to the reported association between the genetic variation of the IL-21R and the presence of elevated serum IgE levels in females only (14), the results from our study do not support a gender-restricted modulating effect of IL-21 on the synthesis of IgE, although we would like to caution this conclusion because of the limited size of the cohort studied here.

Taken together, the results presented in this study show that the differential regulating effects of IL-21 on IL-4-induced human IgE production is, to some extent, associated with the presence of a polymorphism in the *IL-21R* gene. However, IL-21 is only one among many factors with known inhibitory effects on the production of IgE, notably IL-10 and TGF- β that, in contrast to IL-21, strongly inhibit germline C ϵ transcription, thereby acting at a crucial step in the process of CRS. Moreover, the inhibitory effect of IL-21 is strictly dependent on the action of IFN- γ , the production of which in turn, is induced by a large variety of other cytokines, including IL-12, IL-15, IL-18, IL-23, and IL-27, often with strong synergistic effects. Therefore, IL-21 is likely to play a modulating, rather than a critical, role in the regulation of human IgE synthesis.

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Disclosures

The authors have no financial conflict of interest.

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