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CUTTING EDGE

Cutting Edge: IL-21 Is a Switch Factor for the Production of IgG$_1$ and IgG$_3$ by Human B Cells[1]

Jérôme Pène,* Jean-François Gauchat,[†] Sandrine Lécart,* Elodie Drouet,[‡] Paul Guglielmi,[‡] Vera Boulay,* Adriana Delwail,[§] Don Foster,[¶] Jean-Claude Lecron,[§] and Hans Yssel[2]*

IL-21 is a cytokine that regulates the activation of T and NK cells and promotes the proliferation of B cells activated via CD40. In this study, we show that rIL-21 strongly induces the production of all IgG isotypes by purified CD19$^+$ human spleen or peripheral blood B cells stimulated with anti-CD40 mAb. Moreover, it was found to specifically induce the production of IgG$_1$ and IgG$_3$ by CD40-activated CD19$^+$CD27$^-$ naive human B cells. Although stimulation of CD19$^+$ B cells via CD40 alone induced γ1 and γ3 germline transcripts, as well as the expression of activation-induced cytidine deaminase, only stimulation with both anti-CD40 mAb and rIL-21 resulted in the production of Sγ/Sμ switch circular DNA. These results show that IL-21, in addition to promoting growth and differentiation of committed B cells, is a specific switch factor for the production of IgG$_1$ and IgG$_3$. The Journal of Immunology, 2004, 172: 5154–5157.

U pon activation, naive B lymphocytes can switch from the production of IgM to that of IgG, IgE, or IgA as a result of Ig isotype class switch recombination (CSR)[3] (reviewed in Ref. 1), a highly regulated process controlled by cytokines, as well as by cellular interactions, involving B cell-expressed CD40 and its ligand CD154 (reviewed in Ref. 2). Isotype switching in B cells is important in the humoral immune response, because it generates Abs of the same specificity, but with different effector function. Induction of naive IgM- or IgD-expressing B cells to switch to the production of other isotypes occurs via a distinct recombination process during which the *VDJ* gene is moved from its initial position upstream of the C region (C$_H$) gene. This process is a result of deletion of the DNA between the recombination breakpoints that occur within internally repetitive sequences, known as switch (S) regions, which is excised as circular DNA, the presence of which provides direct proof of specific isotype switching (reviewed in Ref. 3).

CSR is invariably preceded by the transcription of specific germline transcripts, which is under the control of promoters upstream of each switch region. The activity of germline promoters is regulated by specific cytokines. For example, it has been demonstrated that IL-4 and IL-10 selectively induce γ4/ε and γ1/γ3 germline promoters and switching, respectively, in human B cells. In the presence of appropriate costimulatory signals, this results in the production of the corresponding Ig: IgG$_4$ and IgE (4, 5) or IgG$_1$ and IgG$_3$ (6, 7).

IL-21, a typical four-helix-bundle cytokine with significant homology to IL-2, IL-4, and IL-15, signals via a class I receptor, selectively expressed in lymphoid tissues (8). The IL-21R has homology to the shared β-chain of the IL-2R and the IL-15R (9) and is associated with the common γ-chain of the IL-2R (8, 10). IL-21, produced by CD4$^+$, but not CD8$^+$, T cells is important, but not required, for mouse NK cell development and has been shown to inhibit IL-15-mediated expansion of resting NK cells (11). IL-21 inhibits the proliferation of mouse B cells induced by IL-4 and anti-IgM Ab. It has the opposite effect on B cells stimulated with anti-CD40 mAb on which proliferation is increased (8).

In the present study, we have analyzed the capacity of rIL-21 to induce the production of Ig isotypes by human B cells.

Materials and Methods

Cells, separation, and culture conditions

Highly purified (purity, >98%) CD19$^+$ spleen B cells were obtained from human spleen fragments (generously provided by Prof. J.-M. Fabre (Centre Hospitalier Universitaire St. Eloi, Montpellier, France) in accordance with the guidelines of the ethical committee of the Montpellier University Hospitals) by positive selection using specific mAb-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), as described (12). Purified CD19$^+$ B cells (purity, >98%) were also isolated from PBMC (Etablissement Français du Sang, Montpellier, France) using the Rosettesep procedure (StemCell Technologies, Meylan, France). CD19$^+$CD27$^-$ and CD19$^+$CD27$^+$ B cells were obtained by staining with an FITC-conjugated anti-CD27 mAb (BD PharMingen, La Jolla, CA) followed by sorting on a FACSVantage (BD Biosciences, San Jose, CA). Two-color sorting was conducted using an additional PE-labeled mouse anti-human surface IgG mAb (BD Biosciences). Reanalysis of sorted cells showed >99% purity.

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[3] Abbreviations used in this paper: CSR, class switch recombination; AID, activation-induced cytidine deaminase.
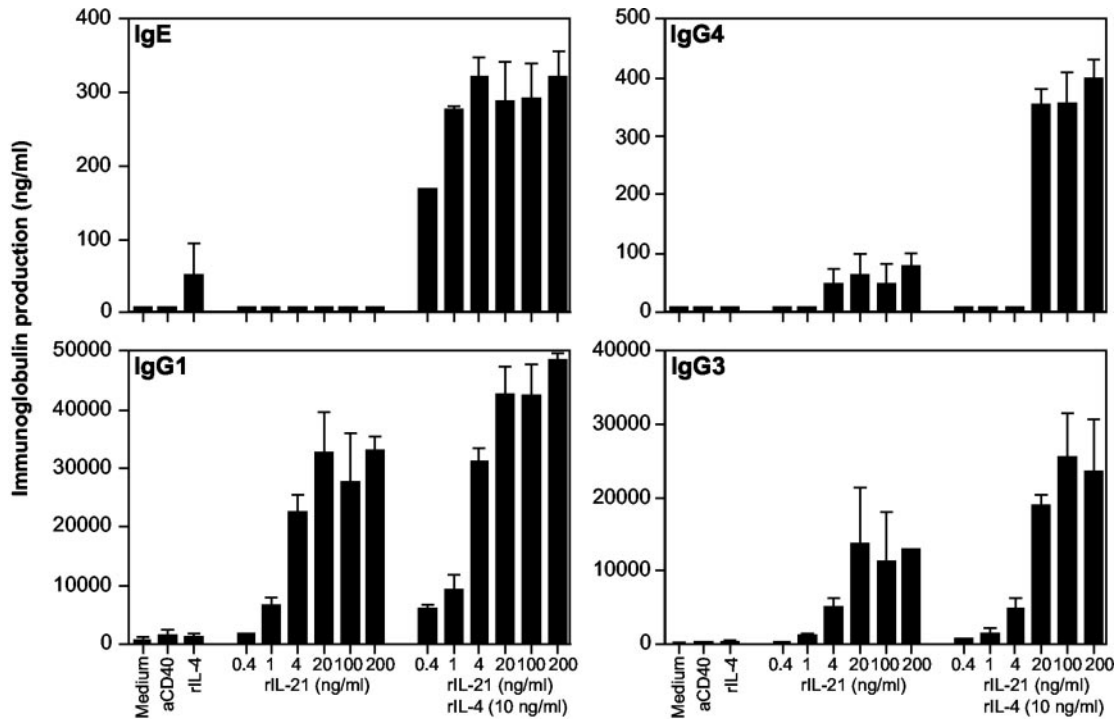
FIGURE 1. Induction of Ig production by rIL-4 and/or rIL-21 in cultures of purified CD19$^+$ splenic B cells. Purified human CD19$^+$ splenic B cells (10^6 per milliliter) were stimulated with anti-CD40 mAb in the presence or absence of rIL-4 (10 ng/ml) and/or increasing concentrations of rIL-21. Culture supernatants were analyzed by isotype-specific ELISA after 12 days of culture. Data represent mean $\pm$ SD of five independent experiments with spleen samples from four different donors.

Induction of Ig production was conducted as follows: whole CD19$^+$, or sorted CD19$^+$CD27$^-$ and CD19$^+$CD27$^+$ human B lymphocytes (10^6 per milliliter) were cultured with 1 μg/ml anti-CD40 mAb 89 (13), in the presence or absence of combinations of rIL-4, rIL-10 (kind gifts from Dr. F. Brière (Schering-Plough, Dardilly, France) and rIL-21 (see Ref. 8) in flat-bottom 96-well culture plates in IMDM, supplemented with 10% FCS in sextuplate in a final volume of 200 μl. PBMC (10^6 per milliliter) were cultured with combinations of cytokines, in the absence of anti-CD40 mAb. After 12 days of incubation at 37°C and 5% CO$_2$, culture supernatants were collected, and Ig content was measured by isotype-specific ELISA. For analysis of various transcripts, purified CD19$^+$ B cells were cultured under the same experimental conditions and were harvested for mRNA isolation after 5 or 7 days of culture.

Measurement of Ig production

IgG$_1$, IgG$_2$, IgG$_3$, IgG$_4$, IgE, and IgA secretion was determined by isotype-specific ELISA (12).

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

Detection of isotype class-specific germline transcripts, activation-induced cytidine deaminase (AID), and spliced Iγ-Cμ transcripts was conducted by RT-PCR. RNA extraction, reverse transcription, and amplification of cDNA was conducted as described (12). The nucleotide sequences of PCR primers (5′-3′) were as follows: Cγ1, sense Iγ1, ACGAGGAACATGACTGGATGC, and antisense Cγ1, TGTGAGTTTTGTCACAAGATTTGGG; Cγ3, sense Iγ3, AGGTGGGCAGGCTTCAGGCACCGAT, and antisense Cγ3, TTGTGTCACCAAGTGGGGTTTTAGC; AID, sense, GAGATTTTTCTGGCCTGAGA, and antisense, CCACTGTCTTCAGCAGAGAT; Iγ-Cμ, sense, GAGGGAGGAGGAGAGGCCCC, and antisense, AGGAAGTCCTGTGCGAGGCAG; and β-actin, sense, GCTGCTGACCGAGGCCCCCCTGAAC, and antisense, CTCCTTAATGTCACGCACGATTTC.

PCR conditions for germline transcripts consisted of 35 cycles of 30 s at 94°C (25 cycles for β-actin), 1 min at 60°C, and 1 min at 72°C, and for AID, as well as spliced Iγ-Cμ transcripts, of 32 cycles of 45 s at 94°C, 30 s at 55°C, and 1.5 min at 72°C. Where indicated, PCR products were sequenced on a 3730XL sequencer (Applied Biosystems, Foster City, CA).

Results and Discussion

To investigate the capacity of rIL-21 to induce the production of Ig, purified human CD19$^+$ splenic B cells were stimulated

with anti-CD40 mAb in the presence of increasing concentrations of rIL-21, and the production of IgG$_1$, IgG$_2$ IgG$_3$, IgG$_4$, IgE, and IgA was analyzed by isotype-specific ELISA. rIL-21 induced the production of all isotypes, with the exception of IgE and IgA, in a dose-dependent fashion (Fig. 1 and data not shown). Optimal IgG production-inducing capacity of rIL-21 was observed at concentrations between 4 and 10 ng/ml cytokine. As expected, addition of rIL-4 to cultures of anti-CD40 mAb-stimulated CD19$^+$ B cells, induced the production of IgG$_4$ and IgE (14), which was strongly enhanced when both rIL-4 and rIL-21 were added. Moreover, rIL-4 also enhanced rIL-21-induced IgG$_1$ and IgG$_3$ production.
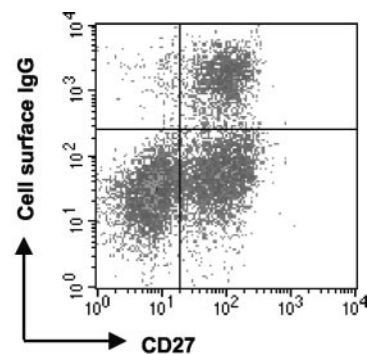

FIGURE 2. Expression of surface IgG and CD27 on CD19$^+$ splenic B cells. Purified CD19$^+$ spleen-derived B cells were stained with an FITC-conjugated anti-CD27 mAb and a PE-conjugated mouse anti-human IgG mAb and analyzed by flow cytometry. Cell surface expression of CD27 (*x*-axis) and IgG (*y*-axis) is represented on a four-decade log scale as dot blots of correlated FITC and PE fluorescence. Quadrant markers were positioned to include >98% of control Ig-stained cells in the *lower left quadrant* (not shown).

Table I. *IL-21 induces the production of IgG$_1$ and IgG$_3$ by CD40-activated CD19$^+$CD27$^-$ human B cells[a]*

Culture Conditions	IgG$_1$			IgG$_2$		IgG$_3$			IgG$_4$	
	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2
CD19$^+$CD27$^-$ B cells										
Medium	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
CD40	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
CD40 + IL-4	7	1	1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
CD40 + IL-21	606	117	236	≤1	≤1	33	12	80	≤1	≤1
CD40 + IL-4 + IL-21	909	216	290	≤1	≤1	122	24	135	≤1	≤1
CD19$^+$CD27$^+$ B cells										
Medium	23	20	47	3	10	1	17	31	10	≤1
CD40	39	46	58	28	11	2	29	35	10	≤1
CD40 + IL-4	172	79	496	80	38	8	56	46	172	≤1
CD40 + IL-21	9,782	2,835	7,069	162	134	362	63	382	512	28
CD40 + IL-4 + IL-21	20,118	7,054	28,094	329	562	888	244	1650	744	109

[a] Purified CD19$^+$CD27$^-$ and CD19$^+$CD27$^+$ splenic B cells (10^6 cells/ml) (Expts. 1 and 2) or surface IgG$^+$-depleted CD19$^+$CD27$^-$ B cells (Expt. 3) were stimulated with 1 μg/ml anti-CD40 mAb in the presence or absence of rIL-4 and/or rIL-21 (both cytokines at 10 ng/ml). IgG levels were determined by isotype-specific ELISA after 12 days of culture and expressed as nanograms per milliliter. Three representative experiments from a total of five, using different spleen samples, are shown.

Splenic and peripheral blood B cell populations consist of both IgM-producing naive B cells that have not yet undergone isotype class switching and isotype-committed memory B cells that produce IgG, IgA, or IgE. According to the literature, naive B cells are characterized by the absence of cell surface CD27 and are surface IgG$^-$, whereas isotype-committed Ig-producing B cells are contained within the CD19$^+$CD27$^+$ population (15, 16). CD19$^+$CD27$^-$ and CD19$^+$CD27$^+$ B cells are about equally represented in human spleen (Fig. 2). IL-21 has been reported to induce the proliferation of human peripheral blood B cells stimulated via CD40 (8), raising the possibility that its Ig production-inducing capacity might be due to its growth-promoting activities on isotype-committed Ig-producing B cells, rather than to a specific induction of class isotype switching in naive B cells. Therefore, the Ig production-inducing effects of rIL-21 were tested on highly purified CD19$^+$CD27$^-$ and CD19$^+$CD27$^+$ splenic B cells.

As shown in Table I, rIL-21 induced the production of all four IgG isotype subclasses by CD19$^+$CD27$^+$ B cells at levels that were comparable to those produced by nonseparated CD19$^+$ B cells (Fig. 1). However, rIL-21 specifically induced the production of IgG$_1$ and IgG$_3$, but neither IgG$_2$, nor IgG$_4$, by CD19$^+$CD27$^-$ B cells. It is of note that ~2% of CD19$^+$CD27$^-$ B were surface IgG$^+$ (Fig. 2) and could therefore contribute to the observed IgG$_1$ and IgG$_3$ production. However, removal of surface IgG$^+$ cells by FACS sorting yielded similar results (Table I), excluding the latter possibility. These results indicate that the production of both the IgG$_1$ and IgG$_3$ isotypes directly results from isotype switching, whereas that of IgG$_2$ and IgG$_4$ may be due to the proliferative effect of rIL-21 on isotype-committed CD19$^+$CD27$^+$ B cells. Indeed, rIL-21 was found to induce strong proliferative responses in CD19$^+$CD27$^+$, but not in CD19$^+$CD27$^-$ B cell subpopulations (data not shown). This dual effect of rIL-21 with respect to the induction of CSR and of IgG$_1$ and IgG$_3$ production by naive B cells is reminiscent to that of rIL-10, the other cytokine known to promote CSR for the production of these isotypes (6). Finally, the IgG$_1$ and IgG$_3$ production-inducing capacity of IL-21 is not related to the origin of the B cells, because production of both isotypes was observed in cultures of PBMC, as well as anti-CD40 mAb-activated purified peripheral blood-derived B cells, in the presence of this cytokine (data not shown). The appearance of small C$_H$-specific germline RNA transcripts is considered to be an obligatory event, preceding isotype

switching in both mouse and human B cells (4, 17). Indeed, Cγ1 and Cγ3 sterile transcripts were detectable in B cells that had been stimulated with anti-CD40 mAb and rIL-21 (Fig. 3A). It is of note that B cells stimulated either via the CD40 molecule or the IL-21R also expressed these transcripts, indicating that, although a prerequisite, their expression alone is not sufficient to warrant IgG production. Because of the high homology among the human γ subclasses, PCR amplification by itself may cause difficulties in the identification of γ subclass-specific transcripts (18). However, RT-PCR products from germline Cγ1 and Cγ3 have unique endonuclease restriction
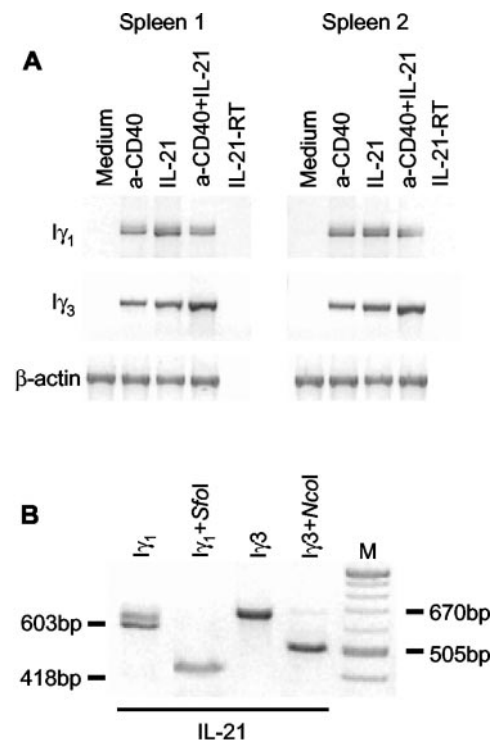

FIGURE 3. Effect of rIL-21 on isotype germline transcription by human B cells. *A*, Human CD19$^+$ B cells were stimulated as described in *Materials and Methods*, and transcripts for Iγ1, Iγ3, and β-actin were amplified by PCR. *B*, Amplification products corresponding to germline γ transcripts of B cells stimulated with rIL-21 and anti-CD40 mAb were digested with *Sfo*I or with *Nco*I. Size and position of the largest expected digestion products are indicated.
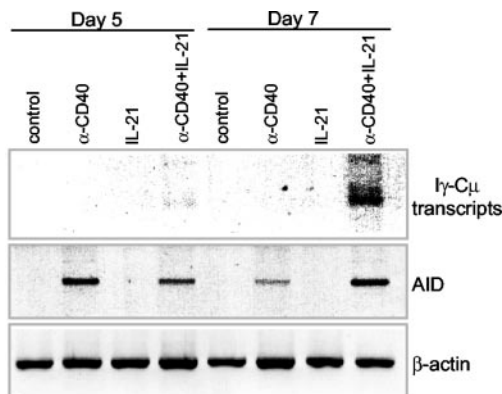
FIGURE 4. Analysis of AID and spliced Iγ-Cμ transcripts in splenic B cells. Purified human CD19$^+$ B cells were stimulated, and *AID* gene and spliced Iγ-Cμ transcripts were analyzed as described in *Materials and Methods*. Representative results of three independent experiments with spleen cells from different individuals.

sites; therefore, their specific germline transcripts can be determined by sequence analysis and identification of the *Sfo*I (*Nar*I isoschizomer) (γ1) or *Nco*I (γ3) restriction endonuclease digestion of the respective PCR products (19, 20). Stimulation of purified CD19$^+$ spleen B cells with anti-CD40 mAb and rIL-21 for 5 days, followed by RNA isolation and RT-PCR analysis of germline transcript synthesis, yielded Iγ1-Cγ1 and Iγ3-Cγ3 PCR products with expected lengths of 603 and 670 bp, respectively (Fig. 3*B*). Direct sequence analysis and *Sfo*I and *Nco*I digestion of the PCR-amplified rIL-21-induced transcripts showed that the amplified product contained the *Nar*I and *Nco*I restriction sites, indicating that IL-21 specifically induces germline γ1 and γ3 transcription (data not shown).

Stimulation of splenic B cells with anti-CD40 mAb and rIL-21 was found to induce transcription of the *AID* gene that could be easily detected after 5 and 7 days of culture (Fig. 4). AID transcripts were also present in B cells treated with anti-CD40 mAb alone. However, despite the presence of sterile transcripts for γ1 and γ3, as well as AID transcripts in B cells stimulated via CD40, the production of IgG was observed only in those cells that had been costimulated with anti-CD40 mAb and rIL-21. These results show that rIL-21, although incapable of inducing AID transcription by itself, is required to promote IgG production, most likely by synergizing with CD40-mediated signaling pathways.

The normal molecular mechanism of isotype class switch involves a nonhomologous recombination event during which the intervening DNA between two S regions is excised as circular DNA (21). To provide direct evidence that the induction of IgG$_1$ and IgG$_3$ production by rIL-21 is associated with this type of DNA recombination, the presence of circular switch DNA in activated B cells was analyzed. At day 7 of culture (and in some experiments at day 5), spliced Iγ-Cμ transcripts resulting from the formation of Sγ-Sμ switch circular DNA were present only in B cells stimulated with both anti-CD40 mAb and rIL-21 and were readily detectable after 25 cycles of amplification. In contrast, further increasing the number of PCR cycles (up to 45) yielded no positive signal in B cells stimulated with anti-CD40 mAb or rIL-21 alone. The class specificity of the recombination events was confirmed by the lack of detectable Iε-Cμ and Iα-Cμ switch transcripts in B cells stimulated with rIL-21 and anti-CD40 mAb (results not shown) in association with the absence of IgE and IgA production, respectively, under these cul-

ture conditions. In theory, only a single nonreplicating switch circular DNA can be generated from each allele in a given B cell. Because this is a one-time event, the observed increase in the number of switch DNA circles cannot be explained by the expansion of already isotype-committed B cells. Therefore, the observed production of IgG$_1$ and IgG$_3$ must be the result of a bona fide CSR event induced by IL-21 in naive surface IgG$^-$ B lymphocytes and is unlikely to be due to the proliferation-inducing effects of this cytokine. The latter notion is underscored by the observation that Iγ-Cμ transcripts were absent in anti-CD40 mAb and rIL-21-stimulated B cells in early cultures and became detectable in increased amount only after 5 days of culture (Fig. 4 and results not shown).

Taken together, the results of this study show that IL-21 plays an important role in the regulation of IgG$_1$ and IgG$_3$ production, in particular due to its capacity to induce CSR in naive human B cells.

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