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Modulation of human B lymphocyte differentiation by therapeutic immunoglobulins: from protein to mRNA levels

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ABSTRACT

Several groups are investigating the mechanisms of action of therapeutic immunoglobulins (IVIg) in order to improve their use. In vitro models such as CD40-CD154 interaction are necessary to study the physiological response of human B lymphocytes to IVIg. Human B lymphocytes treated with IVIg triggers a rapid phosphorylation (<1 h) of extracellular-regulatedkinases 1 and 2 (ERK1/2), which subsequently results in increased differentiation and decreased proliferation. However, the modulation of human lymphocyte physiology by IVIg is a gradual and cumulative process and requires long-term experimentation. Differentiation of human B lymphocytes into Ig-secreting cells can be evaluated both at the transcription and translation levels. The secretion of immunoglobulins can be assessed using ELISA or ELISPOTS whereas expression of immunoglobulin genes can be measured using semi-quantitative or quantitative PCR methods. We hereby report a comparison of these methods to explain how contradictory observations towards IVIg effects could result from their use. Our results indicate that ELISA and ELISPOTS will provide consistent observations by opposition to real-time PCR quantification. Besides, the reliability of each of these methods remained dependent on the stimulation period as well as the preparation of cellular extracts or cell samples following IVIg-treatment.

Keywords: Human B Lymphocytes; CD40-CD154; IVIg; Differentiation; Immunomodulation

1. INTRODUCTION

Therapeutic immunoglobulins are constituted of IgG (≥98%) and are used in the treatment of several inflammatory and autoimmune disorders in which IVIg have been shown to reestablish homeostasis [1]. Following IVIg injection, the concentration of IgG in the patient's serum increases by 5 to 10 mg/mL [2-4]. In many cases, treatment with IVIg results in the reduction or disappearance of pathologic autoimmune antibodies in the patient's serum for prolonged periods of time. Several studies have proposed that anti-idiotypic antibodies, present in IVIg, could interact with the B-cell receptor (BCR). By doing so, IVIg modulate autoreactive B lymphocytes [1]. According to this hypothesis, we showed that IVIg interaction with B lymphocytes results in phosphorylation of ERK1/2, which is downstream to BCR cross-linking [5]. We also showed that IVIg can directly modulate B lymphocytes by inducing secretion of IgG reacting with self- and non-self antigens in cells obtained from healthy individuals [6] as well as from patient with systemic lupus erythematosus [7]. In vitro models are important to study the mechanisms of action of IVIg and several groups are using the CD40-CD154 interaction to delineate their effects on human B lymphocytes. However, all in vitro models using this interaction are not equal [8]. Variations in the level of CD40-CD154 interactions can result in phenotypic and functional differences in human B lymphocyte responses [9,10] as well as in dendritic cells [11] and macrophages [12]. Cell fate determination toward proliferation or differentiation is directly proportional to the average number of CD154 molecules per B lymphocyte [9,13]. Essentially, low levels of CD40 occupancy (500 to 1000 CD154 molecules per B cell) result in high Ig secretion and low proliferation. Conversely, low Ig secretion and high proliferation is observed when using a high level of interaction (5000 to 10,000 CD154 molecules per B cell) [9,13]. Moreover, sub-populations of human B lymphocytes will respond distinctively to variable intensity of CD40 stimulation [9,13-15]. Therefore, models based on *in vitro* CD40-activation of human B lymphocytes may

lead to very contrasting physiological effects, which are as diverse as the nature of the ligands used to bind CD40 (reviewed in [8]). As a result and on the basis of our previous studies, the strength of IVIg modulation on B lymphocytes will depend on the level of CD40-CD154 interaction [7].

For the present work, we have used low and high levels of CD40-CD154 interaction and compared methods to assess the direct effect of IVIg on those B lymphocytes. We specifically targeted the differentiation status by using ELISA and ELISPOT methods to determine the secretion rates and the frequency of secreting cells and we used polymerase chain reaction (PCR), semi-quantitative PCR and real-time PCR (Q-PCR) to evaluate immunoglobulins' expression levels.

2. MATERIALS AND METHODS

2.1. Intravenous Immunoglobulins

Commercial preparation of GAMUNEX® IVIg containing 9% to 11% of protein and 160 to 240 mM glycine, was obtained from Talecris (North Carolina, USA) and albumin from bovine serum (BSA, Cohn fraction V, Sigma-Aldrich Ltd, Oakville, ON, Canada) was prepared at 10% in 10 mM phosphate buffered saline (PBS). IVIg and BSA preparations were dialysed against 10 mM potassium/sodium phosphate containing 136 mM NaCl (PBS) (Gibco, Grand Island, NY, USA) and 40 mM glycine, pH4.5 (Sigma-Aldrich). After dialyse, IgG content in IVIg was mainly monomeric (96% - 98%) and the final concentration was 100 mg/ml [5]. BSA, which does not interfere with B-cell proliferation and IgG secretion [6], was used as a control for protein content.

2.2. Human Peripheral B Lymphocytes

This study has been reviewed and approved by Héma-Québec's Research Ethics Committee. Samples were obtained from healthy individuals after informed consent. Peripheral blood mononuclear cells (PBMNCs) were prepared from leucocytes recovered from leucoreduction system by density centrifugation over Ficoll-Paque (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, QC, Canada) and stored frozen until B-cell purification as described previously [16]. B lymphocytes were purified by negative selection using the Easy Sep CD19 cocktail, according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, BC, Canada) and were pure at 90% to 95% $CD19^+$ cells, as determined by flow cytometry.

2.3. CD40 Stimulation of B Lymphocytes

L4.5 cell line is a genetically modified L929 cell line (CCL-1, American Type Culture Collection, Manassas, VA) [17] expressing 21,000 ± 4000 CD154 molecules per cell [9]. Purified B lymphocytes were seeded in Primaria plates (BD Labware) at ~3.75×10^5 cells/mL in the presence of γ-irradiated L4.5 cells expressing CD 154 [18]. L4.5 cells were seeded as to obtain ratios corresponding to high and low levels of CD40-stimulation, 3 - 5 or 20 - 25 B lymphocytes per L4.5 cell, respectively. B lymphocytes were cultured in a medium based on IMDM supplemented with 10% ultra low IgG FBS containing 10 g/mL insulin, 5.5 g/mL transferrin, 6.7 ng/mL sodium selenite, 100 µg/ml streptomycin and 100 U/ml penicillin G (all from Invitrogen, Burlington, ON, Canada), 50 U/mL IL-2, 25 U/mL IL-10 (PeproTech, Rocky Hill, NJ, USA) and 100 U/mL IL-4 (R&D Systems, Minneapolis, MN, USA). This complete IMDM medium was used in all assays. B lymphocytes were stimulated with high or low level of CD40-CD154 interaction for 9 days in the presence or absence of 10 mg/ml of IVIg. When indicated, cells were cultured in the presence of 10 mg/mL of BSA or 4 mM glycine pH4.5 as controls for IVIg-addition. Cultures were fed by replacing half of the culture medium every 2 - 3 days, while L4.5 cells were renewed every 4 - 5 days. Cell counts and viability were evaluated in triplicates by trypan blue dye exclusion using a hemacytometer. Cultured B lymphocytes were always >96% $CD19^+$ cells. Generation time (T_{gen}) was calculated according to the formula: $\kappa = 1/\ln 2$ $\ln 2[N_{t2}] - \ln[N_{t1}]/t_2 - t_1$ and $T_{gen} = 1/\kappa$.

2.4. ELISA

For the determination of IgG and IgM secretion rate, cells were harvested and washed 5 times to remove residual IVIg with PBS containing 2 g/L of glucose (PBS-sglc). To validate that the secretion by the treated cells was *de novo* protein synthesis, the washed cells were incubated in complete IMDM medium for three hours in the presence or absence of 15 µg/ml cycloheximide (CHX). At this step these 3 h-supernatants were kept and the cells were washed in PBS-glc and seeded at 1 - 2 × 10^6 cells/mL in IMDM medium alone for 18 to 22 hours. IgG and IgM concentrations were determined in a standard ELISA using plastic-adsorbed goat antibodies specific to human γ- and μ-chains and the bound IgG or IgM were both revealed with peroxidase conjugated goat polyvalent anti-human Ig antibodies. All antibodies were obtained from Jackson Laboratories (Mississauga, Canada).

2.5. ELISPOTS

B lymphocytes, untreated or treated with IVIg, were washed 5 times in PBS-glc, transferred in IMDM containing 10% FBS and incubated for 3 hours with or

without 15 µg/mL CHX. B lymphocytes were washed and plated in 96-well Multiscreen IP sterile plate (Milli-pore, Billerica, MA, USA) adsorbed with goat anti-bodyies specific to human γ-chain following manufacturer's instructions. B lymphocytes were added in triplicates using serial dilutions varying from 20,000 to 156 cells/well, and incubated overnight at 37˚C in a CO_2-incubator. Plates were washed with 0.85% NaCl and incubated with peroxidase-conjugated goat polyvalent anti-human Ig antibodies for 2 h at 37˚C in 10% CO_2. All antibodies were obtained from Jackson Laboratories. Spots were revealed by incubation with TrueBlue TMB Peroxidase Substrate (Mandel Scientific Cie Inc., Guelph, Canada) for 30 minutes at room temperature. Membranes were washed and dried overnight in the dark and their numbers were determined using a microscope. The frequency of secreting cells was determined by dividing the number of spot units by the amount of seeded cells for at least two successive dilutions.

2.6. Reverse Transcriptase-PCR and Semi-Quantitative PCR

Purified B lymphocytes stimulated as described above, in absence (CTL) or presence of 10 mg/ml BSA or 10 mg/ml IVIg, were washed 5 times to remove IVIg. RNA extraction was performed using High Pure RNA isolation kit (Roche diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. RNA was treated with DNAse Amp grade (Invitrogen) and first-strand cDNA was synthesized using M-MLV RT (Invitrogen). PCR was performed on PCRExpress (Thermo-Hybaid, Ashford, UK) using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Amplification of gamma regions of IgG_1, IgG_2, IgG_3, IgG_4 [19] and IgG_1-S, $IgG_{2\text{-}4}$ [20], was done using the primer combinations listed in **Table 1**, at 95˚C for 5 minutes followed by 35-cycles at 95˚C for 15 seconds and 60˚C for 30 seconds, with a final elongation step at 72˚C for 6 minutes. Semi-quantitative PCR was performed using the same method, but using an initial denaturation at 95˚C for 5 minutes, followed by 15, 20, 25, 30 and 35 PCR cycles. All PCR products were separated by electrophoresis on a 2% agarose gel (Invitrogen).

2.7. Quantitative Real-Time-PCR

Q-PCR was performed on Stratagene Mx3005P QPCR system using PerfeCta SYBR® Green FastMix™ (Quanta Bioscience Gaithersburg, MD, USA) and master Mix 1 following manufacturer's instructions. Amplification of gamma region of IgG, IgG_1, IgG_2, IgG_3 and IgG_4, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [21,22] or 18S ribosomal RNA [20] as endogenous control genes, was done using the primers listed in **Table 1**. Amplification of gamma region of IgG and GAPDH yielded amplicons of ~300 nt. When indicated quantification of GAPDH or 18S rRNA was performed for each sample; allowing normalization of samples as previously described [23]. Dissociation curve analysis was performed to obtain the amplification of a single PCR product. Quantification of the transcripts was carried out with the software Mx3005P version 2.02 (Invitrogen) using the comparative threshold cycle $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers for PCR and Q-PCR.

IgG	F[1]	CAAGGACTACTTCCCCGAAC	200[2]
	R	TCTTGTCCACCTTGGTGTTTG	
IgG_1	F	GCATGTACTAGTTTTGTCACAAGATTTGGG	300
	R	TCCACCAAGGGCCCATCG	
IgG_2	F	CTCGACACTAGTTTTGCCGCTCAACTGTCTT	300
	R	TCCACCAAGGGCCCATCG	
IgG_3	F	TGTGTGACTAGTGTCACCAAGTGGGGTTTT	300
	R	TCCACCAAGGGCCCATCG	
IgG_4	F	GCATGAACTAGTTGGGGGACCATATTTGGA	300
	R	GCTTCCACCAAGGGCCCATC	
IgG_1-S	F	CATCTCCAAAGCCAAAGG	150
	R	ATGTCGCTGGGATAGAAG	
$IgG_{2\text{-}4}$	F	CATCTCCAAAGCCAAAGG	150
	R	ATGTCGCTGGGGTAGAAG	
GAPDH	F	CGAGATCCCTCCAAAATCAA	300
	R	GTCTTCTGGGTGGCAGTGAT	
18S rRNA	F	AGTCCCTGCCCTTTGTACACA	68
	R	GATCCGAGGGCCTCACTAAAC	

[1]Forward (F); Reverse (R) [2]Amplicon (nts).

2.8. Statistical Analysis

When indicated, the mean values ± standard deviations or standard error of the mean (SEM) were calculated, data distribution was evaluated using the Shapiro-Wilk test and analysis of variance was done using the F-test. Thereafter, statistical significance between tests and controls was determined using Mann-Whitney U-test or two-sided Student's paired t-test.

3. RESULTS

3.1. IVIg and B Lymphocyte Proliferation

Human B lymphocytes, isolated from 6 independent samples were stimulated with high or low level of CD-154 and cultured for 9 days, in the presence or absence of 10 mg/ml IVIg. IVIg were added from the start and

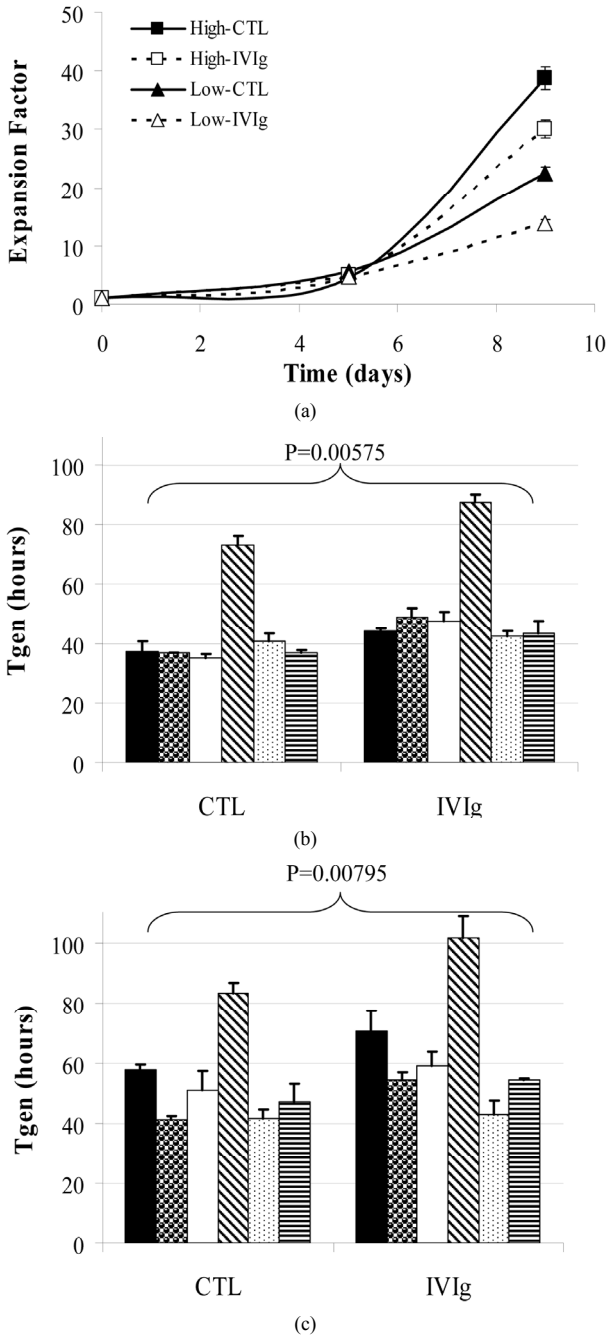
maintained in medium renewal for the duration of the culture (**Figure 1**). Total expansion was determined over the 9-day culture period (**Figure 1(a)**) and T_{gen} was determined for cells receiving high (**Figure 1(b)**) and low (**Figure 1(c)**) CD40-CD154 interaction within the exponential growth phase, namely between days 4 and 9. As previously reported [9,13], T_{gen} mean value appeared higher for cells submitted to low (54 ± 16 hours) than for those submitted to high (43 ± 15 hours) CD154 interaction. However, this tendency was not statistically different ($p = 0.267$). In both cases, addition of IVIg signifycantly increased the T_{gen} by about 20% when compared to untreated cells: 52 ± 17 hours and 63 ± 23 hours for cells submitted to high and low level of CD154 interaction, respectively. Worthy of note, there was no difference in growth rate during the initiation phase, from day 0 to 4, whether the cells were submitted to high or low CD40-stimulation and in presence or absence of IVIg. Essentially, these 6 independent experiments showed that significant IVIg reduction of B lymphocyte proliferation was observed only after a 9-day IVIg treatment in cells maintained in exponential growth.

3.2. Residual IgG in B Lymphocyte Sample Following IVIg Treatment

In vitro IVIg treatment of B lymphocytes using 10 mg/ml or higher concentrations is highly problematic for the determination of IVIg effects on IgG secretion. However, we previously reported [6] that such measurements can be done when using cells that have been washed extensively and seeded back to secrete in absence of IVIg for a short period (<24 h). To illustrate how important and efficient was the repeated washings of B lymphocytes, we have measured the residual IgG content in washing supernatants of IVIg treated-cells (**Figure 2**). A minimum of 5 washing steps were needed to decrease the concentration of IgG (IVIg) from 10 mg/ml to less than 500 ng/mL of IgG. These successive washes also resulted in the recovery of a final amount of $2 - 4 \times 10^6$ cells, representing less than 30% of the cells collected at the end of the culture period. Therefore, when 18 to 21 hours-secretion periods were evaluated, the IgG content coming from IVIg could correspond to a residual concentration of 125 to 250 ng per 10^6 cells.

3.3. IVIg Increase Differentiation into IgG-Secreting Cells

As described in **Figure 1**, cultured B lymphocytes were washed and treated for 3 hours in the presence or absence of CHX. Then, these 3 h-treated B lymphocytes were washed once more and seeded back in culturemedium for 18 hours to 21 hours. This two-step incubation enables us to discriminate between *de novo* synthesis



Human B lymphocytes (>95% $CD19^+$ cells) stimulated for 9 days with high (square symbols) and low levels (triangle symbols) of CD154 interaction in presence (IVIg; empty symbols) of 10 mg/ml IVIg or with an identical volume of 40mM glycine buffer (CTL; filled symbols). Error bars for triplicates, can be smaller than symbols. These results are representative of 6 independent experiments. Generation time values (T_{gen}) for B lymphocytes exposed to high (b) and low (c) level of CD154 are showed for 6 independent samples. P values for T_{gen} comparing IVIg-treated and untreated cells were determined using two-sided Student's paired t-test and correlation coefficient were r = 0.968 (B) and r = 0.982 (c). In B and C, each histogram pattern stands for one sample. For example filled histograms in B and C are the same sample ± IVIg in both conditions.

Figure 1. IVIg inhibit B lymphocyte proliferation.

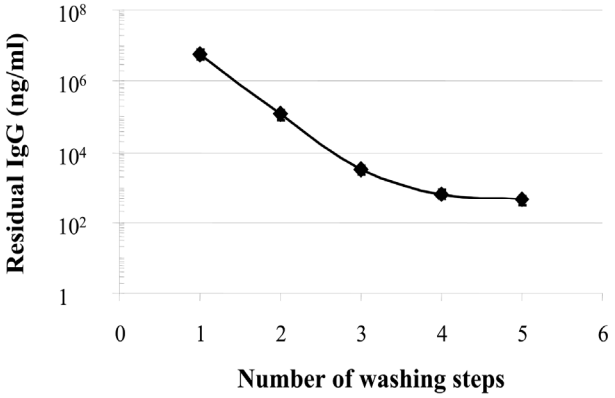


Figure 2. Removal of 99% of residual IgG in IVIg treated cells. B lymphocytes have been stimulated with high or low CD154 interaction, as described in **Figure 1**, in the presence of 10 mg/mL IVIg for 9 days. A total number of 16 - 20 × 10^6 cells were washed with PBS-glc 5 times. IgG content was measured in each washing supernatants by ELISA. Data are presented as the mean ± SD of 3 independent experiments.

of IgG following IVIg treatment and releasing of IVIg, which could occur following spontaneous internalization [24]. IgG and IgM secretion rates were established for 6 independent samples following incubation in the presence or absence of IVIg and compared to CHX-treated cells (**Figures 3(a)-(b)**). This CHX-treatment inhibited IgG and IgM secretion by more than 80% for at least 18 to 21 hours (**Figures 3(a)-(b)**) and inhibition of protein synthesis was already noticeable after 3 hours (**Figure 3(c)**). As suggested by the large SEM (**Figure 3**), such *in vitro* CD40-activation is characterized by inter-individual variability in the human B lymphocytes response [7,9,15,25]. Still, the comparison of 6 experiments using two-sided Student's paired t-test allowed us to highlight significant differences.

Human B lymphocytes submitted to high levels of CD40-CD154 interaction and treated with IVIg were secreting 18 to 208 ng of IgG/h/10^6 cells (mean ± SD; 97 ± 70) while control cells were secreting 6 to 89 ng of

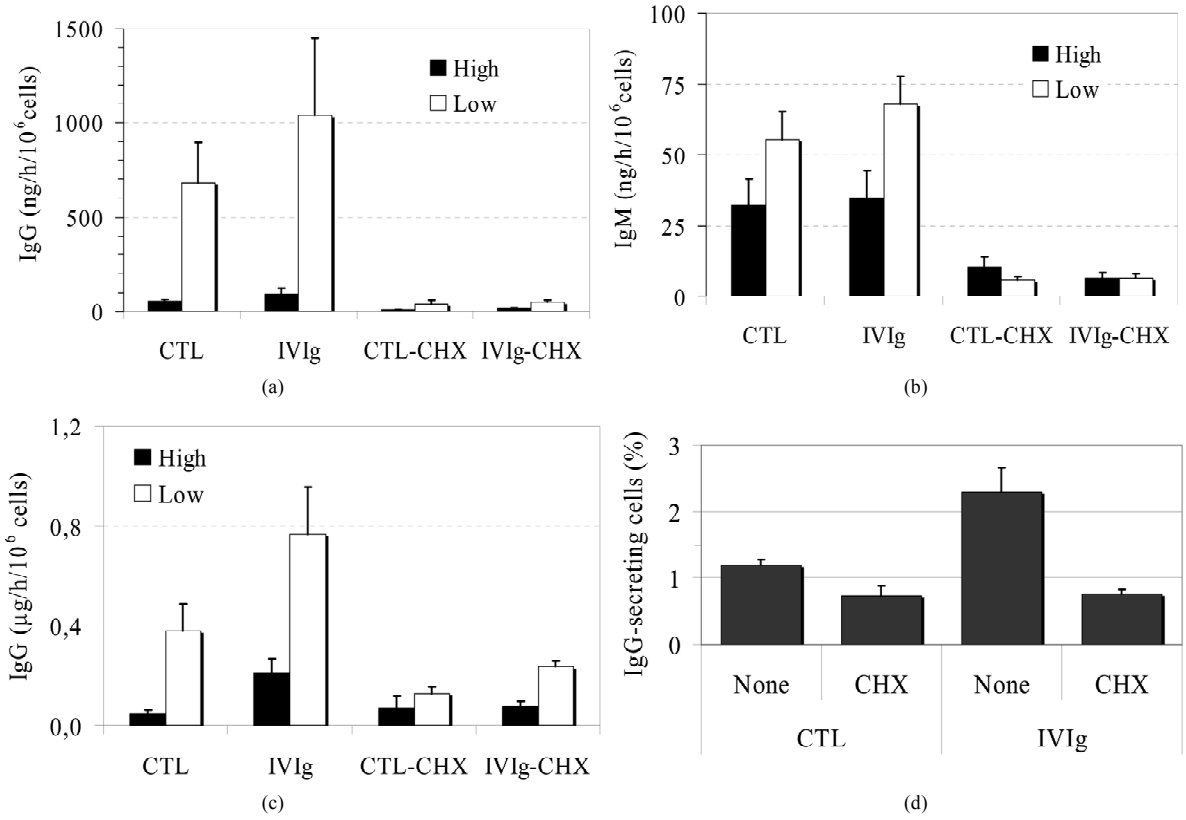


Figure 3. IVIg-increased de novo IgG secretion. B lymphocytes were stimulated as described in **Figure 1**. On day 9, cells were washed 5 times and incubated 3 hours in the absence or presence of 15 µg/mL cycloheximide (CHX). Cells were washed and then seeded back in IMDM alone for a supplemental 18 to 20 h. IgG and IgM contents were determined in these supernatants ((a) and (b)) and in the 3 h-supernatant (c). The frequency of IgG-secreting cells was determined by a standard ELISPOT (d). Data presented in A and B are the mean ± SEM of six independent experiments while results presented in (c) are the mean ± SEM of four independent experiments. The IVIg increase of IgG secretion in B lymphocytes submitted to high ($p = 0.030$) and low ($p = 0.09$) levels of CD40-CD154 interaction is significant according to paired t-test while no significant difference was observed for IgM secretion ((a) and (b)). The IVIg induced IgG secretion during CHX-treatment is significant for cells submitted to high level of CD40-CD154 interaction ($U = 2,309$; $p = 0.0209$).

IgG/h/10^6 cells (mean ± SD; 50 ± 35). Thus, high levels of CD40-CD154 interaction clearly led to a statistically significant IVIg-increase of IgG secretion ($p = 0.0300$). On the other hand, human B lymphocytes submitted to low stimulation in the presence of IVIg were secreting 111 to 2951 ng of IgG/h/10^6 cells (mean ± SD; 1042 ± 1014) while untreated cells were secreting 65 to 1550 ng of IgG/h/10^6 cells (mean ± SD; 681 ± 544). Such an increase of IgG secretion ($p = 0.09$), namely about 1.5-fold, corresponds to a supplemental secretion of 362 ± 513 ng of IgG/h/10^6 cells. In contrast, IVIg did not modulate *de novo* IgM secretion by B lymphocytes submitted to low and high level of CD40-CD154 interaction (**Figure 3(b)**).

IgG content in the supernatant from CHX-treated cells in 4 independent experiments was also assessed (**Figure 3(c)**). IVIg increased IgG secretion by 1.8-fold and 4.6-fold for cells submitted to low and high level of CD40-CD154 interaction, respectively. We have also measured IgG *de novo* synthesis by ELISPOT using CHX treated-cells (**Figure 3(d)**). On day 9, the frequency of IgG secreting cells detected by ELISPOT assay was very low (<3%), nonetheless IVIg still appeared to slightly increase the frequency of IgG-secreting cells.

We have previously shown that IVIg-treatment of human B lymphocytes increases IgG secretion in long-term culture, namely 14 days [5-7]. These results further substantiate that IVIg can increase the *de novo* IgG secretion in CD40-activated B lymphocytes after a 9-day treatment (**Figure 1**).

3.4. IVIg Modulation and mRNA Synthesis

B lymphocytes were cultured for 9 days, as above (**Figure 1**), in the presence or absence of IVIg. In this case, controls were treated with PBS-40 mM glycine pH 4.5 (CTL) and BSA as indicated methods. RT-PCR was used to verify whether all 5 sets of primers used efficiently detected gamma chains as well as subclasses such as IgG_1, IgG_2 and IgG_3 and IgG_4 (**Figures 4(a)** and **(b)**). Except for the primers used to detect IgG_3, all sets of primers used were reliable to detect the various IgG mRNA from controls cells as well as cells treated with BSA or IVIg. IgG_3 mRNA was however detected in some samples but showed no increase in IVIg-treated cells (data not shown). For the set of primers used in to detect IgG_1 and IgG_2 (**Figure 4 (b)**), these analyses, which have been done up to 4 times on 3 independent samples, showed an increase in IgG mRNA content following IVIg treatment. However, these observations were not consistent with the other sets of primers used targeting the same mRNA.

Using semi-quantitative PCR, we evaluated whether IVIg modulation of IgG transcription can be detected (**Figure 4 (c)**). In this case, the two sets of primers used in panel A target IgG_1 ($IgG_{1\text{-}S}$) and IgG_2, IgG_3 and IgG_4 ($IgG_{2\text{-}4}$) [20]. Although the quantitative aspect was improved when compared to the RT-PCR experiments, there was no increase in the IgG mRNA levels following IVIg treatment when compared to untreated cells. BSA-treated cells displayed lower expression levels of IgG_1 mRNA than untreated cells or IVIg-treated cells. These results did not reveal any significant effect of IVIg and were contradictory with the observed increased of IgG protein, as determined by ELISA, for the same experiment and samples. For example, panel A and B correspond to a culture where IgG secretion was increased by 2-fold in IVIg treated cells when compared to BSA-treated cells and untreated cells, which were giving identical IgG secretion.

Q-PCR analyses were performed to compare the mRNA expression of untreated cells and BSA-treated cells to IVIg-treated cells (**Figure 5**). Relative IgG mRNA content varied depending on the housekeeping gene used and depending on whether IVIg-cells were compared to untreated or BSA-treated cells. The twofold increase, which is usually considered as a significant difference in Q-PCR analysis, was not observed when IVIg conditions were compared to untreated cells. Once again, IVIg appeared to significantly increase IgG_1 and IgG_2 expression when compared to the BSA condition.

Overall, neither semi-quantitative PCR nor Q-PCR detected an increased IgG secretion in human B lymphocytes with IVIg. Furthermore, these experiments carried out using 3 independent samples were fluctuating depending on the samples used.

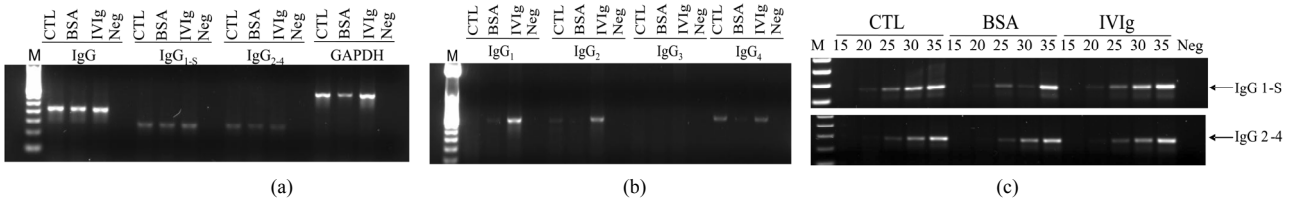


Figure 4. IgG mRNA is not influenced by IVIg. B lymphocytes were stimulated for 9 days with low levels of CD40-CD154 interaction in the presence or absence of 10 mg/mL bovine serum albumin (BSA) or 10 mg/mL IVIg (IVIg) both dialyzed in 40 mM glycine pH 4.5. For controls, 10% (v/v) 40 mM glycine pH4.5 was added in the culture medium (CTL). IgG mRNA levels were evaluated using PCR method ((a) and (b)) and semi-quantitative PCR (c) with primers (**Table 1**) specifically targeting IgG, $IgG_{1\text{-}s}$, $IgG_{2\text{-}4}$, IgG_1, IgG_2 IgG_3 and IgG_4 gamma chains, and GAPDH, as indicated at the top of the panels.

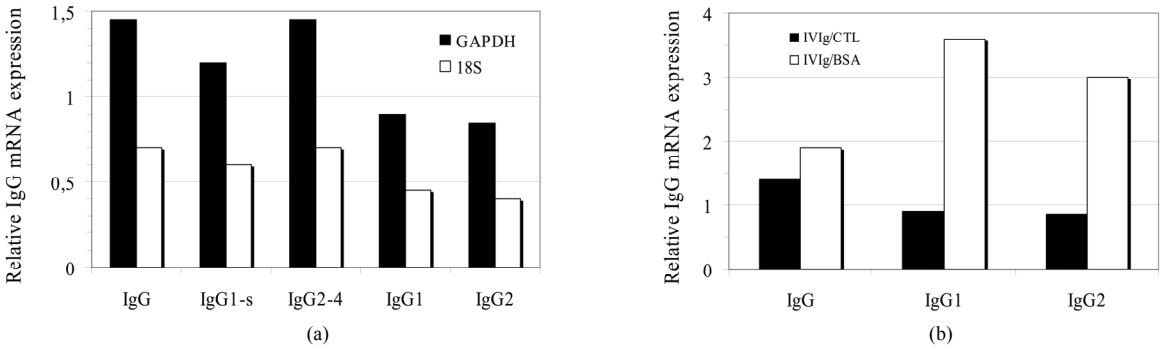


Figure 5. Q-PCR does not correlate with IVIg modulation of IgG**.** B lymphocytes were stimulated as above (**Figure 4**). In (a), two housekeeping genes, GAPDH (filled histograms) and 18S rRNA (Shaded histograms), relative expression values are obtained by comparing IVIg-treated cells to controls cells. In (b), relative values are for IVIg-treated cells compared to control (CTL; filled histograms) or BSA-treated cells (BSA; hatched histograms) using GAPDH as a reference gene.

4. Discussion

We have previously shown that IVIg can have direct effects on B lymphocytes [5-7]. Essentially, IVIg modulation of human B lymphocytes translates into a diminution of proliferation concomitant to an augmentation of Ig secretion. Such an increase in the secretion of human IgG is caused by *de novo* synthesis, which has been shown using metabolic labeling with ^{35}S-methionine [6]. On the other hand, we have also highlighted that variation in the intensity of CD40-CD154 interaction can strongly modulate the evolution of human blood B lymphocytes and alter the outcome of naïve and memory populations [8,9,13] as well as their modulation by IVIg [7]. Therefore, interpretation of IVIg-modulation of human B lymphocytes, using such *in vitro* models, depends on the nature and strength of CD40 interaction and might be a source of discrepancy between studies.

In this study, we used high and low level of CD40-stimulation in a cell-to-cell stimulus provided by CD 154^+ L cells [17] and human B lymphocytes treated with 10 mg/mL of IVIg as previously done [7]. As expected, proliferation was delayed in IVIg-treated cells and differentiation was down-regulated and these changes were also dependent on the level of CD40 stimulation.

The study of IVIg modulation of human B lymphocyte differentiation is limited by the *in vitro* treatment because IVIg interfere with the measurements of IgG secretion in the IVIg-treated cells [20,26]. To minimize the contribution of IVIg in IgG measurements, we constantly perform extensive washing of the treated cells [5-7]. We show that these procedures effectively remove 99.9% of residual IgG, which is less than 500 ng/mL. On the other hand, the extensive washings induce the loss of 60% - 70% of the cells during centrifugation, which is a drawback when the amount of B lymphocytes is somewhat limited. Moreover, IVIg treatment not only precludes direct IgG measurements in culture supernatants, but may also have potential interferences caused by its high protein content, as recently reported for proliferation assays based on BrdU incorporation [27]. Therefore, extensive washings should be done even if the assays are not related to IgG measurements in order to avoid unwanted interferences.

To support the measurements of enhanced differentiation in IVIg-treated cells, we further emphasize on *de novo* synthesis by using protein synthesis inhibitor [28] in combination to ELISA or ELISPOT assays. The cycloheximide treatment confirmed that IVIg induction of IgG secretion and enhancement of IgG secreting cells are mainly caused by novel synthesis.

In this study, we also investigated the effect of IVIg on mRNA levels using PCR, semi-quantitative PCR and Q-PCR as previously done [20]. All analyses were done after 9 days of culture in the presence or absence of IVIg, in conditions where human B lymphocytes exhibit generation times (T_{gen}) ranging from 30 to 70 hours [15]. Although we have used several sets of primers amplifying any gamma chain as well as primers specific to each gamma 1 to gamma 4 subclasses, our results were inconclusive mostly because not reproducible from one samples to another. Worthy of note, such Q-PCR analysis is adequate to monitor the 10-fold increase in IgG secretion in B lymphocytes stimulated with low compared to high CD40-CD154 interaction (data not shown).

The use of BSA as a control for IVIg addition is once more questionable [7]. Semi-quantitative analyses showed lower expression levels of IgG_1 mRNA in BSA-treated cells compared to untreated cells. Similar results were obtained with Q-PCR analyses where BSA inhibits IgG mRNA expression when compared to untreated cells (data not shown). Besides, we observed a down regulation of GAPDH mRNA expression in the BSA-treated cells. GAPDH is considered as a protein content control by many investigators. Therefore, when comparing IVIg-treated cells to BSA-treated cells, increase mRNA expression could be incorrectly interpreted as a relative higher expression for IVIg-treated cells. Those results

confirm previous studies showing that BSA have properties that make it unsuitable as a placebo [29].

On the other hand, the use of Q-PCR to monitor IVIg modulation of IgG secretion is questionable, particularly since a maximum of twofold increase secretion is often observed. Besides, these twofold changes are not valid in Q-PCR determination. In fact, the choice of reference "housekeeping" genes can greatly influence the normalization results and can vary from one type of cells to another. As example, the choice of 18S rRNA to monitor IVIg treatment [20], which reliability may vary from one tissue to another [21,22,30] and is particularly inconsistant in T lymphocytes [31]. Our Q-PCR analyses show a maximum increase of 1.6-fold in IgG mRNA expression following IVIg treatment, which is not considered significant for this method. In a certain point of view, these results are contradictory with the ELISA conclusion but are in agreement with those of Heidt and collaborators [20] stating that IVIg had no effect on IgG secretion according Q-PCR analysis of mRNA levels.

To avoid IVIg interferences, the quantification of IgM secretion have been previously used to monitor IVIg influence on the immunoglobulin production in common variable immunodeficiency, which was quite significant [26]. However, IgM secretion is restricted to naïve and memory subsets of B lymphocytes and is not representative of switched memory B lymphocytes. Moreover, our results indicated that IVIg effects on IgM secretion was less important and not significant compared to that on IgG synthesis. Therefore, the method described here represents an acceptable option to monitor the direct effect of IVIg on B lymphocytes differentiation. Furthermore, variations in the nature and intensity of CD40 binding during *in vitro* activation of human B lymphocytes will result in the emergence of distinct B-cell populations [9], which might in turn give rise to differential responses to IVIg [7].

In conclusion, by delimiting the strength and weakness in ELISA and PCR methods used to monitor IVIg effects on human B lymphocytes, this study intend to contribute to a better understanding of the mechanisms of action of IVIg from the protein to the mRNA level.

5. ACKNOWLEDGEMENTS

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