The regulatory capacity of B cells directs the aggressiveness of CLL

Audrey Mohr, Marie Cumin, Cristina Bagacean, Pierre Pochard, Christelle Le Dantec, Sophie Hillon, Yves Renaudineau, Christian Berthou, Adrian Tempescul, Hussam Saad, Jacques-Olivier Pers, Anne Bordron, and Christophe Jamin

ABSTRACT

Chronic lymphocytic leukemia (CLL) is associated with abnormal T-cell responses responsible for defective anti-tumor activities. Intriguingly, CLL B cells share phenotypical characteristics with regulatory B (Breg) cells suggesting that they might negatively control the T-cell activation and immune responses. We elaborated an in vitro co-culture system with T cells to evaluate the Breg capacities of CLL B cells following innate Toll-like receptor 9 (TLR9) engagement. We demonstrated that B cells from half of the patients exhibited regulatory capacities, whilst B cells from the remaining patients were unable to develop a Breg function. The T cell sensitivities of all patients were normal suggesting that defective Breg activities were due to intrinsic CLL B cell deficiencies. Thus, TLR9-dedicated gene assays highlighted differential signature of the TLR9 negative regulation pathway between the two groups of patients. Furthermore, correlations of the doubling time of lymphocytosis, the time to first treatment, the mutational status of IgVH and the Breg functions indicate that patients with efficient Breg activities have more aggressive CLL than patients with defective Breg cells. Our in vitro observations may open new approaches for adjusting therapeutic strategies targeting the Breg along with the evolution of the disease.

Introduction

Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease characterized by the accumulation of CD5+ tumor B cells originating either from naive B cells, marginal zone B cells, mature CD5+ B cells, or from the CD27+ memory B cell compartment. Development of the disease is associated with abnormal immune responses that may be responsible for the defective anti-tumor activity. T cells display abnormal functions and are unable to trigger, sustain and complete an immune response against the tumor cells. One explanation can be found in the differential sensitivity of CD4+ and CD8+ T cells to be terminated by CLL B cells. The enriched regulatory T (Treg) cells in progressive CLL patients compared with indolent patients and normal individuals also suggests a down-regulation of the tumor-specific response.

It has now been widely demonstrated that some B cells have regulatory properties to control adaptive as well as innate immune responses in autoimmune, infections, allergies and cancers mainly through IL-10-dependent and IL-10 independent mechanisms. Thus, one of the predominant regulatory factors secreted by Breg cells is IL-10. The IL-10 producing Breg cells, called B10 cells in mice, represent less than 5% of the B-cell pool in humans. They differentiate from CD24hiCD38+CD5+ memory B cells expressing the CD5 molecule following BCR and subsequent CD40 signaling, suggesting that antigen exposure and T-cell contact are key components for their generation. However, TLR9 and IL-21R signals are able to generate effector B10 cells indicating that innate stimulations can also activate efficient regulatory activities. Other Breg cells can be functionally defined by their IL-10-independent control of the immune responses. Thus, CD24hiCD38hiCD5+ B cells suppress the proliferative response of T cells and the function of dendritic cells mainly by cellular interactions, TGF-β production and Treg expansion. Their regulatory properties are acquired following CD40 activation during cell-to-cell contacts and are up-regulated following TLR9 engagement.

Breg and CLL B cells share phenotypical characteristics, both expressing CD5, CD24 and CD27, and low levels of surface IgM. They also share physiological analogies. While non-stimulated CLL cells produce low levels of IL-10, in vitro stimulation through CD40L or TLR9 induces significant production of IL-10 similar to human Breg cells. Furthermore, the inability of CLL B cells to stimulate T cell proliferation or their Th1 polarization associated with increased Treg frequencies suggest that they could exhibit regulatory properties like Breg cells inhibiting the T cell proliferation through an IL-10-independent mechanism suppressing the Th1 polarization through the production of IL-10 and expanding the Treg cells. Taken together, the disturbance of the immune responses observed in CLL patients may result from the development of different Breg functions.
While CLL B cells may share IL-10-dependent immunosuppressive functions with B10 cells leading to the control of Th1 polarization, their IL-10-independent regulatory properties to control the T cell proliferation during immune responses have never been identified. To evaluate these capacities, we developed an autologous in vitro co-culture system. We highlight that B cells from half of the patients exhibit efficient regulatory capacities, whilst B cells from the remaining patients are unable to develop regulatory function after TLR9 stimulation. Comparison of the two groups indicates differential gene expression signatures related to the control of the TLR9 pathway. Moreover, Breg activity appears to be associated with the clinical evolution suggesting that the development of the IL-10-independent regulatory control of the CLL B cells may be associated with the aggressive outcome of the disease.

Results

**TLR9-induced Breg activity differentiates two groups of CLL patients**

To assess the IL-10-independent Breg function, purified B cells were incubated for 4 days with autologous T cells activated by anti-CD3 and anti-CD28 mAb to induce their proliferation in the presence of CpG-ODN. TLR9 stimulation of CLL B cells identified two groups of patients (Figure 1(a)). A regulatory activity was observed in the first group, classified as Reg POS CLL patients, for which the T cell proliferation was inhibited by $+8.0 \pm 1.2\%$. In the second group, classified as Reg NEG CLL patients, no inhibition of the T cell proliferation was induced ($-8.2 \pm 1.3\%; p < 0.001$) (Figure 1(a)). Because the control of the T cell proliferative response by the B cells is IL-10 independent, but involves a TGF-β-dependent mechanism as previously demonstrated with blocking Abs, both cytokines were assessed in the two groups. Consistent with these observations, the level of the inhibition of the T cell proliferation was not associated with the concentration of IL-10 but was slightly, though not significantly, correlated with the concentration of TGF-β detected in the co-culture’s supernatants (Figure 1(b)). Furthermore, because CLL B cells are prone to die spontaneously in vitro, the level of the B cell mortality after 4 days of co-cultures was evaluated. Reg POS and Reg NEG CLL B cells exhibited $7.8 \pm 2.1\%$ and $8.2 \pm 2.3\%$ of mortality respectively (Figure 1(c)), indicating that the different Breg behavior cannot be ascribed to different survival levels. Moreover, the level of inhibition of T cell proliferation was not enhanced when the CLL B cell ratio increased ($p < 0.05$) (Figure 1(d)), suggesting that B cell regulatory deficiency in the Reg NEG CLL is independent of lymphocytosis. The ensuing experiments were therefore performed at a 1T:1B ratio for all samples.

**CLL T cell proliferation response is not defective**

It can be argued that T cell differential sensitivities and/or differential CD4:CD8 ratio could be responsible for the discrepancies in the Breg activities. However, the T cells exhibited similar levels of mortality after 4 days of co-culturing ($p > 0.05$) and the CD4:CD8 ratios were found to be identical between Reg POS CLL and Reg NEG CLL patients ($p > 0.05$) (Figure 2(a)). Moreover, the proliferation index of CD4$^+$ and CD8$^+$ T cells (Figure 2(b)) were equivalent between Reg POS CLL ($2.8 \pm 0.2$ and $3.1 \pm 0.1$, respectively) and Reg NEG CLL ($2.7 \pm 0.1$ and $2.7 \pm 0.3$, respectively) ($p > 0.05$), and the CD4$^+$ and CD8$^+$ T cells were similarly regulated by their B cell counterparts in autologous co-culture experiments (Figure 2(c)). Taken together, these results indicate that the distinct Breg activities between the two groups of CLL patients are not due to differential CD4$^+$ or CD8$^+$ T cell sensitivities.

Furthermore, in heterologous co-cultures (Figure 2(d)), healthy control (HC) B cells similarly inhibited the proliferation of Reg POS CLL T cells ($+17.8 \pm 3.5\%$) and Reg NEG CLL T cells ($+13.0 \pm 1.8\%$) ($p > 0.05$). Conversely, Reg POS CLL B cells but not Reg NEG CLL B cells inhibited the proliferation of HC T cells ($5.7 \pm 2.0\%$ vs $-12.2 \pm 7.3\%$, $p < 0.05$, respectively). All these data indicate identical sensitivities of the T cells from Reg POS CLL and Reg NEG CLL patients and emphasize that B cells from Reg NEG CLL patients exhibit intrinsic defective Breg capacities compared to B cells from Reg POS CLL patients.

**Differential efficient signaling pathways in Reg POS and Reg NEG CLL B cells**

To understand the differential Breg capacities of CLL B cells, we first searched for phenotypic discrepancies. As expected, all B cells displayed a unique cell surface phenotype (Figure 3(a)), confirming the CLL diagnosis established by Matutes et al. for both Reg POS and Reg NEG patients with higher level of CD5 and reduced expression of CD22, CD79b, IgM, IgD and FMC7 relative to HC B cells. As previously described, decreased levels of CD19 and CD20 were also found confirming the cellular origin of these cells. However, the densities of molecules previously shown to be associated with regulatory function such as CD23, TLR9, CD24, BR3-IL-21R, CD25, CD38 or TACI were similar on Reg POS and Reg NEG CLL B cells ($p > 0.05$) (Figure 3(b)). Because CD27 naïve B cells were considered to possess a greater regulatory capacity than CD27$^+$ memory B lymphocytes, CD27 expression was also evaluated. All cells were found to be positive with identical CD27 MFI on Reg POS and Reg NEG CLL B cells ($p > 0.05$), suggesting that the presence of CD27 is unlikely associated with the efficacy of the Breg effect of the CLL B cells. Finally, CD40 which is known to be required for the generation of the Bregs was also similarly expressed on Reg POS and Reg NEG CLL B cells ($p < 0.05$), suggesting that this interacting molecule is unlikely responsible for the difference in the Breg activity.

To go further in depth, we then questioned the efficiency of the activating TLR9 signaling. B cells were incubated with CpG-ODN for 48h. The MFI of the CD86 and CD25 molecules were up-regulated without significant differences between Reg POS and Reg NEG CLL B cells (Figure 4(a)). However, while Reg POS CLL displayed higher proportion of CD69$^+$ activated B cells, TLR9 stimulation increased their frequencies in the two groups indicating that Reg POS as well as Reg NEG CLL B cells can be activated by CpG-ODN (Figure 4(a)). The TLR9-induced
Reg NEG CLL B cells exhibited a lower up-regulated expression of CD40 molecules compared to the enhanced density triggered on Reg POS CLL B cells (Figure 4(a)). These results suggest a defective TLR9 signaling pathway in the Reg NEG CLL B cells that may be responsible for their inability to develop Breg activity in close contact with the T cells.

Therefore, we deciphered the TLR9 pathway engaged and controlled following CpG-ODN stimulation and 3 days of co-cultures. Among 66 genes analyzed in the TLR-dedicated TaqMan gene expression assays, 29 genes were over-expressed and 10 genes were under-expressed in the Reg NEG CLL B cells compared to the Reg POS CLL B cells (Figure 4(b)). Among these clusters, 12 genes were relevant to the TLR9 pathways (Table 1). In the Reg NEG B cells, 4 genes associated with activating signals were over-expressed (TBK1, IRAK-4, IRAK-1 and REL) when 6 genes were under-expressed (IRF-7, IRF-8, MAPK14, MAPK13, RELB and UBC). Moreover, 2 genes associated with inhibitory signals of the TLR9 pathway were also over-expressed in the Reg NEG CLL B cells (TOLLIP and TANK) when none were under-expressed compared to the Reg POS CLL B cells. Compared with HC, this differential expression was confirmed at the mRNA and protein levels for IRAK-1, IRAK-4 and TANK molecules for
which specific bands could be identified with the corresponding mAbs on the Western Blot (Figure 4(c)). The imbalance in favor of the inhibitor signal of the TLR9 pathway may be the basis of the inability for the Reg NEG CLL B cells to develop a Breg function following TLR9 stimulation.

**Correlation of the Breg capacities, the time to first treatment and the doubling time of lymphocytosis**

Finally, the correlation of the Breg capacities with clinical aspects was assessed (Table 2). Breg activities were not correlated with either sex, age of diagnosis, the Binet stage, lymphocytosis, or CD38 expression. Identical repartitions of cytogenetic abnormalities (Figure 5(a)) as well as mutational status of IgVH (Figure 5(b)) were also observed in Reg POS and Reg NEG CLL B cells. However, patients with unmutated IgVH status are known to exhibit a more aggressive form of CLL compared with patients harboring a mutated IgVH. We wish to evaluate the impact of the Breg efficiency on the CLL aggressivity. We found that the doubling time of lymphocytosis appeared more quickly for the patients with unmutated IgVH than for the patients with mutated IgVH, and even more quickly for the Reg POS CLL patients than for the Reg NEG CLL patients (p < 0.05) (Figure 5(c)). Furthermore, the time to first treatment occurred earlier for the patients with unmutated IgVH than for the patients with mutated IgVH and even more earlier for the Reg POS CLL patients than for the Reg NEG CLL patients (p < 0.05) (Figure 5(d)). These observations indicate that the detection of Breg activity is associated with a more aggressive form of CLL.
Discussion

Breg cells turn off the anti-tumor T-cell immune responses in solid cancers by the inhibition of the T cell proliferation and the expansion of the Treg cells. Their role in the development of CLL is not fully clarified and the question as to whether CLL B cells can be considered as Breg cells contributing to disease progression has not yet been elucidated. Two functional mechanisms defining distinct subsets of Breg cells have been recognized to date. The first corresponds to B cells expressing IL-10 leading to the control of the Th1 polarization and inflammatory responses. CLL B cells share the functional capacity to express IL-10 although the identification and characterization of IL-10 competent CLL cells remain to be established. The second mechanism is IL-10-independent and requires cell-to-cell contact with CD40/CD40L interaction leading to the control of the T cell proliferation. Herein, TLR9 engagement, known to induce efficient IL-10-independent Breg activities in HC B cells defined two groups of patients classified as Reg POS and Reg NEG CLL. No evident association could be found with either clinical aspect or with the IgVH mutational status of any patients, suggesting that defective IL-10-independent Breg activity is not associated with specific antigen patterns. Heterogeneous responses to CpG-stimulation have already been reported with proliferation of CLL B cells in half of the cases but also with apoptosis in some cases. It is likely that the variation of intra-cellular signaling pathways might determine different CLL B cell behaviours in response to TLR9 engagement as shown for example by the variable magnitude of Akt signaling. Thus, it has been recently demonstrated that the levels of CD19 expression and the associated intracellular pathways are key elements in the efficient TLR9-induced B cell activation. CD19 abrogated expression and an altered PI3K/Akt/BTK-dependent CD19 pathway result in a TLR9 activation defect. As previously observed, we found lower CD19 surface expression on CLL B cell patients compared to HC B cells but the levels were similar on Reg POS and Reg NEG CLL B cells, suggesting that CD19 expression is unlikely involved in the Breg deficiencies of the Reg NEG patients. Furthermore, the over-expression of the inhibitors TOLLIP and TANK in the Reg NEG CLL B cells participating in the inhibition of the TLR9 signals could explain the absence of Breg activities in these patients. Consistent with this hypothesis,
TLR9 stimulation induced a lowered CD40 up-regulation on Reg NEG CLL B cells. This implies defective CD40-mediated transductions that might be inappropriate to efficiently activate the CLL B cells during the close contact with T cells and may impede the development of Breg activities.

Our observations indicate that the TLR9-induced Breg control of the T cell proliferation develops in patients with shorter doubling time of lymphocytosis and with earlier time to first treatment suggesting that IL-10-independent Breg properties may be more readily apparent in more aggressive CLL B cells. These Breg functions may control anti-tumor immune responses over time and could explain why time from diagnosis is associated with the severity of immune suppression, especially when TLR9 signaling is effective.

There are also arguments supporting the concept of CLL B cells that may exhibit IL-10-competence contributing to the immunosuppression observed in these patients. Further studies will be required to evaluate whether IL-10-dependent and -independent regulatory mechanisms may jointly occur or appear separately during disease progression and whether these Breg activities may be the properties of distinct or common CLL cells. The emergence or disappearance of the Breg effects might influence the outcome of the disease's severity. Ultimately, understanding how these Breg mechanisms are controlled may help in the selection of
targeted therapies that could be adjusted along with the evolution of the disease.

Materials and methods

Patients and normal controls

Fifty-six untreated patients fulfilling the criteria for the diagnosis of CLL were enrolled in the study and were scored clinically according to Matutes and colleagues. Twenty males and 15 females ranging in age from 40 to 84 were scored at stage A, 10 males and 8 females ranging in age from 43 to 79 scored at stage B and 3 males ranging in age from 54 to 71 scored at stage C. For some experiments, blood was also taken from 29 aged-matched healthy controls (HC). Consent was obtained from all individuals and the protocol conducted in compliance with the Declaration of Helsinki and approved by the Ethical Board at the Brest University Medical School Hospital (OFICE, nov 26th, 2015, collection 2008–214).

Cell preparation

Peripheral blood mononuclear cells were separated by density-gradient centrifugation on Ficoll-Hypaque (Eurobio, Courtaboeuf, France). B lymphocytes were enriched with a negative pan B-cell isolation kit (Miltenyi Biotec, Paris, France). Due to the weak frequency in CLL, T cells were directly isolated from the patients’ peripheral blood using the positive MACSxpress Pan T cell isolation kit (Miltenyi Biotec) while T lymphocytes from HC were isolated with neuraminidase-treated sheep red blood cells (Thermo scientific, Hampshire, United Kingdom). The purity was over 90% in all conditions.

Cell culture

Cells were cultured at 37°C into 96-well plates coated with a goat affiniPure F(ab’)2 anti-mouse IgG fragment (Jackson Immunoresearch, Baltimore, USA) in RPMI1640 medium (Sigma Aldrich, Saint Louis, USA) supplemented with 2 mM L-glutamine, antibiotics and 10% of human AB serum (Sigma Aldrich). 4.10^4 T cells were stimulated with

Table 2. Clinical characteristics of the CLL patients.

|                         | Reg POS CLL patients | Reg NEG CLL patients | p   |
|-------------------------|----------------------|----------------------|-----|
| Number of patients      | n = 30               | n = 26               |     |
| Sex ratio               | Male/Female          |                      | 0.36|
| Binet's Stage           |                      |                      |     |
| A                       | 17                   | 18                   |     |
| B/C                     | 13                   | 8                    |     |
| ≥ 65 years old at diagnosis | 13/30               | 14/26                | 0.43|
| Lymphocytosis           |                      |                      |     |
| ≤50 Giga/L              | 13                   | 5                    |     |
| ≤50 Giga/L              | 17                   | 21                   |     |
| IgVH mutational status  |                      |                      | 1.00|
| Mutated                 | 23                   | 15                   |     |
| Unmutated               | 6                    | 3                    |     |
| ND                      | 1                    | 8                    |     |
| Cytogenetic abnormalities|                      |                      |     |
| normal                  | 2                    | 3                    | 0.52|
| Del13q                  | 22                   | 11                   |     |
| Tri12                   | 4                    | 2                    |     |
| Del11q                  | 3                    | 0                    |     |
| Del17p                  | 1                    | 1                    |     |
| UND                     | 0                    | 8                    |     |
| CD38 MFI ± SEM          | 1.5 ± 0.2            | 1.9 ± 0.5            | 0.86|

Figure 5. Patients with regulatory B cell activity present a more aggressive form of CLL. Comparisons of chromosomal abnormalities (a) and IGVH mutational status (b) between Reg POS CLL and Reg NEG CLL patients. (c–d) Kaplan-Meier graphs for the comparisons between Reg POS CLL and Reg NEG CLL patients according to their IGVH mutational status. (c) Comparisons of the doubling time of lymphocytosis. (d) Comparisons of the time to first treatment. ns, not significant.
mouse anti-human CD3 (0.2 µg/ml, Clone OKT3, Biolegend, San Diego, USA) and anti-human CD28 (0.2 µg/ml, Clone CD28.2, Beckman Coulter, Marseille, France) mAb to induce their proliferation. TLR9 of B cells was activated with CpG-ODN 2006 (0.25 µM, Invivogen, Toulouse, France). T and B cells were co-cultured at a 1:1 ratio as previously described, unless otherwise indicated, for four days. For the proliferation assays, T cells were first labeled with the CellTrace™ Violet Cell Proliferation Kit (ThermoFisher scientific, Waltham, USA) and analyzed by flow cytometry (Navios, Beckman Coulter, Villepinte, France). The proliferative response was evaluated using the FlowJo software (FlowJo, Ashland, USA) as a proliferation index into two conditions: when T cells were cultured alone and when T cells were co-cultured with B cells in the presence of CpG-ODN. The inhibition of proliferation in the presence of B cells was then calculated according to the following formula: \((\text{Proliferation index of T cells cultured alone}) - \text{Proliferation index of T cells co-cultured with B cells})/\text{Proliferation index of T cells cultured alone} \times 100\%\).

To study the B cell activation, 5.10^5 B cells (from CLL patients and HC) were positively selected with the CD19 MicroBeads kit (Miltenyi Biotec) for qRT-PCR and Western Blot studies. Total RNA was extracted with the RNeasy Plus Micro kit (Qiagen, Courtaboeuf, France) and reverse transcribed to cDNA with Superscript II reverse transcriptase (Invitrogen). Gene expression patterns of TLR pathways were evaluated using the TaqMan Array Plate (Applied Biosystems, Foster City, USA). For each group defined below, 3 samples of cDNA were diluted (1/40), mixed and added on one plate. Relative expression was normalized with the mean of all CTs (Cycle Threshold) and compared to each other with the fold change method. CT values greater than 35 reflected no expression and were excluded from analysis.

After washing at 4°C in TNE buffer (25mM Tris-HCl pH 7.5, 140 mM NaCl and 1 mM EDTA), positively selected B cells were incubated for 30 min in 1% Triton X-100 in TNE buffer containing anti-proteases cocktail (Sigma). Samples were separated by SDS-PAGE electrophoresis and proteins transferred on polyvinylidene difluoride (PVDF) membranes. After 1 h of saturation with 5% milk in 0.1% Tween 20 buffer, PVDF membranes were incubated in the presence of either rabbit anti-IRAK-1, anti-IRAK-4 (Cell signaling) or anti-TANK (Abcam) antibodies. After washes, HRP-conjugated anti-rabbit immunoglobulins (Jackson) were added, revealed using the ECL Advance Kit (GE Healthcare) and membranes analyzed with Quantity One Software (BioRad).

**Flow cytometry**

All antibodies were from Beckman Coulter unless otherwise specified: FITC-conjugated anti-TLR9 (Imgenex, San Diego, USA), CD268 (B-cell activating factor-receptor (BAFF-R, Biolegend), IgD, CD19, CD25 (BD Biosciences, San Jose, USA), phycoerythrin (PE)-conjugated anti-CD5, CD19, CD86, IL21R (BD Biosciences, Grenoble, France), IgM, CD267 (TACI, Biolegend), CD69, CD40, PE-cyanin(Cy)5.5-conjugated anti-CD4, anti-CD11b, CD25, CD79b, PE-Cy7-conjugated anti-CD19, CD23, CD38, allophyocyanin (APC)-conjugated anti-CD40, CD24, APC-Alexa Fluor 700 (AF700)-conjugated anti-CD19, APC-AF750-conjugated anti-CD5, Pacific blue (PB)-conjugated anti-CD22, CD20 and FMC7, Krome Orange (KO)-labelled anti–CD8 mAbs. 1 x 10^6 lymphocytes were incubated for 30 min at 4°C with saturating concentrations of mAbs. The determination of the mean fluorescence intensity (MFI) of all markers required a minimum of 5,000 events. The results were standardized to those obtained with isotype controls. For intracellular staining, a preliminary permeabilization was performed with the cytotox/cytoperm kit (BD Biosciences). For the proliferation evaluation after co-culture, the fluorescence intensity of Cell Trace Violet was analyzed on CD19-negative T cells. Their viability was assessed using the DRAQ7 DNA staining dye (DRAQ7 Biostatus, Beckman Coulter).

**Elisa**

Concentrations of IL-10 and TGF-β from the supernatants of co-cultures were measured with OptEIA™ IL-10 (BD Biosciences) and Human TGF-beta 1 Quantikine (R&D System) ELISA kits, respectively.

**Real time quantitative PCR (qRT-PCR) and western blot**

After 3 days of co-culture with autologous T cells, CpG-simulated B cells from CLL patients and HC were positively selected with the CD19 MicroBeads kit (Miltenyi Biotec) for qRT-PCR and Western Blot studies. Total RNA was extracted with the RNeasy Plus Micro kit (Qiagen, Courtaboeuf, France) and reverse transcribed to cDNA with Superscript II reverse transcriptase (Invitrogen). Gene expression patterns of TLR pathways were evaluated using the TaqMan Array Plate (Applied Biosystems, Foster City, USA). For each group defined below, 3 samples of cDNA were diluted (1/40), mixed and added on one plate. Relative expression was normalized with the mean of all CTs (Cycle Threshold) and compared to each other with the fold change method. CT values greater than 35 reflected no expression and were excluded from analysis.

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**Mutational status of IgVH**

The immunoglobulin variable heavy chain (IgVH) gene mutation status was determined after PCR amplification and sequencing based on the BIOMED-2 consortium guidelines. 100 ng of genomic DNA isolated with the BioSprint 15 DNA Blood kit (Qiagen), 0.25 µl of AmpliTaq Gold DNA Polymerase (Applied Biosystem), 10 pmol of each primer, 0.2 mM dNTP Mix, 1.5 mM MgCl2, 1x PCR Buffer II, and water adjusted to 50 µl were used to perform the multiplex PCR amplifications. PCR products were controlled through 2% agarose gel, purified with ExoSAP-IT PCR product cleanup kit (Affymetrix, High Wycombe, United Kingdom) and sequenced with a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem). Results were analyzed with the database IMGT/HighV-Quest (The international ImMunoGeneTics information system, Montpellier). A homology sequence >98% defined an un-mutated status.

**Statistics**

All the results were analyzed with GraphPad Software and presented as Mean ± SEM. Analyses were performed using Mann-Whitney and Chi-square statistical tests, with significances assessed at p < 0.05.
Abbreviations

MFI  mean fluorescence intensity
ND  not determined
UND  undetermined. p: Comparison between Reg POS and Reg NEG CLL patients.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORCID

Sophie Hillion  http://orcid.org/0000-0002-3354-0981
Christophe Jamin  http://orcid.org/0000-0002-9494-3223

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