Functional studies of chronic lymphocytic leukemia B cells expressing β₂-integrin type complement receptors CR3 and CR4

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Abstract

The expression and role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in B cells are not yet explored in contrast to myeloid cells, where these β_2-integrin type receptors are known to participate in various cellular functions, including phagocytosis, adherence and migration. Here we aimed to reveal the expression and role of CR3 and CR4 in human B cells. In B cells of healthy donors CR3 and CR4 are scarcely expressed. However, two patients with chronic lymphocytic leukemia (CLL) characterized by a peculiar immune-phenotype containing both CD5-positive and CD5-negative B cell populations made possible to study these molecules in distinct B cell subsets. We found that CD11b and CD11c were expressed on both CD5-positive and CD5-negative B cells, albeit to different extents. Our data suggest that these receptors are involved in spreading, since this activity of CpG-activated B cells on fibrinogen could be partially blocked by monoclonal antibodies specific for CD11b or CD11c. CpG-stimulation lead to proliferation of both CD5-positive and CD5-negative B cells of the patients with a less pronounced effect on the CD5-positive cells. In contrast to normal B cells, CLL B cells of both patients reacted to CpG-stimulation with robust IL-10 production. The concomitant, suboptimal stimulus via the BCR and TLR9 exerted either a synergistic enhancing effect or resulted in inhibition of proliferation and IL-10 production of patients' B cells.

Our data obtained studying B cells of leukemic patients point to the role of CR3 in the interaction of tumor cells with the microenvironment and suggest the involvement of IL-10 producing B cells in the pathologic process.

1. Introduction

The expression and function of CD11b and CD11c on myeloid cells is known for long. In association with CD18, they compose complement receptor type 3 (CR3, comprising CD11b/CD18) and type 4 (CR4, comprising CD11c/CD18), which belong to the family of β_2 integrins. These receptors mediate several important cellular functions, including adhesion, trans-endothelial migration and interaction with extracellular matrix proteins [1]. By interaction with iC3b, the complement-derived natural ligand, CR3 and CR4 mediate the uptake of opsonized cells and particles by monocytes, macrophages, neutrophils and dendritic cells [2]. Although CR3 and CR4 were thought to exert similar functions, recently we showed that they have different roles in various human myeloid cells. Namely, while CR3 dominates phagocytosis in the case of dendritic cells [3], CR4 is more important in mediating adherence by human monocytes macrophages and dendritic cells [2].

Regarding human B cells, the expression and function of CD11b and CD11c by various subsets is just being discovered. Appearance of CD11c but not CD11b on activated human tonsillar B cells was described in 1991 by Postigo et al. [4]. These authors proved that CD11c mediates binding to fibrinogen and may be involved in B cell proliferation. Later, Kawai et al showed that CD11b expressed by human memory B cells mediate migration [5].

Expression of CD11b, CD11c and CD18 has been reported in various B cell malignancies, including B cell chronic lymphocytic leukemia (B-CLL), or chronic lymphocytic leukemia (CLL) [6]. B-CLL represents the most common adult B cell tumor, which is characterized by the monoclonal expansion of CD5-, CD19- and CD23-positive B cells, usually with weak surface Ig expression [7]. CD5 is a pan-T-cell marker, which is constitutively expressed also on the long-lived subset of B-1 cells. CD5 is coexpressed with CD11b on a smaller population of B-1 cells, which have been shown to spontaneously produce IL-10 [8]. CLL demonstrates considerable heterogeneity at clinical, phenotypic as well as genomic level, including lack of CD5 expression in 7-20% of the cases [9]. Since the survival of the CLL cells is strongly dependent on the stimuli from the microenvironment, CD11b, CD11c and CD18 may play a role in proliferation and trafficking of CLL cells. The expression of CD11b by B cells of CLL patients was demonstrated by [10]. Later, the appearance of CD11c was detected on neoplastic B cells in the case of 52 out of 106 patients studied by [11]. Importantly, CD18 was found to be associated with advanced disease in CLL (Domingo, Leukemia Research 1997).

In the present study, we aimed to further characterize and identify the role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in CLL B cells. Selecting two patients with CLL characterized by a peculiar immune-phenotype containing both CD5-positive and CD5-negative B cell populations, provided us with the opportunity to study these molecules in distinct B cell subsets. The expression of CR3 and CR4, the proliferative capacity of the cells upon BCR and TLR9 triggering and their IL-10 production was thus assessed and compared on separated CD5-positive and CD5-negative subpopulations of the patients' B cells. Furthermore, the role of CR3 and CR4 in the capacity of B-CLL cells to adhere and spread over fibrinogen coated surface was also investigated.

2. Materials and methods

2.1. Healthy donors

Blood samples of healthy donors were purchased from the Hungarian Blood Transfusion Service with written informed consent of the donors. Venous blood treated with EDTA of two CLL patients was used in the experiments after giving informed consent. The study was conducted in accordance with the Declaration of Helsinki and has been approved by the Hungarian Medical Research Council Scientific and Research Committee (ETT TUKEB, permission number: 21655-1/2016/EKU).

2.2. Patients

Patient 1 was diagnosed with chronic lymphocytic leukemia (CLL, Rai stage 0) in 2010 at age of 71 presenting with 65% lymphocytosis with 40% CD19+ CD5+ CD23+ λ monoclonal B cells. These B cells showed low CD38 and intermediate CD20 and CD22 expression. In 2012, FISH analysis with probes specific for 17p deletion, 11q deletion, 13q deletion, 6q deletion and chromosome 12 trisomy was negative. In 2015, lymphocytosis increased to 75% with 46% malignant B cells and in addition to the classical CD5+ CLL B cells with low surface light chain expression, a CD5- B cell population with normal surface

light chain expression could be detected. These CD5- B cells did not show clonal restriction, but were considered malignant due to their high CD23 expression. The patient required no therapy, she is currently monitored using a watch and wait strategy.

Patient 2 is a 78-year-old female. CLL was diagnosed in 2009, classified as Rai stage 0. Characteristics at the diagnosis: 74% lymphocytosis with 35% CD19+ CD5+ CD23+ κ monoclonal B cells. These B cells showed low CD38 and intermediate CD20 and CD22 expression. In 2012, lymphocytosis increased to 81% with 56% CD19+ B cells. By flow cytometry analysis, B cells could be divided in two subpopulations. 27% of the B cells showed high CD23, intermediate CD19, CD20, CD22, CD5 and no CD81 expression, whereas 73% of the B cells showed high CD19, CD20, CD22, intermediate CD23 and partial CD5 expression. CD38 expression was still low on all B cells. FISH analysis with probe specific for p53 deletion was negative. In 2015, lymphocytosis increased, 88% lymphocytes in whole blood with 73% malignant B cells. Two B cell populations were identified, 40% of the B cells showed high CD19, CD20, intermediate CD200, CD23, κ light chain and no CD5 and CD38, whereas the other 60% showed intermediate CD19, CD20, CD200, high CD5, CD23 and no surface light chain expression. Similar to Patient 1, this patient required no therapy either, she is currently monitored using a watch and wait strategy.

Samples were available from both patients on five occasions within a period of 15 months.

2.3. Cell isolation, flow cytometry and cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma-Aldrich Inc., St Louis, MO) density gradient centrifugation from buffy coat or patients' EDTA-treated venous blood. Patients' B cells were purified by negative selection using the Miltenyi B-CLL Cell Isolation Kit achieving > 99% purity. B cells of healthy controls were purified by negative selection using the Miltenyi B Cell Isolation Kit II achieving ~80-95% purity.

For flow cytometry analyses, cells were labelled with mouse monoclonal antibodies against CD11b (clone TMG6-5, provided by István Andó at BRC Szeged, Hungary and clone ICRF44, BioLegend), CD11c (clone BU15, ImmunoTools GmbH, Friesoythe, Germany), CD21 (clone LT21, ImmunoTools) and CD35 (clone UJ11, ImmunoTools) and after washing cells were stained with Alexa488-conjugated donkey anti-mouse antibodies (Thermo Fisher Scientific Inc., Waltham, MA). After blocking with mouse serum, patients' B cells were stained with APC-conjugated anti-CD5 (clone LT1, ImmunoTools), and B cells of healthy controls with APC-conjugated anti-CD19 (clone LT19, ImmunoTools. B cell receptor expression was analysed by labelling the cells with goat anti-IgD F(ab')$_2$ (Southern Biotech) or goat anti-IgM F(ab')$_2$ (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and Alexa488-conjugated rabbit anti-goat antibodies (Thermo Fisher Scientific) or FITC-conjugated goat anti-IgG F(ab')$_2$ (Jackson ImmunoResearch Laboratories). Measurement was performed on a FACS Calibur cytometer (BD Biosciences, San Jose, CA) using the CellQuest software and data were analysed using the FlowJo software (Treestar, Ashland, OR, USA).

For analysis of CD5-positive and CD5-negative B cell populations, cells were stained with FITC-conjugated anti-CD19 (clone LT19, ImmunoTools) and APC-conjugated anti-CD5 and sorted using a FACS Aria III instrument and the FACSDiva software.

2.4. Quantitative real-time PCR analysis

Total RNA was extracted from sorted CD19-positive CD5-negative and CD19-positive CD5-positive cells of the patients obtained at three independent time-points as well as sorted CD19-positive B cells of healthy controls using Trizol reagent (Ambion) as recommended by the manufacturer. One microgram of RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. mRNA levels were quantified using the following TaqMan® assays: CD11b: Hs01064805_m1, CD11c: Hs00174217_m1, CD21: Hs01079100_m1, CD35: Hs00355835_m1. Samples were run in duplicates on a QuantStudio™ 3 Real-Time PCR System (Life Technologies) and results were obtained as threshold cycle (Ct) values. Expression values were calculated as the mean of duplicates and normalized to the expression of β-glucuronidase (Hs99999908_m1) using the ΔCt method.

2.5. Cell culture and proliferation assay

Purified B cells of healthy donors and sorted CD5-negative and CD5-positive B cells of the patients were seeded onto 96-well flat bottom culture plates (Greiner Bio-One, Kremsmünster, Austria) at 2×10^5 cells/well density in 200 µl RPMI-1640 medium containing 10% FCS and gentamycin. CpG-activated cells were cultured in the presence of 50 ng/ml recombinant human IL-2 (ImmunoTools) and 0.5 µg/ml CpG-ODN 2006 (Sigma) for 5 days. In proliferation assays, purified B cells of healthy donors and sorted CD5-positive and CD5-negative B cells of the patients were plated at 2×10^5 cells/well density in 100 µl medium containing 50 ng/ml IL-2. Cell culture wells were precoated with 2.5 µg/ml goat anti-human IgG+A+M F(ab')$_2$ (Jackson ImmunoResearch Inc.) plus 2.5 µg/ml goat anti-human IgD F(ab')$_2$ (Southern Biotech) and cells were stimulated with 0.5 µg/ml CpG ODN 2006 where indicated. Proliferation was assessed by measuring ^{3}H-thymidine incorporation. After 48 hours cells were pulsed with 1 µCi/well ^{3}H-thymidine (NEN, Boston, MA) for 18 hours. Incorporated radioactivity was measured with a Wallac 1409 liquid scintillation beta counter (Wallac, Allerod, Denmark). Data are expressed as cpm (count per minutes) values and are mean of triplicates + SD.

2.6. Measurement of adherence

96-well cell culture plates were coated with 10 µg/ml fibrinogen (Merck, Budapest, Hungary) for 1 h at 37°C and washed with phosphate buffered saline (PBS). 10^5 freshly isolated or activated (50 ng/ml IL-2 and 0.5 µg/ml CpG-ODN for 5 days) B cells were seeded onto the wells and let adhere for 1 h at 37°C. After fixing with 2% paraformaldehyde, unbound cells were washed away. The cells were stained with mouse anti-CD19 (Pharmingen, BD) and Alexa647-conjugated goat anti-mouse Ig (Thermo Fisher Scientific)

and the actin cytoskeleton was stained with Alexa488-conjugated phalloidin (Molecular Probes). In blocking experiments, activated B cells were incubated with FcR blocking reagent (Miltenyi Biotech) for 10 minutes at 4°C then labelled with APC-conjugated anti-CD5 for 20 min at 4°C. After washing, cells were incubated in medium alone, with 10 µg/ml anti-CD11b (clone ICRF44) or with anti-CD11c (clone BU15) for 30 min at 4°C. To ensure that recycled integrins from the cytoplasm are also blocked, the antibodies were not washed out. Cells were then allowed to adhere to fibrinogen-coated wells for 1 h at 37°C. After fixing and washing, nuclei were stained with Draq5 (Molecular Probes) and the actin cytoskeleton with Alexa488-conjugated phalloidin. Images were taken by an Olympus IX81 laser scanning confocal microscope using the FluoView500 software. Ten representative fields were scanned in each well and nuclei were counted using ImageJ software.

The area, perimeter and circularity parameters were measured for 100 cells/treatment based on the actin staining using ImageJ software. Circularity is calculated by the following formula (the value is between 0-1, with a perfect circle's value being 1): $4\pi \times$ [Area] / [Perimeter]^2. Cells with a circularity value < 0.35 were counted as "spread out".

2.7. Measurement of IL-10 production

Purified B cells of healthy donors and sorted CD5-positive and CD5-negative B cells of the patients were plated onto 96-well cell culture plates at 2×10^5 cells/well density in duplicates and stimulated as described for the proliferation assay. After 48 hours, IL-10 content of the supernatant was measured using IL-10 Duoset ELISA system (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Complement receptor expression of CLL B cells

Although sporadic data are available regarding the expression of complement receptors binding various activation fragments of component C3, namely CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in CLL [6, 12, 13], so far the B cell populations of the same patient were not screened in parallel. To get a complex picture, in our studies complement receptor expression was assessed employing the isolated B cells of each patient. We determined CR expression on the CD5-negative as well as the CD5-positive B cell populations.

We measured CD11b and CD11c expression on the patients' B cells by flow cytometry. In the case of Patient 1, CD11b was present on the majority of B cells, and only a small subset of CD5-positive cells showed low expression of the receptor (Figure 1). By contrast, CD11c was generally weakly expressed, whereas the CD5-positive subset showed strong expression. CD5-positive B cells of Patient 2 had very weak CD11b but strong CD11c expression, whereas CD5-negative B cells expressed CD11b but not CD11c. Gating on CD19-positive B cells of healthy donors, neither CD11b nor CD11c could be detected.

To get a complex picture of complement receptors, expression of CD35 and CD21 was also measured. CD35 was expressed on both the CD5-negative and CD5-positive B cells of Patient 1, while in the case of Patient 2 only CD5-negative B cells expressed CD35. CD21 was present on all B cells of both patients. CD19-positive B cells of a healthy donor expressed both CD21 and CD35.

Expression of CD11b, CD11c, CD21 and CD35 at mRNA level was also determined and compared to that of normal B cells of healthy donors. Patients' B cells were sorted and CR expression of the CD19-positive CD5-negative and CD19-positive CD5-positive populations were analysed. B cells of healthy donors were sorted based on CD19-positivity. Results were normalized to the expression of the housekeeping gene β-glucuronidase. All CR mRNAs were detectable in both the CD5-negative and CD5-positive B cell populations of the patients as well as in B cells of healthy donors, even when surface expression of the CR proteins were not detectable by flow cytometry (Figure 2).

3.2. Proliferation of CLL B cells

Next, the proliferative capacity of the CD5-positive and CD5-negative subpopulations of B-CLL patients and B cells of healthy individuals was compared. CLL B cells with mutated Ig genes are known to become anergic in response to stimulation via BCR [14] while unmutated CLL retains sIg mediated signalling [15]. Recently, Chatzouli et al. showed that concomitant engagement of BCR and TLR9 exerts a heterogeneous effect depending on the mutational status of sIg [16].

Our results show that CpG induces strong proliferation of CD5-negative B cells of both patients, as well as B cells of healthy controls. CD5-positive CLL B cells also proliferate in response to CpG, however, to a lesser extent (Figure 3). The suboptimal dose of anti-BCR stimulus used in these experiments resulted in a weak activation of Patient 1's B cells and B cells of healthy individuals, and had no effect in the case of Patient 2. Simultaneous, suboptimal stimulus by CpG and anti-BCR synergistically enhanced proliferation of Patient 1's CD5-negative B cells, similar to B cells of healthy controls, but not of CD5-positive B cells. Interestingly, both CD5-positive and CD5-negative B cells of Patient 2 showed weaker proliferation in response to the combined stimulus than to CpG alone. This might have been caused by differences in BCR expression of the CD5-negative and the CD5-positive populations of the patients' B cells, as detailed in the section of Materials and Methods.

3.3. CpG stimulation leads to IL-10 production

CLL B cells are known to secrete high amounts of the anti-inflammatory cytokine IL-10, mainly due to their elevated CD5 expression [17]. To test whether there is a difference between the CD5-negative and CD5-positive B cells of the patients regarding this ability, IL-10 production was measured from cell culture supernatants after stimulation with anti-BCR, CpG and their combination.

We found that unstimulated B cells of both patients and those of healthy controls produced negligible amount of IL-10, similarly to their response to a suboptimal BCR-stimulus (Figure 4). By contrast, CpG caused a robust IL-10 production by the patients' B lymphocytes with a more pronounced effect on the CD5-positive B cells, but only slightly increased IL-10 secretion of normal B cells. The effect of the combined stimulus did not differ from that of CpG alone in case of CD5-positive cells of the patients as well as of normal B cells of healthy controls. However, CD5-negative B cells of Patient 1 produced higher amount of IL-10 when CpG stimulus was combined with anti-BCR stimulus, while it had an inhibitory effect on CD5-negative B cells of Patient 2.

3.4. CpG stimulation leads to changes in CR3 and CR4 expression

Next we set out to investigate whether stimulation of the cells induces any changes in the expression of the β_2-integrin family members, CR3 and CR4. As seen in Figure 5, CpG-stimulation lead to changes in CR-expression by the patients' B cells. In the case of Patient 1, the CD5-positive and the CD5-negative B cells had similar characteristics. Flow cytometry analysis revealed decreased CD11b expression compared to the freshly isolated cells, while no staining with CD11c was seen. CpG-stimulated Bcells of Patient 2 showed a similar pattern to that of freshly isolated cells, however, expression of CD11b and CD11c was less intensive. CD5-negative cells were still negative for CD11b.

3.5. Blocking CR3 or CR4 on CpG-activated CLL B cells decreases spreading on fibrinogen

CLL B cells of both patients adhered to fibrinogen (Figure 6), whereas adhesion of normal B cells could not be seen under the conditions used (data not shown). Freshly isolated CLL B cells were small and round, showing typical B cell morphology. Although the cells expressed CR3 and/or CR4 (Figure 1), blocking CD11b or CD11c had no influence on the adhesion of the cells (data not shown). By contrast, CpG-activated cells were significantly larger and prone to spread, shown by actin cytoskeleton staining (Figure 6). CR3- and CR4-expression in response to CpG decreased (Figure 5), though their role in adhesion of the activated cells to fibrinogen could be demonstrated. Preincubation of the cells with monoclonal antibodies specific to the ligand binding site of CD11b and CD11c partially inhibited spreading of the cells (Figure 7).

4. Discussion

While the expression and function of CD11b and CD11c on myeloid cells is known for long, the possible role of CR3 and CR4, the β_2-integrin type complement receptors in

various functions of B cells is just being discovered. In order to get a deeper insight into the proliferative capacity, IL-10 production and adherence of CR3 and CR4 expressing B lymphocytes, we selected for our studies two CLL patients with a peculiar immune-phenotype containing both CD5-positive and CD5-negative B cell populations. Our results are summarized in Table 1.

In contrast to healthy individuals, where we could detect neither CD11b nor CD11c on the CD19-positive population, B cells of both CLL patients expressed CR3 and CR4, although with different intensity (Figure 1 and Table 1). CD5-positive and CD5-negative B cell populations of Patient 1 had similar characteristics with strong CD11b and weak CD11c expression, except for a small CD5-positive population that was positive only for CD11c. B cells of Patient 2, however, were different: CD5-positive cells expressed CD11c, while CD5-negative cells CD11b. This difference between the patients is probably explicable by the different origin of the CD5-negative populations. CD5-negative B cells of Patient 1 do not show clonal restriction, whereas CD5-negative B cells of Patient 2 are thought to originate from the CD5-positive clone (see Patient description). Although in our present study we did not aim to investigate the expression and function of complement receptor type 1 (CD35) and 2 (CD21), to get a more complex view, these receptors were also included in the screening both at the mRNA and protein levels. CD5-positive CLL B cells are reported to partially or completely lose CD35 and decrease CD21 expression. In accordance with this, we found that CD21 was expressed on the cell surface of both the CD5-positive and CD5-negative subpopulations of the patients and in the case of Patient 2 CD35 was lost on the CD5-positive cells. CD5-positive B cells of Patient 1, however, strongly expressed CD35.

In the proliferation assay, CD5-negative B cells of both patients showed robust proliferation in response to CpG-treatment, similar to normal B cells (Figure 3 and [18]). Furthermore, in agreement with the observation of Decker et al. [19], CD5-positive CLL B lymphocytes of both patients' showed remarkable proliferation to CpG stimulation, which might correlate with these cells' higher expression of TLR9, the main receptor for CpG [20]. In contrast to the strong response to CpG, triggering via the BCR did not induce a notable proliferation of B cells under the experimental conditions used. The low response to the BCR-mediated stimulus partially might be due to the anergic state of B cells, which is a characteristic feature in mutated CLL. In addition, we used suboptimal dose of BCR-stimulus to analyze the synergistic effect of the anti-BCR and CpG stimuli. The combined trigger enhanced the proliferation of Patient 1's CD5-negative B cells – similarly to the results obtained with cells of healthy donors –, and induced a weaker response in the case of Patient 2's B lymphocytes when compared to the trigger by CpG alone. Our data point to the modulation of BCR-mediated trigger by additional stimuli to induce proliferation of CLL B cells, a phenomenon which might be particularly important under pathological conditions.

Our finding that CD5-positive B cells are less reactive than the CD5-negative ones might partially be due to the low expression of surface immunoglobulins and the negative regulatory effect of CD5 molecule. This latter had already been proven in the case of the BCR signaling pathway [21-23]. Furthermore, Jahrsdörfer et al. found that in response to CpG, the CD5-negative population of CLL B cells undergo more divisions than the CD5-positive cells [24]. In line with these data, we found that CpG treatment alone promoted mainly the proliferation of the CD5-negative B cells, particularly in the case of Patient 2.

In healthy individuals, IL-10 secreting B cells – termed B10 cells – normally represent only a very small proportion of blood B cells [25, 26]. In contrast to this, B cells of CLL patients possess the capacity to secrete substantial amounts of IL-10 that can promote leukemic cell survival via immunosuppression [25, 27]. In agreement with these data, we found a much higher – up to ten-fold – amount of IL-10 in the supernatant of stimulated B-CLL cells than in the case of healthy controls. Our data demonstrate that in addition to – or parallel with – the strong proliferative effect of CpG, this agonist also induces a significant IL-10 production by both the CD5-positive and CD5-negative subpopulations of the patients' B cells, supporting the data of Chen et al. [20]. In response to the combined anti-BCR and CpG trigger, the CD5-positive B cells of the patients did not produce much higher amount of IL-10 than to CpG treatment alone – which might be again due to the suboptimal BCR-trigger, decreased surface immunoglobulin expression and anergic state of the malignant cells. Data on IL-10 secretion of CD5-negative CLL B cells are not available in the literature. The pattern of IL-10 production of the CD5-negative B cells in response to the combined anti-BCR and anti-TLR9 stimulus is remarkably similar to that of proliferation. This might suggest that the higher amount of secreted IL-10 is not the result of differentiation but is rather due to the increased number of cells in the culture. In agreement with the known IL-10 synthesis promoting effect of CD5, we detected elevated IL-10 secretion of CD5-positive cells in the case of Patient 2 in response to CpG-treatment alone or its combination with BCR ligation [17].

Activation of patients' B cells via TLR9 leads to changes in expression of CR3 and CR4, as well as changes in morphology of fibrinogen-adhered cells. Stimulation with CpG induced spreading of the cells measured by the area of adhered cells (Figure 6). Expression of the analyzed integrins CR3 and CR4 was reduced on CpG-treated cells (Figure 5) still, there was no difference in the number of adhered cells on fibrinogen coat between freshly isolated and CpG-stimulated cells. This suggests that adhesion of CLL B cells to fibrinogen is mediated mainly via other integrins. Since spreading of CpG-activated B cells of the patients on fibrinogen, which could be incompletely blocked by monoclonal antibodies specific for CR3 or CR4, we suppose that these receptors are partially responsible for mediating spreading in response to activating stimuli (Figure 7). CR3 was also implicated in the protection against fludarabine-induced apoptosis of CLL B cells when binding another of its natural ligands, the complement fragment iC3b [28]. Similar roles, namely mediating adhesion/spreading and protection against apoptosis against anti-cancer drugs are attributed to another integrin, CD49d (VLA-4) [29].

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Table 1. Characteristics of CLL B cells from Patients 1 and 2.

Patient	CD5 expression	CR3 (CD11b/ CD18) expression	CR4 (CD11c/ CD18) expression	Proliferation in response to CpG	Proliferation in response to CpG +BCR [1]	IL-10 secretion in response to CpG	IL-10 secretion in response to CpG +BCR [2]	Spreading of CpG-stimulated cells on fibrinogen [3]
1.	CD5-negative	+++	+	++	+++	++	+++	++ partial inhibition by anti-CD11b
	CD5-positive	+++/- [4]	+/+++ [4]	+	+	++	++	
2.	CD5-negative	++	-	+++	++	++	+	++ partial inhibition by anti-CD11b and anti-CD11c
	CD5-positive	(+)	++	++	(+)	+++	++	

[1] Cells were stimulated with suboptimal anti-BCR stimulus, which alone did not induce notable proliferation
[2] Cells were stimulated with suboptimal anti-BCR stimulus, which alone did not induce notable IL-10 production
[3] Unseparated CD5-negative and CD5-positive cells were investigated
[4] Signs after '/' denote a small CD5-positive subset showing characteristics distinct from the majority of CD5-positive cells

Figure 1

Patient 1

Patient 2
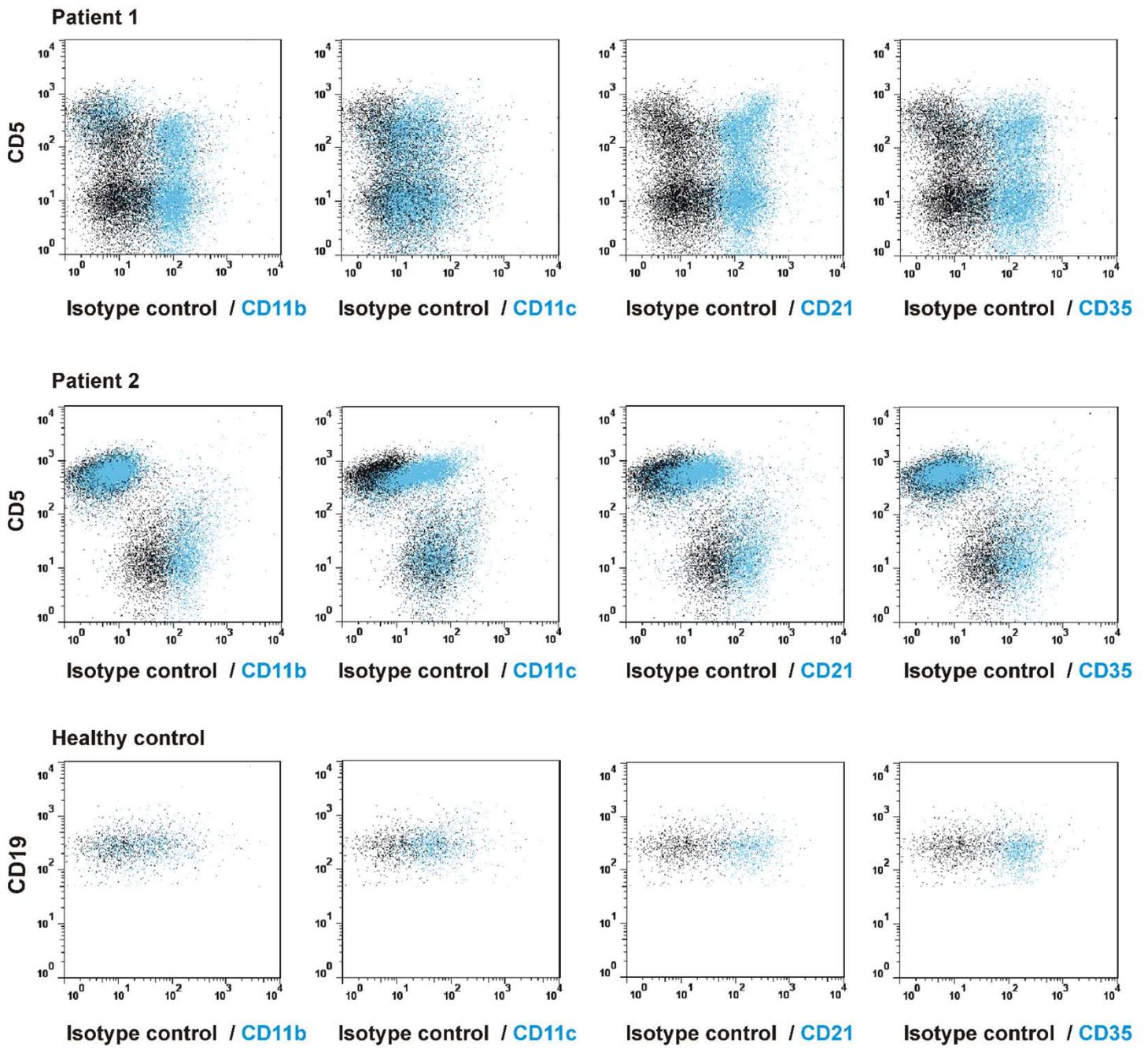
Healthy control
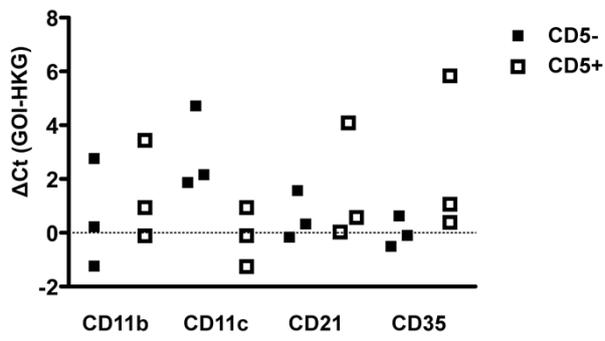
Figure 2

Patient 1

Patient 2
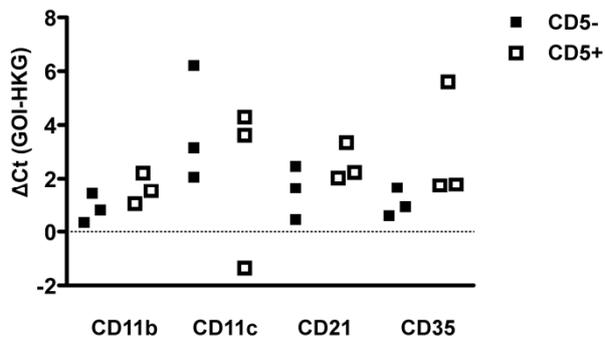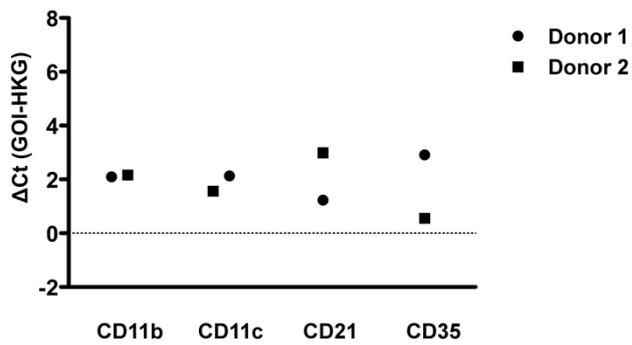
Healthy controls

Figure 3

Patient 1
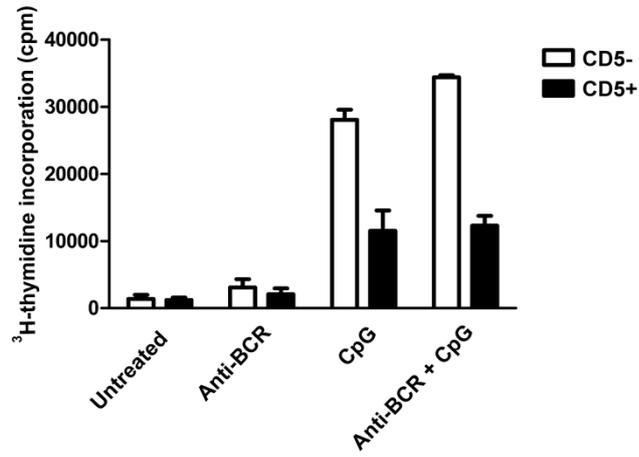
Patient 2
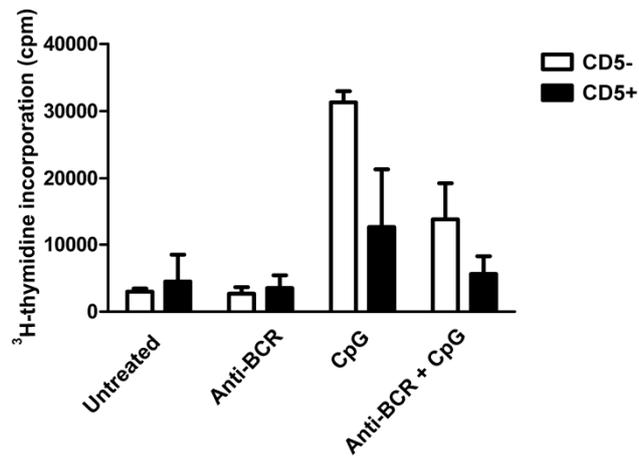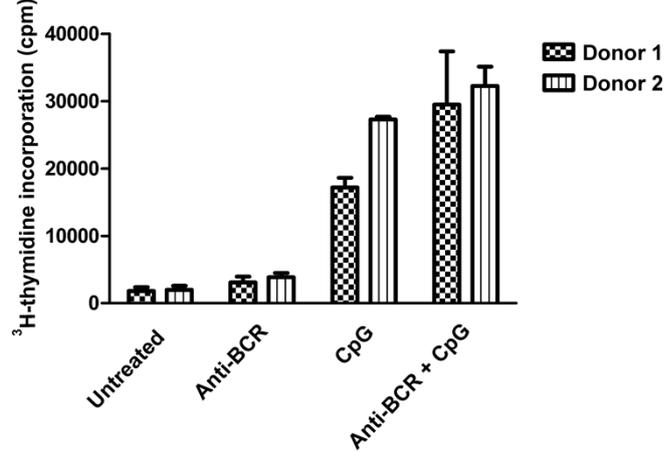
Healthy controls

Figure 4

Patient 1

Patient 2

Healthy controls

Figure 5

Patient 1

Isotype control / CD11b   Isotype control / CD11c

Patient 2

Isotype control / CD11b   Isotype control / CD11c

Healthy control

Isotype control / CD11b   Isotype control / CD11c
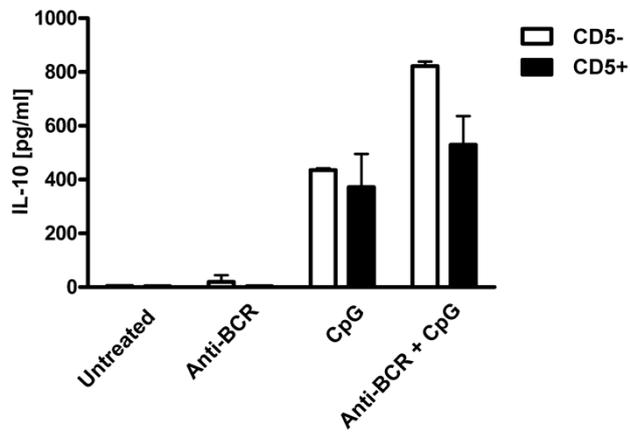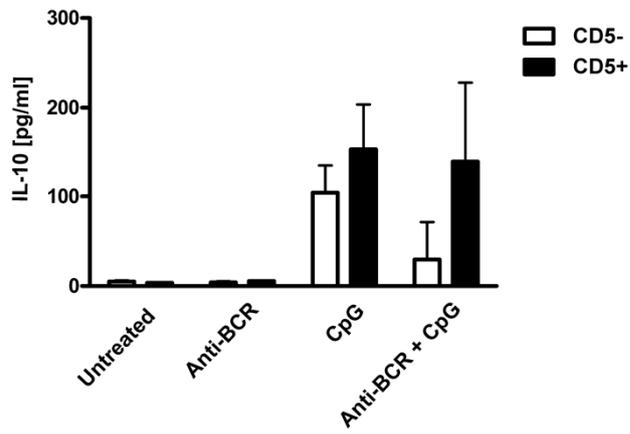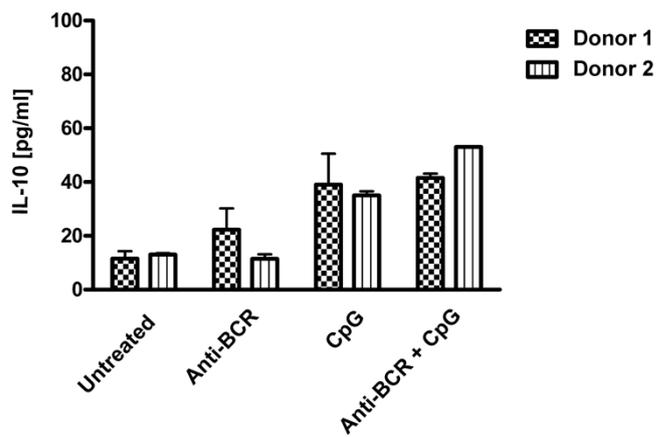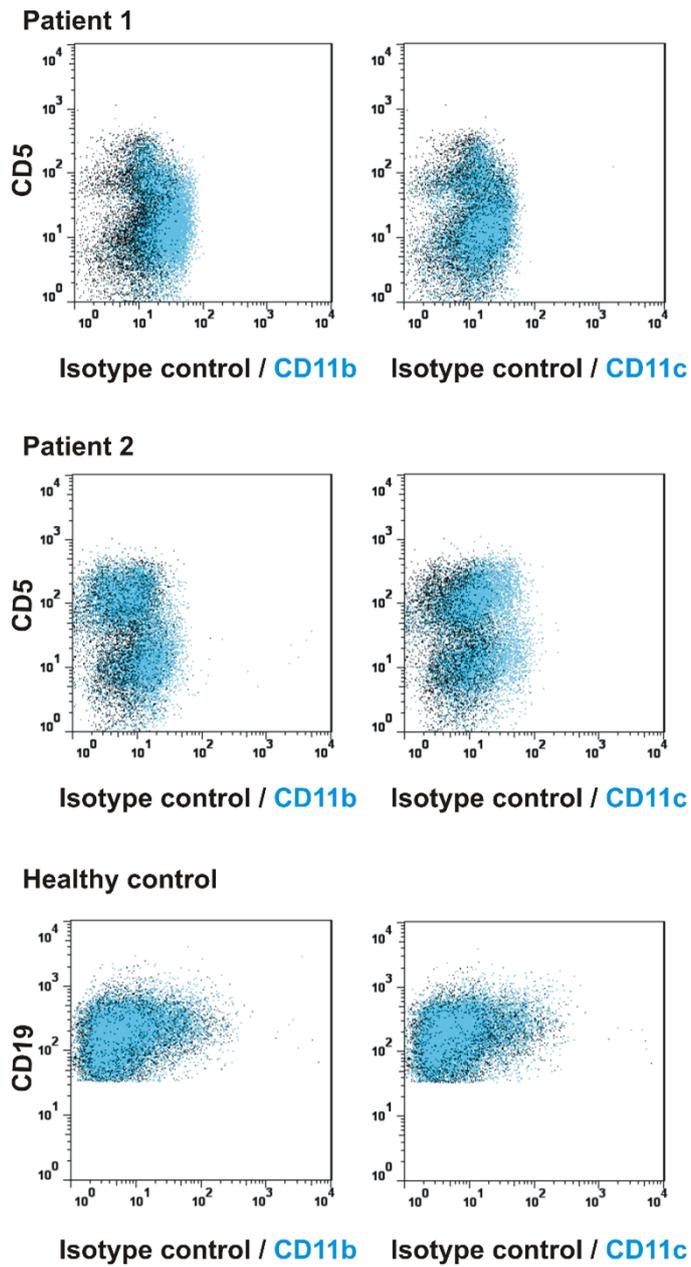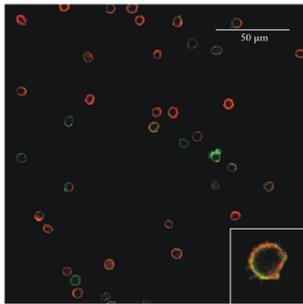
# Figure 6

Patient 1
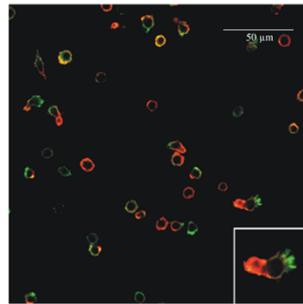
Freshly isolated   CpG-stimulated
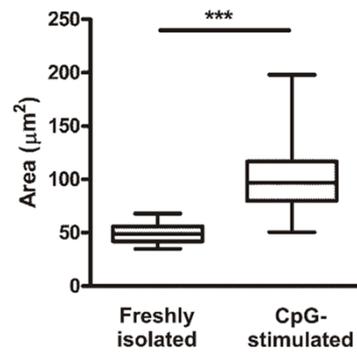
Patient 2
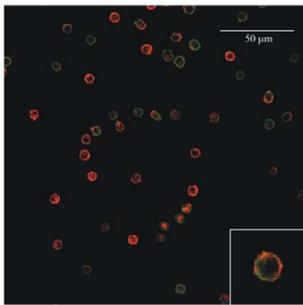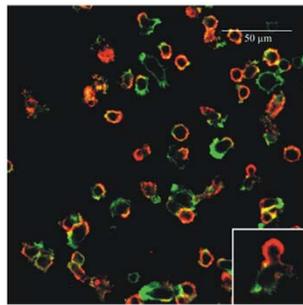
Freshly isolated   CpG-stimulated
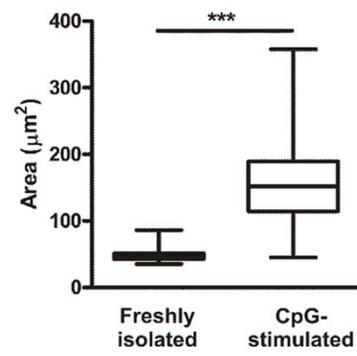
Figure

Patient 1
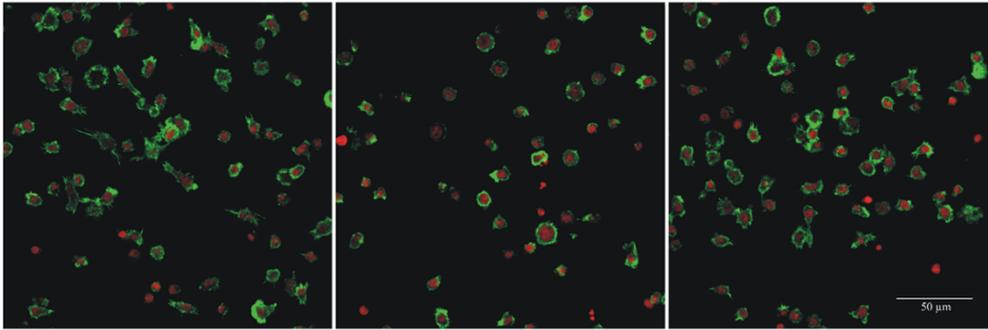
Untreated   Anti-CD11b   Anti-CD11c
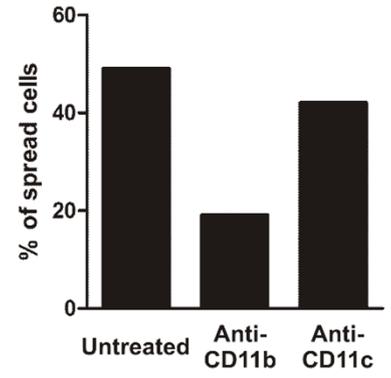
Patient 2

Untreated   Anti-CD11b   Anti-CD11c