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CD150 and CD180 are negative regulators of IL-10 expression and secretion in chronic lymphocytic leukemia B cells

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Chronic lymphocytic leukemia (CLL) is a strikingly heterogeneous disease both at the molecular level and clinical disease course. The malignant B cells obtain key proliferation and survival signals within lymph nodes or bone marrow. Moreover, CLL B cells and tumor microenvironment dynamically co-evolve organizing inflammatory and immunosuppressive microenvironment by direct contact with surrounding cells and secretion of cytokines, growth factors, or extracellular vesicles. Finding a way to weaken obtaining by malignant B cells supportive signals may improve CLL outcome and optimize treatment strategies. The aim of this study was to evaluate whether CD150 and CD180 cell surface receptors could be involved in the regulation of CLL B cells-derived cytokines (CCL3, CCL4, IL-6, and IL-10). The study was performed on malignant B cells isolated from peripheral blood of primary CLL patients. Flow cytometry, qPCR, ELISA, western blot, *ex vivo* cell surface ligation assay, *ex vivo* drug sensitivity assay, and cell viability assay were used in this study. The CCL3, CCL4, IL-6, and IL-10 mRNA expression levels were heterogeneously presented among studied CLL cases. The elevated CCL3/CCL4 and decreased IL-6/IL-10 expression level are specific features of CLL B cells with positive CD150 and CD180 expression status. Ligation of CD150 and CD180 receptors did not affect CCL3/CCL4 mRNA expression level in CLL B cells, while IL-6 and IL-10 were significantly decreased. After malignant B cells stimulation via CD150 and CD180 observed reduced IL-10 but not IL-6 levels in culture supernatants. Ligation of CD150 and CD180 was linked to the classical NF-κB pathway via regulation of phosphorylation level of NF-κB inhibitor IκBα. We found several correlations between basal mRNA expression levels of CCL3, CCL4, IL-6, and IL-10 in CLL B cells and their sensitivity to chemotherapeutic drugs *ex vivo*. High CCL3/CCL4 and low IL-10 mRNA expression levels are associated with malignant B cells' sensitivity to BEN, while high IL-6 levels are a sign of CLL B cells' resistance to FC. The revealed involvement of CD150 and CD180 in cytokine regulation expands our knowledge of the role of CD150 and CD180 in the pathobiology of CLL and their contribution to a favorable clinical outcome. Determining the cytokines expression levels together with CD150 and CD180 expression status may help to predict the responsiveness of CLL B cells to chemotherapeutic drugs and optimize personalized chemotherapy scheme.

Key words: B cells, cytokines, CD150, CD180, expression, chemosensitivity

Chronic lymphocytic leukemia (CLL) progression is strongly dependent on a combination of extracellular signals, which malignant B cells obtain in local tissue microenvironment [1]. Within lymph nodes and bone marrow, CLL B cells obtain supportive pro-survival and proliferation signals by cross-talk with monocyte-derived nurse-like cells (NLCs), mesenchymal stromal cells, T cells, natural killers, macrophages through direct cell-cell interaction, and via secreted growth factors and cytokines [2, 3]. The inability of CLL B cells to survive without feeder layer of NLCs or fibroblasts *in vitro*, the resistance of CLL B cells co-cultured with NLCs to chemotherapeutic drugs, the association between high serum level of several cytokines, and worse clinical outcome of CLL patients are direct indications that local tumor microenvironment significantly contributes to CLL pathogenesis [4, 5]. These suggest that finding a way of therapeutic targeting the components of the tumor microenvironment is a perspective direction as an additional treatment strategy for relapsed/refractory CLL.

Target cell perception of tumor microenvironment signals depends on its cell surface receptors' expression profile. Combination of input signaling through BCR, Toll-like receptors, adhesion molecules, receptors of tumor necrosis factors' family, cytokines receptors, etc. define malignant B cell fate. That is why cell surface receptors are active players of CLL pathogenesis and could be considered as poten-

tial targets for the regulation of CLL B cells' signaling and biological properties. Cell surface receptors CD150 and CD180 that were previously described as prognostic markers of favorable CLL outcome could contribute to the signaling network of malignant B cells based on their function [1, 6]. Our previous data showed that simultaneous activation of CD150 and CD180 cell surface receptors on CLL B cells led to inhibition of the main pro-survival Akt and MAPK signaling pathways [7]. Moreover, activation of CD150 and CD180 receptors on CLL B cells led to changes in mRNA expression levels of transcription factors (TFs) that regulate B cells' identity and differentiation (EBF1, IRF4, IRF8, BCL6, and PU.1) [8]. Since both CD150 as adhesion and costimulatory molecule and CD180 as a pattern recognizing receptor could be activated in the tumor microenvironment by a pattern of common microbial, neo- and autoantigens, we tested a hypothesis whether CD150 and CD180-mediated signaling could be directly linked to modification of CLL microenvironment through regulation of cytokines expression and secretion by CLL B cells.

Cytokines and their receptors are one of the abundant components of the tumor microenvironment. Numerous data show that high levels of cytokines expression in B cell malignancies are associated with disease progression, drug resistance, and unfavorable clinical outcome [4, 9–13]. In particular, high serum levels of CCL3, CCL4, and IL-6 correlate with poor CLL outcome [14–16]. Elevated IL-10 serum level is also a sign of CLL progression and in addition, is associated with overall immunosuppression [17].

Based on the above, in the current study, we examined whether it is possible to regulate the expression and secretion levels of main driver cytokines (CCL3, CCL4, IL-6, and IL-10) of CLL B cells' propagation via CD150 or/and CD180 receptors.

Patients and methods

Patients and PBMCs isolation. Blood samples from 20 previously untreated patients were obtained from the Department of Oncohematology at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of National Academy of Science of Ukraine (IEPOR, NASU, Kyiv, Ukraine) after verifying CLL diagnosis. All experimental procedures were performed with the patient's prior consent and in accordance with the Declaration of Helsinki, the International Review Board, and Research Ethics Committees of IEPOR NASU. Peripheral blood mononuclear cells (PBMCs) from CLL patients were isolated by Lymphoprep (Axis-Shield PoCAS, Norway) density gradient centrifugation according to the manufacturer's protocol. The malignant cells represented at least 90% in samples of PBMCs.

Flow cytometry analysis. PBMCs from CLL patients were immunophenotyped on the subject of CD5, CD23, CD43, CD19, CD20, CD150, and CD180 as described earlier [7]. Results were analyzed according to GeoMean MFI ratio of antigen to isotype control, where <1.3 r.u. were considered as negative cell surface expression of antigen, GeoMean ratio ≥1.3 r.u. means positive cell surface expression.

Cell stimulation *ex vivo*. CLL B cells obtained from patient`s blood samples with $CD150^{+}CD180^{+}$ phenotype were incubated with 10 μg/ml anti-CD150 (IPO3, IEPOR NASU, UA, Cat No 12601) or/and 10 μg/ml anti-CD180 (G28-8, Thermo Fisher Scientific, USA, Cat No MA1-10125, kindly provided by Prof. Edward Clark, University of Washington, Seattle, WA, USA) monoclonal antibodies (mAbs) in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a CO_2 incubator. Stimulation was stopped after 5, 15, 30 min, and 48 h of incubation with ice-cold PBS + 0.1% NaN_3.

Western blot analysis. PBMCs' precipitates obtained from CLL patients were lysed in Triton lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris, pH 8.0, 1% Triton X100) containing cocktails of protease and phosphatase inhibitors (Sigma, USA). Lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described earlier [18]. Primary goat anti-actin (Santa Cruz Biotechnology, USA, Cat No sc-1615), rabbit anti-pIkBα (Cell Signaling Technology, USA, Cat No 2859), biotin-conjugated IL-6 (Gen-PROBE, USA, Cat No L80703), and IL-10 (Abcam, UK, Cat No ab84031) antibodies were used in this study with following secondary goat anti-rabbit (Abcam, UK, Cat No ab205718), donkey anti-goat (Cat No sc-2030) HRP-conjugated antibodies, and HRP-conjugated streptavidin (Cat No sc-363872) (both from Santa Cruz Biotechnology, USA). Visualization of results was carried out using Clarity Western ECL substrate (Immuno-Star HRP, BioRad, USA) and medical X-ray film (AGFA, Belgium).

Real-time quantitative PCR. Total RNA was isolated from 5×10^6 PBMCs of CLL patients using NucleoZOL reagent (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's protocol. Quality and concentrations of RNA were measured on a spectrophotometer NanoDrop™ 1000 (Thermo Scientific, USA). The cDNA synthesis and real-time PCR were made by a scheme that was reported in previous publications [7, 8]. The sequences or primers used in our research are listed in Table 1. The comparative ΔΔCt method was used to calculate target gene expression levels normalized to internal control (TBP).

Table 1. The primers sequence used in study.

Gene	Forward primer 5′→3′	Reverse primer 5′→3′
TBP	ccactcacagactctcacaac	ctgcggtacaatcccagaact
CCL3	gaaggacacgggcagcagaca	gcagcaagtgatgcagagaactgct
CCL4	cttttcttacaccgcgagga	gcttgcttctttttggtttgg
IL-6	tcaatgaggagacttgcctggtga	tcatctgcacagctctggcttgtt
IL-10	ctttaagggttacctgggttgc	ccttgatgtctgggtcttggt

Enzyme-linked immunosorbent assay (ELISA). To determine the effect of CD150 or/and CD180 ligation on IL-6 and IL-10 secretion levels by CLL B cells, PBMCs previously cultivated during 48 hours under the conditions as described above were centrifuged at 354×g for 5 min at room temperature and culture supernatants were collected. The IL-6 and IL-10 concentrations in culture supernatants were performed using IL-6 and IL-10 ELISA kits (both from Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The optical density was read at 490 nm. The reaction was performed in triplicates.

***Ex vivo* analysis of drugs cytotoxicity.** The sensitivity of CLL B cells to chemotherapeutic drugs was determined as described earlier [19]. The following cytotoxic drugs were used: fludarabine (S. C. Sindan-Pharma S.R.L, Bucharest, Romania), cyclophosphamide (Baxter Healthcare Corporation, Germany), and bendamustine (S.C.Sindan-Pharma S.R.L, Bucharest, Romania). The viability of PBMCs from CLL patients under the cytotoxic drug treatment *ex vivo* was determined by resazurin assay.

Statistical analysis. Statistical significance between groups was evaluated by nonparametric Mann-Whitney U test using Prism Software Version 8.0. Statistical significance between examined groups was assessed as $p<0.05$. Pearson's coefficient was used for the determination of correlation between variables. Results of basal mRNA and protein expression are presented in box plots where whiskers mean maximum and minimum values, upper and lower borders of rectangles match the third and first quartiles respectively. ELISA and mRNA expression levels' data after stimulation are represented as mean ± SEM.

Results

All tested CLL cases were diagnosed as a typical form of CLL with classic $CD5^+CD19^+CD23^+$ malignant B cells phenotype. Since CD150 and CD180 are differentially expressed on CLL B cells, all CLL cases were grouped into $CD150^-CD180^-$ and $CD150^+CD180^+$ (n=10 in each group). The first step in our study was to identify any association between the basal mRNA expression levels of CCL3, CCL4, IL-6, and IL-10 cytokines and CD150/CD180 expression status on malignant B cells. We established that basal mRNA expression levels of CCL3 and CCL4 were significantly higher in $CD150^+CD180^+$ CLL B cells than in $CD150^-CD180^-$ ones ($p=0.01$; Figures 1A, 1B). High mRNA expression levels of IL-6 and IL-10 were found in CLL cases with $CD150^-CD180^-$ B cells but not in $CD150^+CD180^+$ ones ($p=0.01$ and $p=0.02$ respectively; Figures 1C, 1D). Thus, CLL cases are characterized by the heterogeneous mRNA expression level of CCL3, CCL4, IL-6, and IL-10 cytokines in malignant B cells. High mRNA expression levels of CCL3/CCL4 and low levels of IL-6/IL-10 were associated with $CD150^+CD180^+$ CLL B cell phenotype.

Could the revealed link between cytokine expression levels and CD150/CD180 presence on the cell surface of CLL B cells have a functional feature? To clarify the hypothesis that CD150 or/and CD180-mediated signaling are directly involved in cytokine regulation, we performed *ex vivo* ligation of CD150 or/and CD180 receptors on malignant B cells using mAbs against CD150 and CD180 respectively with further evaluation of cytokine's RNA expression levels. Ligation of CD150 or/and CD180 on CLL B cells did not cause statistically significant changes in CCL3/CCL4 mRNA expression level (Figures 2A, 2B). The IL-6 mRNA expression level was considerably downregulated after stimulation of CLL B cells via CD150 or/and CD180 receptors ($p<0.04$; Figure 2C). Both single and dual CD150 and CD180 ligation led to decreased IL-10 mRNA expression levels in CLL B cells ($p<0.01$; Figure 2D). Consequently, signaling initiated through CD150 and CD180 receptors negatively regulates IL-6 and IL-10 mRNA expression, however, is not involved in the regulation of CCL3/CCL4 mRNA expression.

Since CD150 and CD180 are involved in the regulation of IL-6 and IL-10 mRNA expression levels, but not CCL3/CCL4, our following studies were focused just on IL-6 and IL-10. The next step was to verify whether obtained dependence between IL-6 and IL-10 mRNA expression levels and the CD150 and CD180 cell surface receptor status would be confirmed on protein level. According to densitometry analysis, IL-6 and IL-10 protein expression levels were significantly different between $CD150^+CD180^+$ and $CD150^-CD180^-$ CLL B cells (Figure 3). $CD150^+CD180^+$ CLL B cells are characterized by notably lower IL-10 protein expression level ($p=0.03$; Figures 3A, 3C) compared to $CD150^-CD180^-$ ones that reflect the results of basal IL-10 mRNA expression level in malignant B cells (Figure 1D). Low IL-6 protein expression was also detected in $CD150^+CD180^+$ CLL B cells in contrast to $CD150^-CD180^-$ ones as it was observed at basal mRNA expression level ($p=0.02$; Figure 1C and Figures 3A, 3B).

Malignant B cells secrete a plethora of cytokines to affect the surrounding microenvironment and facilitate tumor progression. Thus, the main question was to check whether CD150 and CD180 receptors are involved in the regulation of IL-6 and IL-10 secretion levels. To answer this question, CLL PMBCs were cultivated during 48 hours with added anti-CD150 and anti-CD180 mAbs alone or in combination with the culture medium for CD150/CD180 *ex vivo* ligation. After that, the culture supernatants were collected and concentrations of IL-6 and IL-10 were defined using enzyme-linked immunosorbent assay (ELISA). Ligation of CD150 or/and CD180 on CLL B cells did not influence the IL-6 concentration in culture supernatants compared to the unligated CLL B cells (Figure 4A). At the same time, a statistically significant reduction was noted for the IL-10 level in culture supernatant after CD150 or/and CD180 ligation on CLL B cells ($p<0.001$; Figure 4B). Therefore, CD150- and CD180-mediated signaling lead to downregulation of the immunosuppressive IL-10 secretion level in CLL B cells but at the same time do not affect the IL-6 secretion.

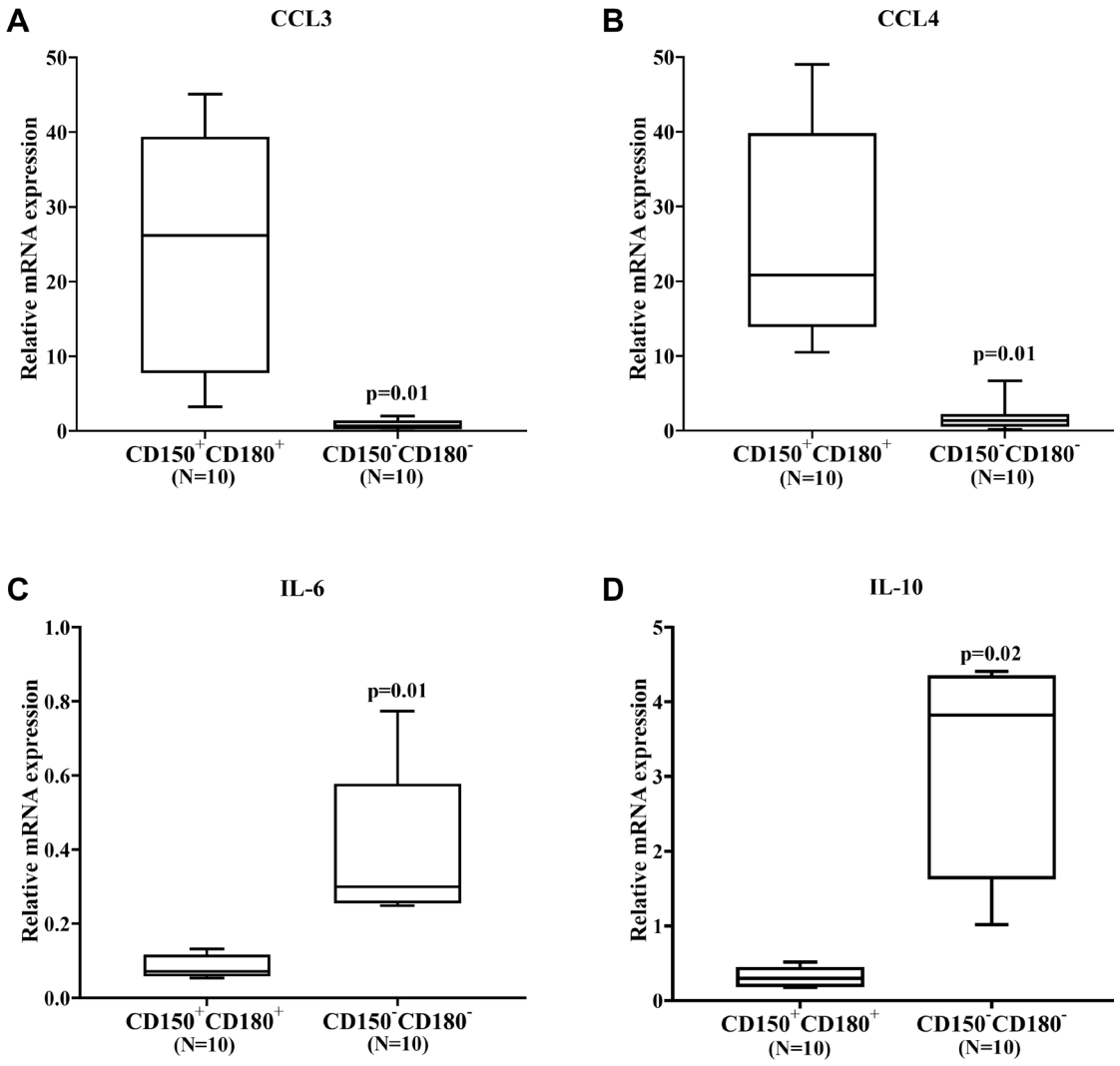

Figure 1. Basal mRNA expression level of cytokine CCL3 (A), CCL4 (B), IL-6 (C), and IL-10 (D) in CLL B cells according to CD150 and CD180 expression. High basal mRNA expression levels of CCL3/CCL4 and significantly downregulated IL-6/IL-10 mRNA expression levels are features of the CD150^{+}CD180^{+} malignant B cells. Results of qRT-PCR. Expression of cytokines was normalized to TBP expression level. Box plots showed quartiles, median, minimum, and maximum values.

Cytokines' expression levels are controlled by the combinations of numerous TFs including B-cell lineage-specific and members of the NF-κB family. Previously it was described in detail the involvement of CD150 and CD180 in the regulation of CLL B-cell specific TFs' expression [8]. Besides, malignant B cells are characterized by high expression levels of NF-kB family members of TFs and their constitutive activity compared to normal B cells through the CLL course [20]. Since the classical NF-κB signaling pathway is a known cytokines' regulator, we checked if it is possible to change the NF-kB activation status via CD150 or/and CD180 *ex vivo* ligation on CLL B cells. Based on the basal phosphorylation level of NF-κB inhibitor IκBα in malignant B cells, two subgroups of CLL cases were revealed (Figure 5). In a part of CLL cases, a phosphorylated form of IκBα was not detected in B cells before receptors' ligation that corresponds to inactivated classical NF-κB signaling pathway (Figure 5A). The activated classical NF-κB signaling pathway was found in another part of cases, supported by a high level of pIκBα in untreated with mAbs CLL B cells (Figure 5B). Ligation of CD150 or/and CD180 caused increasing of pIκBα level just on 30 minutes of CLL B cells stimulation in cases with the basal inactivated NF-κB signaling (Figure 5A). It's important to note that simultaneous ligation of CD150 and CD180 on CLL B cells resulted in a significantly lower level of IκBα phosphorylation compared to these receptors' single effect. The level of pIκBα was slightly decreased after CD150 or CD180 ligation and significantly reduced after these

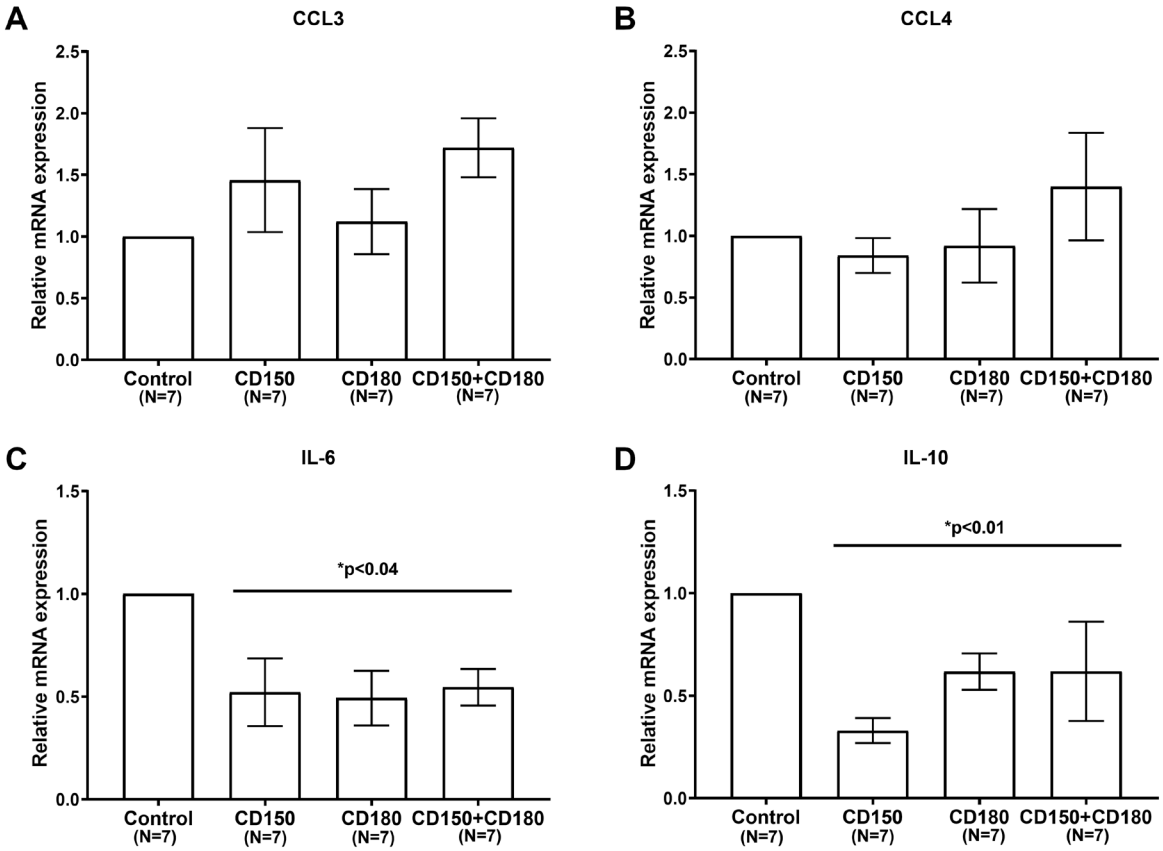

Figure 2. CD150- and CD180-mediated regulation of cytokine CCL3 (A), CCL4 (B), IL-6 (C), and IL-10 (D) mRNA expression level. Ligation of CD150 or/and CD180 on CLL B cells did not cause statistically significant changes in CCL3/CCL4 mRNA expression level but led to the downregulation of IL-6 and IL-10 mRNA levels. Results of qRT-PCR. mRNA expressions of cytokines were measured after the ligation of CD150 or/and CD180 receptors in CLL B cells *ex vivo*. *p-value was measured compared to the control (cultivated CLL B cells without added mAbs).

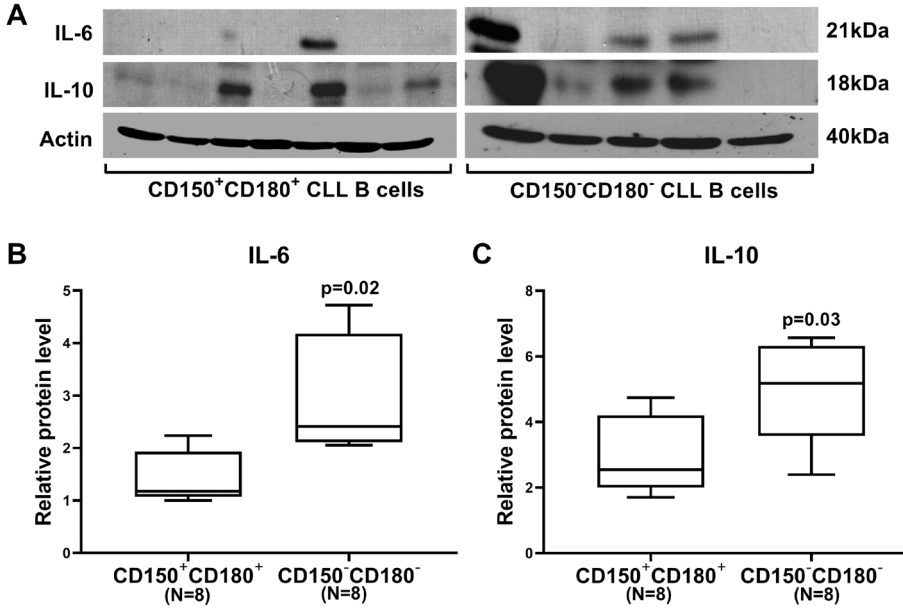

Figure 3. Basal protein expression level of cytokine IL-6 and IL-10 in CLL B cells considering CD150 and CD180 expression status. The significantly higher IL-6 and IL-10 protein expression were found in CLL cases with $CD150^{-}CD180^{-}$, than $CD150^{+}CD180^{+}$ B cells. A) western blot results; B, C) densitometric analysis of IL-6 and IL-10 expressions, respectively.

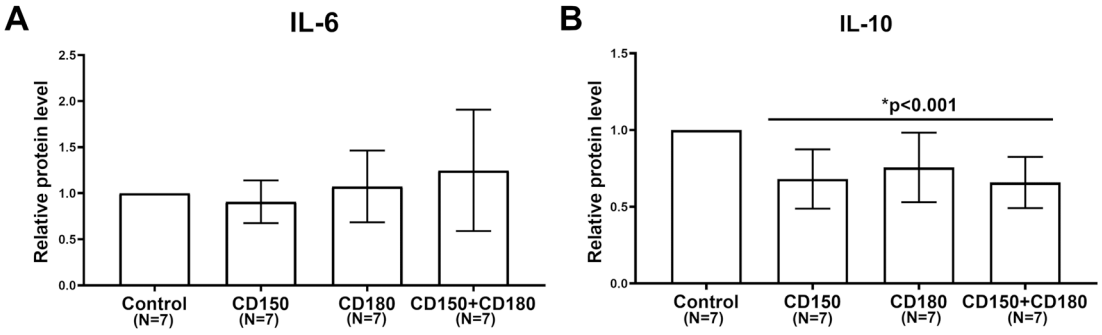

Figure 4. The CD150- and CD180-mediated regulation of cytokine IL-6 (A) and IL-10 (B) secretion by CLL B cells. Ligation of CD150 or/and CD180 on CLL B cells did not influence the IL-6 concentration in culture supernatants compared to unligated CLL B cells, while a statistically significant reduction of IL-10 level was noted in culture. Changes in secretion levels were defined as the relation of cytokines' levels in culture medium after ligation of CD150 or/and CD180 receptors on CLL B cells *ex vivo* to those in control. *p-value was measured compared to control (cultivated CLL B cells without added mAbs).

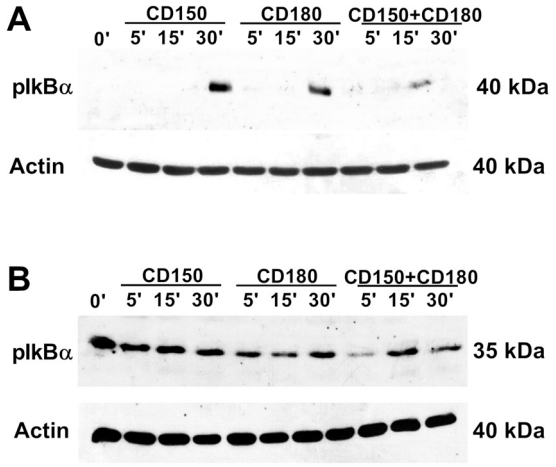

Figure 5. Kinetics of IκBα phosphorylation after CD150 or/and CD180 ligation in CLL B cells. Western blot results. A, B) reflect CLL cases with initially inactivated and active NF-κB signaling in CLL B cells respectively.

Table 2. Correlation between sensitivity of CLL B cells to cytotoxicity drugs and their CCL3, CCL4, IL-6, and IL-10 basal mRNA expression levels.

Cytokine	Chemotherapy drugs			
	FLU	CP	BEN	FC
CCL3	–0.15	–0.12	**–0.68***	0.36
CCL4	–0.12	–0.09	**–0.67***	–0.17
IL-6	0.06	0.35	–0.08	**0.89***
IL-10	0.49	0.20	**0.71***	0.19

Note: Data represent as Pearson correlation coefficients, * - p<0.05

receptors co-ligation on CLL B cells with constitutively active NF-κB signaling pathway (Figure 5B). Thus, the outcome of CD150 and CD180-mediated regulation of classical NF-κB signaling pathway was depended on initial NF-κB activation status. Simultaneous ligation of CD150 and CD180 leads to partial inactivation of the classical NF-κB signaling pathway in CLL B cells that could be a possible reason for downregulation of cytokines' expression.

One of the important aspects in determining the prognosis of the CLL course is patients' response to treatment. In our study, we also try to find out whether there are any correlations between the basal mRNA expression levels of investigated cytokines and the sensitivity of CLL B cells to chemotherapeutic drugs *ex vivo*. For our research, we chose the cytotoxicity drugs which are classically used in the first-line therapy of CLL patients – fludarabine (FLU), cyclophosphamide (CP), and bendamustine (BEN). The combined effect of fludarabine and cyclophosphamide (FC) was also studied because these drugs are usually used together during the chemotherapy course of CLL. We found several correlations between basal mRNA expression levels of CCL3, CCL4, IL-6, and IL-10 in CLL B cells and their sensitivity to chemotherapeutic drugs *ex vivo* (Table 2). In particular, a negative correlation was found between basal CCL3 and CCL4 mRNA expression levels and CLL B cells' viability after BEN exposure (r=–0.68 and –0.67 respectively; p<0.05). On the other hand, high basal IL-6 and IL-10 mRNA expression levels were associated with CLL B cells' resistance to FC and BEN correspondingly (r=0.89 and r=0.71, respectively; p<0.05). The sensitivity of CLL B cells to CP or FLU did not correlate with the basal mRNA expression levels of any studied cytokines. Therefore, high mRNA expression levels of CCL3/CCL4 were a marker of CLL B cells' sensitivity to BEN, while high IL-10 mRNA expression level was a feature of CLL B cells' resistance to BEN. High IL-6 mRNA level was associated with a worse response of CLL B cells to FC.

Discussion

The local tissue microenvironment plays an active role in CLL development, malignant B cell proliferation, survival, sensitivity to chemotherapy, and disease progression. Within the CLL microenvironment, malignant B cells receive activa-

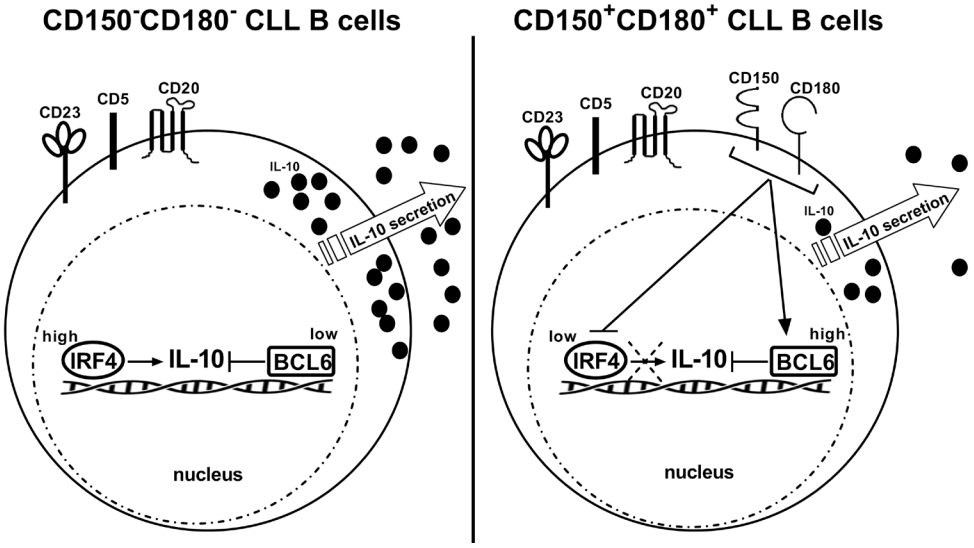

Figure 6. IL-10 expression in CLL B cells taking into consideration the CD150 and CD180 expression status. Double negative CD150⁻CD180⁻ CLL B cells in contrast to CD150⁺CD180⁺ ones are characterized by elevated mRNA and protein levels of IL-10 expression as well as IL-10 secretion level. CD150 and CD180 receptors-mediated signaling are directly involved in the downregulation of IRF4 and in upregulation of BCL6 TFs which are positive and negative regulators of IL-10 mRNA expression respectively that may partially explain the favorable outcome in CLL patients with positive CD150 and CD180 expression.

tion and pro-survival signals via direct cell contacts and reacting to soluble factors as cytokines. Upon interaction with NLCs as well as a result of BCR and CD40 activation CLL B cells express and secrete CCL3 and CCL4 chemokines [21]. The function of CCL3 and CCL4 in CLL remains poorly defined, but based upon the function of these B cell-derived chemokines in normal immune responses, increased CCL3 and CCL4 secretion by CLL cells attracts T lymphocytes and monocytes. The CCL3- and CCL4-mediated attraction of T lymphocytes to the tumor local niche could support the neoplastic process via CD40 signaling initiated on malignant B cells after interaction with CD40-ligand (CD154) expressing T lymphocytes [22]. It was shown that the plasma level of CCL3 was strongly associated with established prognostic markers of worse CLL outcome and time to treatment [21]. Here, we also showed that the determination of CCL3 and CCL4 mRNA expression levels could be used as a predictor of malignant B cells' response to BEN. Despite that high serum CCL3/CCL4 levels are considered as poor outcome markers, the BEN inclusion in personalized chemotherapy scheme could increase treatment effectivity of CLL patients with high CCL3/CCL4 level. Since the NF-κB mediates enhancing of several cytokines expression levels including CCL3/CCL4 and IL-6, it could be hypothesized that elevated CCL3/CCL4 expression levels in CD150⁺CD180⁺ B cells are associated with revealed in some CLL cases a basal phosphorylated form of IκBα that reflects the active NF-κB signaling. At the same time, the absence of changes in CCL3/CCL4 expression after CD150 and CD180 ligation, despite partial receptors-mediated IκBα dephosphorylation, indicates the possible involvement of additional signaling pathways and TFs that are CD150 and CD180 independent. As was reported, NLCs-mediated increasing of CCL3 and CCL4 expression and secretion levels by malignant B cells managed to block using a selective inhibitor of Syk kinase. Such fact indicates that the BCR signaling is the main driver of CCL3/CCL4 production [23]. Our previous results showed the involvement of CD150/CD180 receptors in the regulation of Akt and MAPK signaling pathways that are also activated via BCR [7]. This evidence explains the existence of crosstalk between signals from CD150/CD180 receptors and BCR in malignant B cells. Considering the BCR-mediated strong upregulation of CCL3/CCL4 levels and absence changes after CD150 or/and CD180 *ex vivo* ligation, it could be assuming the necessity of additional costimulatory signals for reaching the CCL3/CCL4 downregulation via CD150 and CD180. For testing this hypothesis, further experiments should be performed.

In CLL, as is common in many hematological malignancies, a more aggressive disease course is associated with systemic immunosuppression [24]. The existence of a separate subpopulation of regulatory B cells (known as suppressor B10 cells) was described which are characterized by the active IL-10 secreting. A key difference of B10 cells is in providing inflammatory and autoimmune reactions as well as the promotion of cancer progression. CLL B cells also secrete IL-10 and share features of suppressor B10 cells, suggesting a contribution of neoplastic B cells to immunosuppression in CLL patients [25]. Development and function of regulatory B10 cells subset with active IL10 secretion are regulated via BCR and CD40 signaling [26]. In addition,

IL-10 expression in B10 and CLL B cells is upregulated via TLR and BAFF receptor TACI [27]. Thus, autocrine production of BAFF is not only facilitating CLL B-cell survival but also promotes B10 cells' activity and immunosuppression in CLL patients via IL-10 production. How this effect can be diminished? May the receptor-mediated signaling events downregulate these chemokines' expression? Here we showed that CD150 and CD180-mediated signaling events downregulate the expression and secretion of IL-10 (Figures 2 and 4). In B10 cells transcription factors Blimp1, XBP1, and IRF4 which are known positive regulators of IL-10 expression are enhanced whereas negative regulators PAX5 and BCL6 are reduced [28, 29]. According to our previous data, CD150 or/and CD180 ligation on CLL B cells *ex vivo* led to a decrease in IRF4 mRNA expression up to 4 times. At the same time, after CD150 or CD180 ligation the BCL6 mRNA expression level was increased compared to control although CLL B cells were characterized by lower basal BCL6 mRNA expression in both $CD150^-$ and $CD150^+$ subgroups of CLL [8]. Considering the above, it could be hypothesized that CD150 or/and CD180 mediated inhibition of IL-10 expression by CLL B cells is connected with the regulation of BCL6 and IRF4 mRNA expression via CD150 and CD180 signaling pathways. The *IL10* gene is not a target of p50/p65 NF-κB subunits [30], so it is unlikely that NF-κB is involved in IL-10 regulation. However, partial silencing of NF-κB signaling after CD150 and CD180 ligation may explain the downregulation of IL-6 mRNA expression level in CLL B cells because of IL-6 inclusion in NF-kB target genes [31]. Considering the above, it could be hypothesized that CD150 or/and CD180 mediated inhibition of IL-10 expression is linked to regulation of BCL6 and IRF4 mRNA expression levels in CLL B cells, while IL-6 with NF-κB signaling (Figure 6).In our previous study, we have shown that the sensitivity of CLL B cells to treatment using cytotoxicity drugs is dependent on the cell surface phenotype of malignant B cells [19]. Herein, we showed that CCL3, CCL4, and IL-10 mRNA expression level determination could be useful for the evaluation of CLL B cells' sensitivity to BEN, while IL-6 for the FC sensitivity (Table 2). High IL-6 mRNA expression was observed in CLL B cells which were more resistant to FC therapy. It's important to note that $CD150^-CD180^-$ phenotype is an independent indicator of worse malignant B cells' response to FC combinatory application [19]. *Ex vivo* ligation of CD150 or/and CD180 on CLL B cells led to a decrease of IL-6 mRNA expression level (Figure 2C) that could explain the increased sensitivity of CLL B cells to FC observed in $CD150^+CD180^+$ CLL cases via IL-6 downregulation. CLL B cells with high basal mRNA expression levels of CCL3 and CCL4 but low mRNA expression level of IL-10 is more sensitive for cytotoxicity impact of BEN *ex vivo* that supported by high rates of Pearson's correlation coefficients between these values (Table 2). Moreover, CD150 or/and CD180 receptors are negative regulators of IL-10 expression and secretion by malignant B cells. So, signaling pathways triggered by CD150 and CD180 receptors can contribute to CLL B cells' better response to BEN therapy via inhibition of IL-10 expression and secretion.

Summarizing all of the above, CLL B cells are characterized by a different profile of CCL3, CCL4, IL-6, and IL-10 expression levels that are dependent on CD150 and CD180 receptors' expression status. High mRNA expression levels of CCL3/CCL4 and significantly downregulated IL-6/IL-10 expression levels are features of the $CD150^+CD180^+$ B cells. The CD150 and CD180 receptors are negative regulators of IL-6 and IL-10 mRNA expression in CLL B cells. For the first time, it was shown that CD150 and CD180 ligation leads to a reduction of immunosuppressive IL-10 secretion. Determination of CCL3, CCL4, IL-6, and IL-10 cytokines mRNA expression level could be used as additional predictive markers of CLL B cells' response to chemotherapeutic drugs. High CCL3/CCL4 and low IL-10 mRNA expression levels point to malignant B cells' sensitivity to BEN, while high IL-6 levels are a sign of CLL B cells' resistance to FC. Our studies not only broaden the knowledge about CD150 and CD180 involvement in CLL pathobiology but also suggest an additional CD150 and CD180-directed approach for improvement of CLL patients' survival and quality of life.

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