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Different expression of CD180, CD284 and CD14 receptors on the CD19$^+$ subpopulation of normal and B-CLL lymphocytes

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Abstract: Numerous experimental data indicate that B-CLL development and progression are influenced by antigenic pressure. It can not be excluded that these antigens may originate from bacteria and viruses. Toll like receptors (TLRs) interact with pathogen associated molecular patterns as part of innate immunity. TLRs are currently used to target different subclasses of B-cell leukemia, and TLR agonists are being evaluated in clinical trials. It is little known regarding the repertoire and function of TLR in B-CLL. The aim of the study was to assess the CD180, CD284 and mCD14 levels in CD19$^+$ subpopulation of B-CLL peripheral blood lymphocytes and compare them with respective levels in the normal B-cells of adult volunteers, before and after LPS stimulation. We investigated the percentage of the CD19$^+$CD180$^+$, CD19$^+$CD284$^+$, CD19$^+$CD14$^+$ cells and the mean fluorescence intensity (MFI) of CD180, CD284 and CD14 antigens among CD19$^+$ B-CLL as well as in the normal B cells for comparison. MFI analysis revealed that CD180, CD284 and CD14 expression was higher on normal B cells then on CD19$^+$ B-CLL (MFI CD180: 99.16 *vs*. 25.3, MFI CD284: 7.37 *vs*. 5.79 and MFI CD14 25.07 *vs*. 8.32). After 24-hour LPS activation of B-cells, CD180 MFI appeared to decrease, in both healthy and B-CLL patients. CD284 MFI in healthy controls decreased after LPS stimulation while slight increase of MFI was observed in leukemic cells. CD14 MFI in leukemic cells was moderately higher after LPS in comparison to CD14 MFI without LPS stimulation, whereas CD14 MFI in normal CD19$^+$ cells after LPS stimulation decreased over three times. Variations observed in expression of both normal and leukemic receptors may be due to their different sensitivity to antigenic stimulation.

Key words: B-CLL, CD180, CD284, CD14

Introduction

B-cell chronic lymphocytic leukaemia (B-CLL) is characterized by accumulation of monoclonal long-living CD5$^+$/CD19$^+$, CD23$^+$, CD21$^+$, CD40$^+$ B lymphocytes in bone marrow, peripheral blood and lymphoid organs. Irrespective of its phenotypic homogenity, B-CLL is clinical heterogeneous. The mutation status of immunoglobulin heavy chain (IgV_H) genes, expression of ZAP70, and CD38, are used as markers of the clinical heterogenity [1,2]. Numerous experimental data indicate that B-CLL development and progression are influenced by antigenic pressure. It can't be excluded that these antigens may originate from bacteria and viruses.The humoral immune system senses microbes via recognition of specific microbial molecular motifs by Toll-like receptors (TLRs) [3]. There are evidences that endogenous TLRs ligands or chronic infections promote tumor growth [4]. TLRs are currently used to target different subclasses of B-cell leukemia, and TLR agonists are being evaluated in clinical trials. It is little known regarding the repertoire and function of TLR in B-CLL. The family of TLR includes 11 different transmembrane proteins answerable for recognition of specific pathogen-associated molecular patterns [5].

Lipopolysaccharide (LPS) is a major gram-negative bacterial component that stimulates innate response and also induces B-lymphocyte activation [3,5]. LPS activates B cells through two known receptors: TLR4 (CD284) and CD180 (previously called

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RP105). TLR4 is essential for signaling via LPS and requires another molecule, MD-2 which is associated with extracellular domain of TLR4 [6]. CD180 structurally resembles TLRs (except Toll/IL -1 receptor (TIR) domain) and it promotes LPS-driven B-cell responses. CD180 creates a complex with MD1 which is structurally related to TLR4/MD-2 complex [7] CD180 is B-cell specific while TLR4 is universally expressed, and each of them uses distinct pathways. LPS binding to CD180/MD1 induces Lyn activation and CD19 phosphorylation whereas LPS binding to TLR4/MD-2 activates MyD88/IRAK and MyD88-independent TIRAP pathways to activate JNK and NFκB [8,9,10]. CD14 functions as a co-receptor for LPS [11]. The role of CD14 in TLR4 signaling consists of binding LPS and presenting it to MD-2 and TLR4 [12,13].

The aim of the present study was to assess the CD180, CD284 and mCD14 levels in $CD19^+$ subpopulation of B-CLL peripheral blood lymphocytes and compare them with respective levels in the normal B-cells of adult volunteers, before and after LPS stimulation.

Materials and methods

Patients. Fifteen patients with newly diagnosed or untreated chronic lymphocytic leukemia (B-CLL) were included in this analysis (median age 64, range: 45-79 years). Patients were diagnosed at the Department of Haematooncology and Bone Marrow Transplantation of the Medical University in Lublin. The diagnosis was made according to standard criteria: clinical, morphological and immunophenotypic features of B-CLL. The total lymphocyte count ranged from 10.54 to 638 × 10^9/L. Stage of the disease was determined according to Rai classification (14). Patients were graded as follows: stage 0 (2 cases), stage I (3 case), stage II (5 cases) stage III (3 cases), and stage IV (2 cases). A group ten of healthy blood donors (median age 55, range: 46-67) was used as control group.

Peripheral blood mononuclear cells (PBMCs) were obtained from patients with B-CLL, and from healthy controls by density gradient centrifugation over Lymphoprep (Nycomed, Pharma AS, Norway).

Flow cytometry analysis. For two-color and triple direct staining flow cytometry, (FACSCalibur, Becton Dickinson Immunocytometry System) PBMCs (1×10^6 cells) suspended in staining buffer (phosphate buffered saline supplemented with 1.0% fetal calf serum and 0.1% sodium azide, pH 7.4) were incubated with commercial mouse monoclonal antibodies [anti-human CD19/Cy-Chrome (PE-Cy5), anti-human CD5/FITC, anti-human CD14/FITC, anti-human CD180/PE anti-human CD284/FITC] and respective immunoglobulin isotype controls. Staining was performed according to supplier instructions for 20 min on ice.

The percentage of cells was calculated on the basis of data obtained from two-color immunofluorescence dot-blots. Analyses were carried out using CellQUEST Software (Becton Dickinson) on gated lymphocytes. Quadrant markers were set relative to negative immunoglobulin isotype controls in such a way that 98-99% of the cells labeled with the control antibodies were located in lower left (LL) quadrant.

CD180, CD284, and mCD14 expression was evaluated as mean fluorescence intensity (MFI) on gated $CD19^+CD180^+$, $CD19^+/CD284^+$ and $CD19^+/CD14^+$ lymphocyte population. MFI was calculated as a ratio of mean value of the fluorescence of the test over that of the control.

Lymphocyte cultures. Human peripheral blood (PB) lymphocytes and B-CLL cells were cultured in RPMI-1640 medium (Gibco) containing FCS (fetal calf serum) (10%), HEPES (10 mM), glutamine 2 mM, streptomycin (100μg/ml), penicillin (100 IU/ml) (Polfa, Poland), on culture plates (10^6 cells/ml) (Corning Inc, USA) at 37°C in humidified 5% CO_2 atmosphere for 24 hours. Lymphocytes were stimulated by LPS (50 μg/ml) (Sigma).

Statistical analysis. All results are shown as mean value, with standard deviation. Statistical significance of the differences observed was determined with the U Mann-Whitney test (Statistica 8.0 PL).

Results

Immunophenotypes

Immunophenotyping was performed by dual- and triple- color flow-cytometry analysis of peripheral blood samples $CD19^+$. The mean percentage of $CD19^+$ lymphocytes in normal controls was 7.2% (SD: 2.57) and in CLL patients was 91% (SD: 5.01).

As demonstrated by $CD19^+/CD5^+$ co-expression analysis, the vast majority of B cells analyzed were malignant CLL cells (mean 80.44%) (Fig.1). $CD19^+/CD5^+$ co-expression was found only in 2.75% of normal PBL, the difference was statistically significant (p=0.0027).

We determine the percentage and MFI of the CD180, CD284, and CD14 positive cells among $CD19^+$ B-CLL normal B cells. The percentage of subpopulation $CD19^+CD180^+$ did not differ significantly between analyzed groups of patients and healthy donors (87.4% in B-CLL and 90.9% in normal cells). CD284 expression was found only in 1.8% of $CD19^+$ normal B cells and 1.1% of $CD19^+$ B CLL cells. Similarly, the percentage of $CD19^+/CD14^+$ population was very low. In normal control was 1.5% and only 0.2% in B-CLL (Fig. 2)

Using flow cytometry MFI was analyzed. The analysis included of CD180, CD284 and CD14 MFI on the $CD19^+$ cell surface. We have shown that CD180 expression of unstimulated normal B cells was four time higher in comparison with unstimulated CLL B cells (MFI: 99.16 *vs* 25.3; p=0.0007). Moreover, the expression CD284 in normal B cells was insignificantly higher than in CLL (MFI: 7.37 *vs*. 5.79; p=0.37). However CD14 MFI was significantly higher in normal B cells (25.07 *vs*. 8.32; p=0.001). Table 1 contains detailed results.

After 24 hour LPS activation of B-cells, the significant differences of CD180 expression were observed. CD180 expression decreased after 24 hour LPS stimulation in normal and leukemic $CD19^+$ cells. CD284 after LPS stimulation insignificantly decreased in healthy controls (mean 6.16 *vs*. 7.37 before LPS stimulation). In contrast, we observed very slight increase of MFI (mean 6.26 after *vs*. 5.79 before LPS stimulation) in leukemic cells. CD14 MFI in leukemic cells

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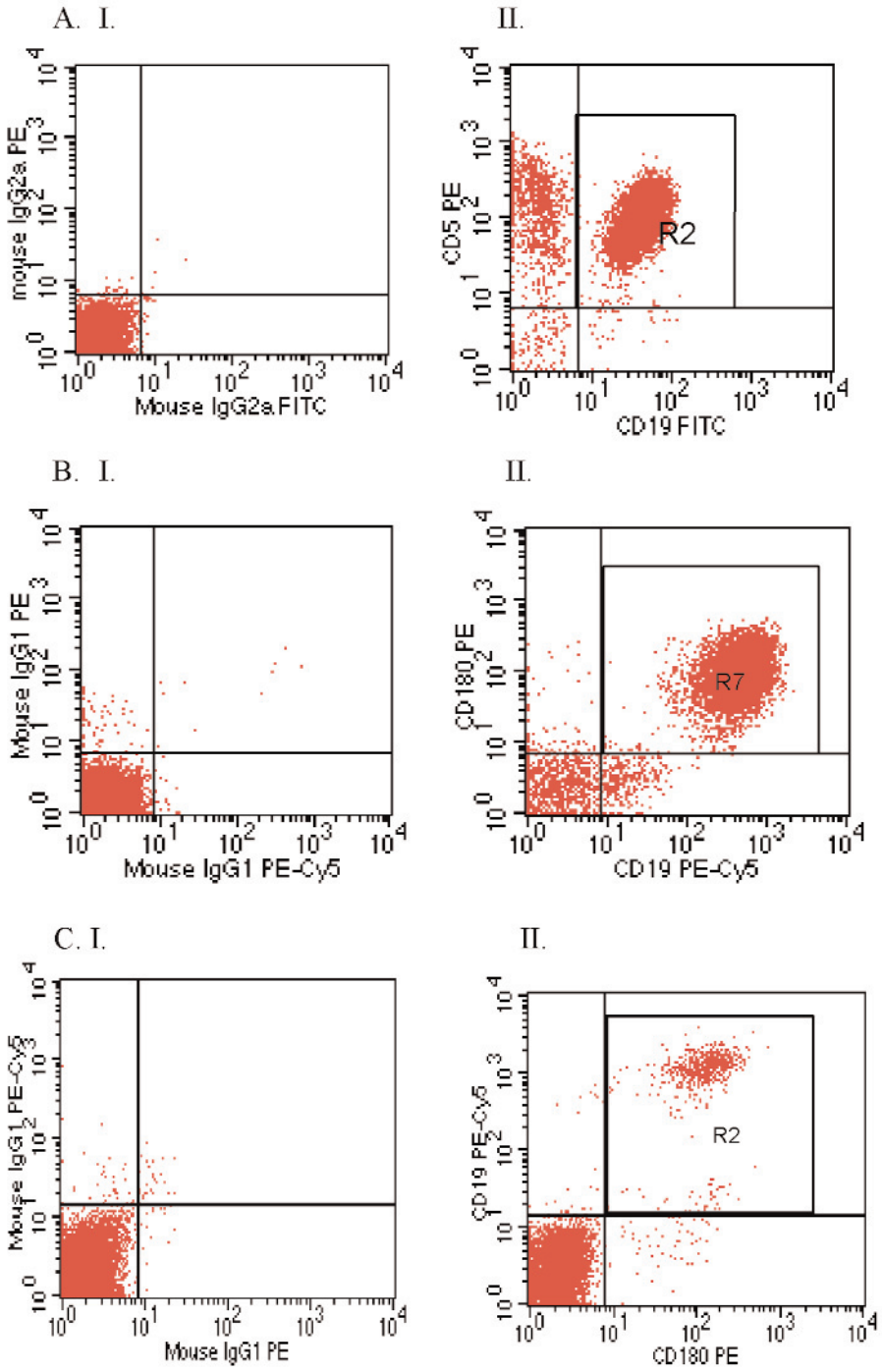

Fig. 1. Expression examples of $CD19^+/CD5^+$ (A) and $CD19^+/CD180^+$ (B) in CLL B cells, CD19+/CD180+ (C) in normal B cells (I – an isotype control, II – B cells of CLL population).

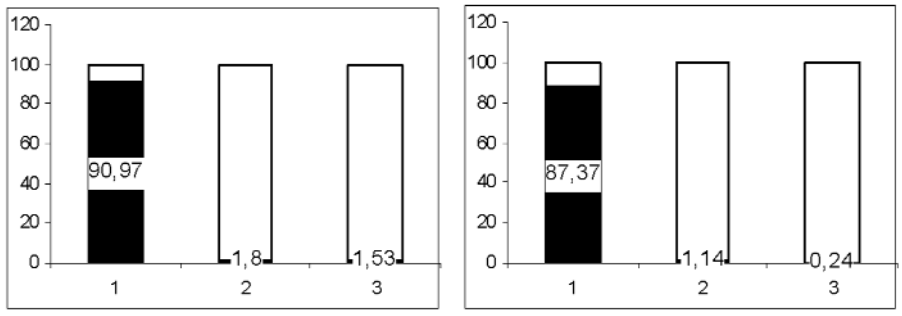

Fig. 2. The mean percentage of 1) $CD19^+/CD180^+$, 2) $CD19^+/CD284^+$, 3) $CD19^+/CD14^+$ cells among $CD19^+$ normal (A) and CLL (B) B cells.

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Table 1. Mean percentage of $CD19^+$, $CD19^+CD180^+$, $CD19^+CD14^+$ and $CD19^+CD284^+$ of normal and leukemic lymphocytes, without and after 24 hour LPS stimulation. MFI of CD180, CD284 and CD14 on the $CD19^+$ surface lymphocytes.

	% CD19+	% CD19+CD180+$^+$	MFI CD180/19	% CD19+/ CD284+	MFI CD284/19	% CD19+/ CD14+$^+$	MFI CD14/19
Control	7.2 (SD 2.57)	6.55	99.16	0.13	7.37	0.11	25.07
Control+LPS		3.65	40.48	0.33	6.16	0.23	7.48
CLL	91.41 (SD 5.01)	79.87	25.3	1.04	5.79	0.22	8.32
CLL+LPS		71.34	15.7	0.64	6.26	0.16	10.63

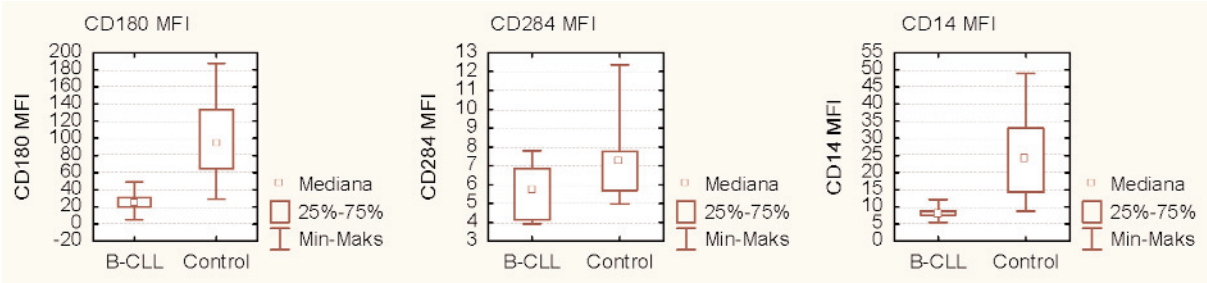

Fig. 3. Mean MFI of CD180, CD284 and CD14 on the $CD19^+$ surface lymphocytes

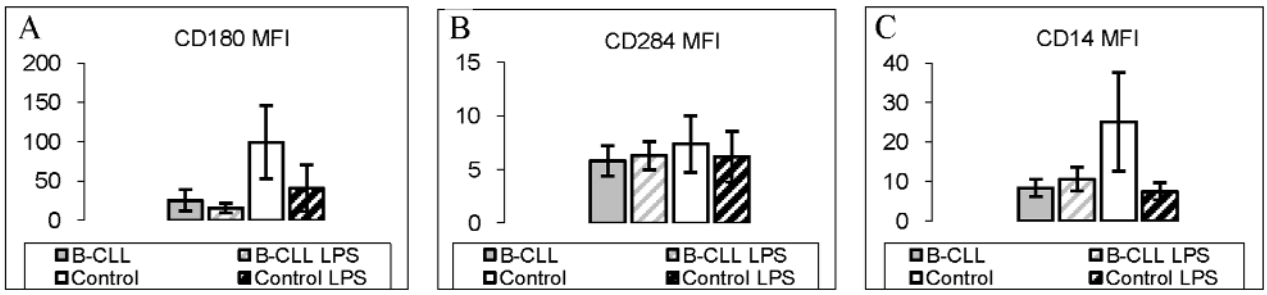

Fig. 4. MFI of cell surface CD180 (A), CD284 (B), CD14 (C) molecules expression in unstimulated and 24 hours LPS stimulated B-CLL and control (normal) B cells.

was somewhat higher after LPS in comparison to CD14 MFI without LPS stimulation (10.63 *vs*. 8.32), whereas CD14 MFI in normal $CD19^+$ cells after LPS stimulation decreased over three times (25.07 *vs.* 7.48).

Discussion

The recent view claims that CLL is accumulative disease associated with higher level of leukocytes proliferation [15]. New evidences suggest that antigenic stimulation, along with lymphocyte interaction with accessory cells and cytokines, are promoting factors that stimulate proliferation of CLL cells and allow them to avoid apoptosis. The existing hypothesis suggests that antigen stimulation is generally a prerequisite for the evolution of B-CLL [15]. These promoting antigens are unknown. However, it is possible that latent viruses or commensal bacteria repetitively activate particular B-cell clones through the B-cell receptor (BCR). Mature B-cells can recognize microbial antigens also via Toll-like receptors in costimulatory manner at least in the context of autoimmune reactions. However, it is little known regarding the repertoire and function of TLR in CLL cells. The TLR family includes 11 different transmembrane proteins which are engaged in recognition of specific pathogen-associated molecular patterns [6]. TLR receptors also take part in alarming immunocompetent cells to trigger an immune response.

The endogenous TLR ligands or chronic infections are suggested to promote tumor growth [4]. Muzio *et al.* [16] found that CLL cells express several TLR pattern-recognition molecules and that the specific TLR

expressed by CLL cells are functional [4,17]. The susceptibility to infections induced by Gram-negative bacteria is largely determined by innate immune responses to bacteria cell wall lipopolysaccharide (LPS). The stimulation of B cells by LPS enhances their antigen-presenting capacity and is accompanied by B cell proliferation and secretion of large quantities of LPS-neutralizing antibodies. Ogata *et al.* [8] demonstrated the functional cooperation between CD284 and CD180 in LPS-mediated nuclear factor κB activation. The CD180 protein plays significant role in B cell development acting both as LPS sensor and regulator of B cell proliferation [8]. The LPS effect on regulation of TLR may be due to either direct impact or to autocrine stimulation by secreted factors such as cytokines. A variety of cytokines induced by LPS may also alter TLR expression.

We assessed levels of the CD284, CD180 and co-receptor for LPS – mCD14 in $CD19^+$ subpopulation of B-CLL lymphocytes and compared them with respective levels obtained from the normal B cells, before and after LPS stimulation. Mentioned molecules employ signals from LPS [18].

CD284 expression in normal as well as leukemic $CD19^+$ cells was poor. The mean fluorescence intensity (MFI) in normal lymphocytes decreased after LPS stimulation while slight increase of MFI was observed in leukemic cells. Increased mRNA levels CD284 in the monocytic leukemia line THP-1 after LPS treatment was also observed by Zarember and Godowski [19]. CD14 MFI in leukemic cells was moderately higher after LPS in comparison to CD14 MFI without LPS stimulation, whereas CD14 MFI in normal $CD19^+$ cells after LPS stimulation decreased over three times. However, the significant differences of CD180 expression were observed. CD180 expression was decreased after 24 hour LPS stimulation in normal and leukemic $CD19^+$ cells.

Our study confirm the results of Zarember and Godowski [19], who observed 100-fold down regulation of CD180 expression in monocytic leukaemia cell line THP-1 after LPS stimulation. The observed in the present study decrease of CD180 protein on lymphocytes surface after LPS stimulation, correlating with mentioned decrease of mRNA expression [19] seems to be natural reaction on ligand-receptor binding. Four-time lower CD180 expression in unstimulated peripheral blood $CD19^+$ CLL cells, in comparison with normal lymphocytes $CD19^+$, may indicate constant stimulation of CD180 receptor in CLL by unknown antigen or auto-antigen. It can not be excluded that chemical structure of unknown antigen resembles lipopolysaccharide of Gram- negative bacteria.

Recently CD180 was found to have regulatory function and inhibits TLR4-signalling [20]. CD180/MD-1 interacts directly with TLR-4/MD-2, inhibiting the ability of this signaling complex to bind LPS [17,20,21]. Double increase of CD284 MFI after LPS stimulation in CLL cells may imply impairment of CD180 inhibiting function in leukaemia.

TLR transduction signaling pathway in CLL has been the subject of intensive study for the last several years. The hypothesis of low-affinity antigen or auto-antigen existence, exerting constant impact on B-CLL cells, has become more and more popular [15,22].

Muzio *et al*. [23] provides molecular evidence, that constitutive activation of B cells may be responsible for anergic state of human B lymphocytes. At the same time Chiron *et al*. [4] suggested that repeated polyclonal activation of leukemic B cells by microbial molecules during natural infections or inflammation may initiate the oncogenic process .

The discovery and defining potential antigens and their function in interacting with both BCR or/and TLR receptors might be useful in specific B-CLL therapy. Many existing information in literature about possible role of antigenic stimulation in CLL seem to confirm this idea.

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