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Research paper

MD-1 downregulation is associated with reduced cell surface CD180 expression in CLL

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ABSTRACT

CD180 is a toll-like receptor that is highly expressed in complex with the MD-1 satellite molecule on the surface of B cells. In chronic lymphocytic leukaemia (CLL) however, the expression of CD180 is highly variable and overall, significantly reduced when compared to normal B cells. We have recently shown that reduced CD180 expression in CLL lymph nodes is associated with inferior overall survival. It was therefore important to better understand the causes of this downregulation through investigation of CD180 at the transcriptional and protein expression levels. Unexpectedly, we found *CD180* RNA levels in CLL cells (n = 26) were comparable to those of normal B cells (n = 13), despite heterogeneously low expression of CD180 on the cell surface. We confirmed that *CD180* RNA is translated into CD180 protein since cell surface CD180-negative cases presented with high levels of intracellular CD180 expression. Levels of *MD-1* RNA were, however, significantly downregulated in CLL compared to normal controls. Together, these data suggest that changes in CD180 cell surface expression in CLL are not due to transcriptional downregulation, but defective post-translational stabilisation of the receptor due to MD-1 downregulation.

1. Introduction

Chronic lymphocytic leukaemia (CLL) is a malignancy of mature B cells which exhibits highly heterogenous clinical and biological features. Interactions between CLL cells and their microenvironment, particularly within the lymphoid organs, play a significant role in determining the heterogeneity of biological function of CLL cells [1]. Microenvironmental signals are received and transduced by receptors, many of which can themselves be heterogeneously expressed by CLL cells.

An example of one such receptor is the orphan toll-like receptor (TLR) CD180, which is co-expressed with the MD-1 molecule on the surface of B cells, monocytes/macrophages and dendritic cells [2,3]. The

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expression of CD180 on the cell surface is highly dependent on MD-1 since cell lines generated to express CD180 but not MD-1 do not express CD180 on the cell surface but rather retain the molecule intracellularly [3]. This was later confirmed *in vivo* through the generation of MD-1-null mice which were found not express CD180 on the surface of their B cells [4]. We have previously established that CD180 is expressed by around two thirds of CLL cases, with a third of CLL samples being negative for cell surface CD180 (csCD180) [5]. The csCD180 on peripheral blood (PB) CLL cells is heterogeneous compared to normal B cells which express consistently high densities of CD180 [5]. Furthermore, CLL lymph node (LN) paraffin sections from different patients have been shown to express variable levels of CD180 [6].

CD180 has also been shown to play a significant role in determining the behaviour of CLL cells . Ligation of CD180 using monoclonal antibodies (mAbs) leads to CLL cell activation in around 60 % of CD180-positive CLL samples, whilst the remaining samples are unresponsive [7]. Among CLL samples which respond to CD180 ligation, dichotomous signalling pathways become activated, which correspond with CLL cell survival or apoptosis [8]. Notably, we have also determined an association between CD180 expression and CLL disease outcome whereby increased CD180 expression in CLL LNs is associated with superior overall survival (OS) and Binet stage A disease [6].

The mechanisms which underpin differential CD180 expression and signalling are, however, presently unclear. Given the role of CD180 in CLL pathobiology, and emerging data which indicates its potential prognostic utility, it was important for us to investigate the causative mechanisms of the reduced csCD180 expression in a third of CLL cases, by looking at the transcriptional and post-transcriptional levels. We considered the possibility that the heterogeneity of csCD180 expression could be underpinned by selective expression of the gene. Surprisingly, we found that levels of *CD180* RNA expression by CLL cells were comparable to those of normal controls and in fact CLL cells harbour significant levels of intracellular CD180. Our data indicate that downregulation of csCD180 is instead due to limited export of the receptor. We found that low csCD180 expression was associated with reduced levels of MD-1 expression. Given that it has been previously established that MD-1 is required for stable CD180 expression on the surface, we use our data to hypothesise that loss of MD-1 may block CD180 export to, and expression on, the surface of CLL cells. We also report here for the first time, that *MD-1* RNA expression is downregulated in CLL.

2. Methodology

2.1. Patients

Twenty-six patients with CLL, aged 52–82 (median age 73) attending the Haematology clinic at University College London hospital were enrolled in this study. All samples were obtained after written informed consent in accordance with the Declaration of Helsinki and the UCL Ethical Committee.

None of the patients suffered from autoimmune diseases and were untreated for at least 4 months prior to the study. White blood cell counts (WBC) varied from 30 to 200 $\times 10^6$/ml. Details of the included patients are found in supplementary Table 1. Thirteen healthy age-matched volunteers aged 62–79 (median age 65) were recruited for the control group.

2.2. Isolation of PBMCS and CD19+ cell separation

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers and CLL patients were isolated using density gradient separation (Histopaque (1.077 g/ml) (Sigma; USA) by centrifugation at 700 g for 20 mins and washed in Hanks Balanced Salt Solution (HBSS, Sigma, UK) and in RPMI-1640 medium (Lonza, Switzerland) supplemented with 10 % FCS (Gibco, USA). Cells were finally resuspended in RPMI (10 % FCS) at 5×10^6cells/ml. Further enrichment of B lymphocytes was achieved by positive magnetic separation of CD19+ cells using microbeads according to the manufacturer's instruction (Miltenyi Biotech; Germany). Enriched CD19+ B lymphocytes (with >96 % purity) or PBMCs were adjusted at 2×10^6 cells/ml for further studies.

2.3. Cell lines

A range of cell lines were used to model CLL disease. HG-3 cells were purchased from the German Collection of Microorganisms and Cell Cultures. HG-3 is a lymphoblastoid B1 cell line established by EBV transformation from a clone from a patient with unmutated *IGHV* CLL [9]. U-RT1 cells were established in the laboratory of Dr Kevin Mellert and Dr Peter Möllert the University of Ulm, from a patient with unmutated *IGHV* CLL that subsequently transformed into diffuse large B cell lymphoma (Richter's Transformation) [10]. MEC-1 cells were purchased from the American Type Culture Collection. The MEC-1 cell line was established in spontaneous culture from a CLL patient with unmutated *IGHV* genes with progression towards prolymphocytic leukaemia. The patient was positive for EBV [10]. All cell lines were cultured in RPMI-1640 supplemented with 10 % foetal calf serum (FCS) and 1 % Penicillin Streptomycin (all - Lonza, Switzerland).

2.4. Extracellular and intracellular immunophenotyping

2 $\times10^6$ PBMCs were incubated with 25 μg/ml of human IgG (Sigma, UK) to prevent non-specific binding of antibodies, and were treated with either unconjugated monoclonal mouse anti-CD180 (BD, USA, clone G28–8), unconjugated polyclonal goat anti-MD-1 (R&D Systems, Minneapolis, USA) or the relevant unconjugated mouse monoclonal IgG1 (BD) or goat polyclonal IgG isotype control (R&D Systems). Rabbit anti-mouse-FITC (Dako, Ely, UK) or donkey anti-goat IgG PE (R&D Systems) were used as the secondary antibodies. Cells were then stained with anti-CD19 PE-Cy5 to identify $CD19^+$ cells, as previously described [5]. Samples were also routinely stained with anti-human IgM (BD, US) to characterise levels of the BCR expression to ensure quality of the cell samples.

For intracellular staining, cells were first stained with anti-CD19 PE-Cy5, washed twice with HBSS by centrifugation and then fixed in fixation solution A (Invitrogen, US) for 20 minutes before being washed in HBSS and then centrifuged. Cells were treated with permeabilization solution B (Invitrogen, USA) for 20 minutes, washed once in HBSS and centrifuged. Samples were then resuspended in HBSS and stained with anti-CD180 or an appropriate isotype control, as above. Rabbit anti-mouse-FITC was used as the secondary antibody.

Cell lines were extracellularly and intracellularly immunophenotyped using direct staining with PE-conjugated anti-CD180 mAb (BD, US, clone G28–8). For both cell surface and intracellular staining, results were expressed as a mean fluorescent intensity (MFI) or as the percentages of positive cells on the $CD19^+$ populations, relative to the isotype control, as described previously [5]. Flow cytometry analysis was conducted on a Beckman Coulter CyAn (Beckman Coulter, California, USA). For intracellular immunophenotyping of CLL cells and cell lines, a BD Fortessa X20 (BD, USA) was used.

2.5. CD180 expression by quantitative PCR

Total RNA was extracted from $CD19^+$ B cells from the blood of CLL patients and healthy controls using RNeasy mini kit (Qiagen) [11]. The RNA yield was assessed and quantified using bioanalyse with nano-quantification. A fixed total RNA amount of 1 μg was used for $CD19^+$ cells. cDNA synthesis was performed using random hexamer priming using Superrscript RT (ABI, Biosciences). Subsequent PCR was set up with 25 μL reactions and Taq Man PCR kit (ABI Biosciences) and amplified according to the conditions prescribed for ABI thermocycler. Relative expression of the *CD180* was assessed by comparison of

amplification of a housekeeping gene (*GAPDH*, Table 1). Values were expressed as a ratio of the mean cycle threshold number (Ct) of *CD180* divided by the mean of the Ct of *GADPH*. The values of gene expression are therefore inversely related to the level of expression of the specific gene. Gel electrophoresis (2 %) was used to detect product amplification. Forward and reverse primers were set to have melting point at 60℃ and the probes at 70℃. All probes were labelled with FAM as a reporter on the 3' end and TAMRA as the quenching dye.

2.6. Data extraction from the Gene Expression Omnibus (GEO)

Data on *CD180* and *MD-1* RNA expression in CLL samples and clinical data were extracted from two different studies in the gene expression omnibus (GEO) under accession numbers GDS4167 [12] and GDS4176 [13]. RNA expression was measured by Affymetrix (U133A / U133 Plus 2.0) chips on healthy B cells or CLL cells purified by $CD19^+$ selection on samples from different tissue compartments. All CLL patients were untreated and characteristics of the CLL patients can be found in the respective publications associated with the GEO datasets. Data from GDS4167 contained RNA expression data on $CD19^+$ peripheral blood B cells from 41 patients with CLL and 11 age-matched controls. For certain analyses of dataset GDS4176, we selected 12 patients (out of 24) for which there was matched data on the levels of RNA expression by $CD19^+$ B cells from the peripheral blood, bone marrow and lymph nodes.

2.7. Statistical analysis

Differences between qPCR data and MD-1 expression were analysed using the Mann-Whitney U-test. Data extracted from the GEO was analysed similarly but using the student t-test to conform to the analysis completed in the original publications. Matched data was analysed using a paired t-test. Correlations between variables were analysed using the Pearson's correlation coefficient. P values were considered significant at 0.05 or below.

3. Results

3.1. CD180 RNA levels in CLL cells are comparable to normal B cells and do not correlate with cell surface expression of CD180 receptor

We found that the levels of *CD180* RNA transcripts in CLL samples were not significantly different from healthy age-matched controls ($p = 0.0655$). However, it was notable that levels of *CD180* RNA appeared to be more heterogeneous within the CLL group and showed a greater range (Fig. 1a). In order to substantiate these findings, we supplemented our own data with data from studies accessible via the GEO. Dataset GDS4167, which contained a larger cohort than our own, also revealed that levels of *CD180* RNA were not significantly different in CLL cells compared to B cells from healthy age-matched controls ($p = 0.0628$; Fig. 1b). Furthermore, we confirmed that there was more heterogeneity within the CLL group compared to the control individuals (Fig. 1b).

We further analysed cell surface CD180 receptor expression levels as measured by flow cytometry in CLL cells and found no correlation between *CD180* RNA Ct and the percentages of CD180-positive CLL cells ($r = 0.2243$, $p = 0.3710$) or CD180 as measured by MFI ($r = 0.1475$, $p = 0.5518$).

3.2. CD180 RNA levels do not differ by IGHV mutational status

CLL cases can be stratified into two prognostic subgroups based on the mutational status of the *Immunoglobulin heavy chain variable gene* (*IGHV*). CLL cases with mutated *IGHV* genes (M-CLL) have superior outcomes to cases with unmutated *IGHV* genes (U-CLL) [14,15]. We have shown previously that csCD180 is preferably expressed by PB mutated M-CLL cells compared to the U-CLL subgroup [5]. We therefore investigated the possibility that levels of *CD180* RNA may also be different between M-CLL and U-CLL samples. However, we found no difference in *CD180* RNA Ct between these subgroups ($p = 0.4754$; Fig. 2a). Furthermore, there was no correlation between *CD180* RNA Ct and csCD180 expression among M-CLL ($r = 0.1771$, $p = 0.4966$) or U-CLL ($r = -0.5401$, $p = 0.2686$) subgroups (data not shown). Analysis of dataset GDS4167 also confirmed that there was no significant difference in *CD180* RNA expression between M-CLL and U-CLL subgroups ($p = 0.4867$; Fig. 2b).

Since we previously found that *CD180* RNA levels were consistent across different CLL tissue compartments through analysis of the GEO dataset GDS4176 [6], we decided to investigate whether CD180 expression could differ by *IGHV*-mutational subgroups within different lymphoid tissues. Like above, *CD180* RNA expression did not differ significantly according to the *IGHV*-mutational status within the PB ($p = 0.3240$), LN ($p = 0.4804$) or bone marrow (BM) ($p = 0.3176$; Fig. 2c).

3.3. CD180 surface negative CLL cells often express CD180 intracellularly

Given the observed lack of correlation between the stable expression of *CD180* RNA and heterogeneous expression of the csCD180 receptor [5,16,17], we explored the possibility that CD180 may be accumulating intracellularly in csCD180-negative cells. For this purpose, we permeabilised and stained CLL samples with an anti-CD180 mAb to measure CD180 expression on the cell surface and intracellularly (ic). The csCD180 was compared to measurements obtained on unpermeabilised samples.

Although the level of increase varied between individual CLL samples, overall, upon permeabilisation, the percentages of positive cells increased in almost all cases, and even in instances where csCD180 was appreciably high ($p < 0.0001$; Fig. 3a). Notably, csCD180 MFIs were appreciably low across in all CLL cases, however, upon permeabilisation, there was an average 10.9-fold increase in CD180 MFI ($p = 0.0002$; Fig. 3b & c).

We next conducted the same experiments on two csCD180-negative CLL cell lines, HG-3 [9] and MEC-1 [10] and the csCD180-negative U-RT1 [18] cell line which was established from a patient with diffuse large B cell lymphoma Richter's syndrome. As with primary CLL cells, we found that the cell lines harboured intracellular CD180, since upon permeabilisation, the percentages of CD180-positive cells increased significantly for the HG-3 ($p = 0.021$; Fig. 4a) and U-RT1 ($p = 0.029$; Fig. 4d) and MEC-1 cell line ($p = 0.0373$; Fig. 4g). There were also similar increases in CD180 MFI in the HG-3 ($p = 0.0284$; Fig. 4b & c) and the U-RT1 ($p = 0.117$; Fig. 4e & f) and MEC-1 ($p = 0.0582$; Fig. 4h & i) cells upon permeabilisation.

Table 1
Oligonucleotide sequences for the TaqMan PCR identified by the Primer Express Software (Applied Biosciences).

Gene	Primers	Probes	Product size (kb)
GAPDH	5'gaaggtgaaggtcggagtc3' 3'caagcttcccgttctcagcc5'	gaagathhtgatgggatttc	207
CD180	5'ctggactgcacttgctcgaatat3' 3'cacaggaaagcttgacatcagatag5'	cacacggtggtctcctccgagcctt	144

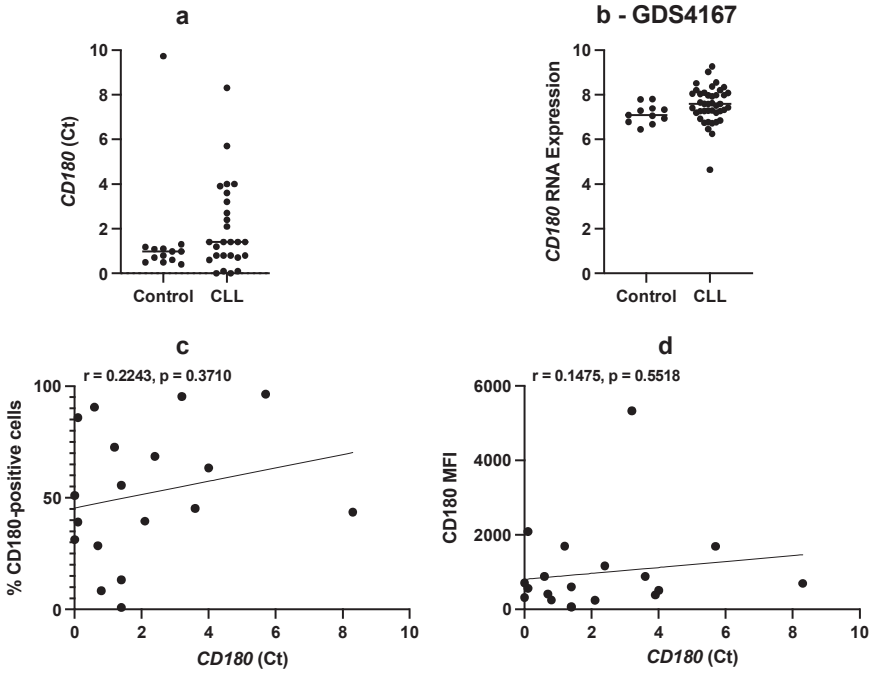

Fig. 1. The Expression of *CD180* RNA in B cells from patients with CLL and healthy age-matched controls. (**a**) Levels of *CD180* RNA Ct were measured in $CD19^+$ cells from CLL samples (n = 26) and normal controls (n = 13) and analysed by Mann-Whitney U test. (**b**) Levels of *CD180* RNA $CD19^+$ B cells from CLL samples (n = 41) and normal controls (n = 11) (GDS4167; t-test). (**c-d**) Levels of *CD180* RNA Ct did not correlate with either the percentages of CD180-positive CLL cells or with the CD180 MFI, as determined by Pearson's correlation (n = 19). MFI – mean fluorescence intensity, Ct – threshold cycle.

3.4. MD-1 expression in CLL may limit cell surface expression of CD180

We next turned to exploring the potential pathways which could contribute to defective csCD180 expression in CLL cells by analysing the expression of the small satellite molecule MD-1 which is required for the expression and stability of CD180 on the cell surface in both mice and humans [3,4,19].

MD-1 RNA expression was significantly reduced in CLL compared to normal controls, when analysing dataset GDS4167 ($p < 0.001$; Fig. 5a). Moreover, an analysis of a small cohort of control samples revealed that cell surface MD-1 was also highly expressed by control B cells compared to CLL cells ($p = 0.0003$; Fig. 5b).

Throughout the various tissue compartments: peripheral blood (PB), bone marrow (BM) and lymph nodes (LN), levels of *MD-1* transcripts did not differ significantly when analysing dataset GDS4176 ($p = 0.5587$; Fig. 5b). However, importantly, our analysis of MD-1 cell surface expression revealed that csCD180-negative CLL cases (defined as cases where less than 35 % of the CLL cells were positive for CD180) were characterised by significantly reduced levels of MD-1, compared to csCD180-positive cases ($p = 0.0247$; Fig. 5c).

We also considered that packaging of the CD180/MD-1 complex could be limited in CLL due to defective post-translational modifications and/or packaging of the two molecules in together. In support of this, we found that the mRNA expression of the chaperone protein *gp96*, of which CD180/MD-1 are clients, was downregulated in CLL compared to control B cells ($p = 0.0002$; Fig. 5f).

4. Discussion

We have previously shown that normal B cells, CD5-negative as well as CD5-positive, express a high density of csCD180, whilst there is a substantial heterogeneity in csCD180 expression in the CLL PB [5] and LN [6] CLL tissue compartments. Moreover, around 30 % of CLL samples do not express csCD180. Since csCD180 was found to be preferably expressed by PB M-CLL cells [5] and its expression in LN was associated with improved overall survival of the patients [6], the level of its expression appears to hold prognostic value. Furthermore, CD180 has been shown to negatively regulate TLR9 and TLR7 [20,21], meaning that should this occur in CLL, CD180 may be able to suppress powerful proliferative and migratory signals [22,23]. Hence, it was important to establish the reasons for the heterogeneity of CD180 expression in CLL.

We first hypothesised that the low/no csCD180 expression might be limited at the transcriptional stage. However, our data indicates that levels of *CD180* RNA of CLL cells did not differ significantly from normal control B cells (Fig. 1a). This was consistent across our own experiments using qPCR and through analysis of a larger dataset from a different centre obtained by microarray which was deposited in the GEO (GDS4167; Fig. 1b), confirming previous observations that levels of *CD180* RNA are high in CLL cells [24]. Furthermore, *CD180* RNA levels do not differ by CLL tissue compartment [6] or by *IGHV* mutational status (Fig. 2). These data therefore indicate that *CD180* is consistently transcribed by CLL cells sampled from different patients.

Therefore, the basis for heterogeneity in csCD180 expression is likely to be found at the post-transcriptional level. Indeed, our data confirm a discordance between *CD180* RNA and its surface expression on CLL cells (Fig. 1c & d). Notably, discordance between the expression of RNA and protein of other TLRs (both cell surface and endosomal) have been reported. These include TLR6 and TLR9 in CLL [24] and TLR2 and TLR5 in multiple myeloma [25].

Of note however, we did not have access to the *TP53* status of these patients and therefore is it presently unclear whether deletion and/or mutations in the *TP53* gene may delineate populations of CLL patients with different levels of *CD180* transcripts. Furthermore, given the high levels of heterogeneity in CLL, in terms of the expression of CD180 [5], larger sample sizes are required to confirm the high levels of *CD180* RNA expression by CLL cells.

Another hypothesis to test was that *CD180* is readily translated in csCD180-negative CLL cells, but there is a defect in its transport/assembly on the cell membrane. This would be tested by determining whether CD180 is accumulated in cytoplasm of csCD180-negative CLL cells. Indeed, we demonstrate here that the majority of csCD180-negative primary CLL cells and csCD180-negative CLL and Ritcher's

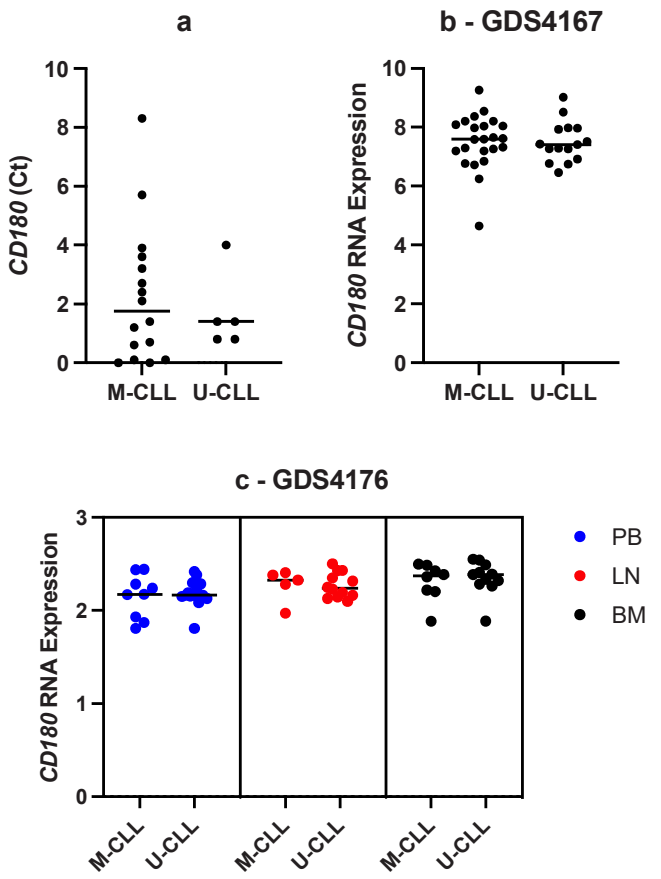

Fig. 2. *CD180* RNA expression by *IGHV* mutational subgroups. (**a**) Levels of *CD180* RNA in CD19^{+} cells from M-CLL (n = 16) or U-CLL (5) samples (t-test). (**b**) Levels of *CD180* RNA in CD19^{+} cells from M-CLL (n = 23) or U-CLL (n = 15) samples as extracted and analysed from the GEO under accession number GDS4167 (t-test; p = 0.4867). (**c**) Levels of *CD180* RNA in CD19^{+} B CLL cells across PB (n = 24), LN (n = 17) and BM (n = 19) tissue compartments stratified by *IGHV* mutational status (t-test). Data was extracted and analysed from the GEO under accession number GDS4176. Differences between groups were determined by t-test. Ct – Cycle threshold; PB – Peripheral blood; LN – Lymph node; BM – Bone marrow.

syndrome cell lines express CD180 intracellularly (Fig. 3 & 4). This supports an earlier study which determined that CD180 was intracellularly expressed in up to 90 % of csCD180-negative CLL cases using immunofluorescence microscopy [17]. Using flow cytometry to better quantify the cs/icCD180 expression, our data further demonstrates that the CLL cells harbour substantial amounts of CD180 protein intracellularly, across a spectrum a CLL cases with varying levels of csCD180 (Fig. 3c & d). Together, these data therefore advocate for dysregulation of CD180 expression by CLL cells at the post-translational level.

In cases where CD180 expression is detected intracellularly, with an appreciably low surface expression (Fig. 3 & 4), inhibited transport of the translated CD180 to the cell surface could be suggested due to a possible instability of the CD180 complex with MD-1. This is because importantly, cell surface expression of CD180 is vastly dependant on co-expression of the satellite molecule MD-1, which is a limiting factor for CD180 expression and/or its natural conformation. This has been previously demonstrated using various approaches in mice and through the use of cell lines [3,4,19]. In support of this concept, we found that MD-1 is downregulated in CLL compared to normal controls both at the RNA level and on the cell surface (Fig. 5). Furthermore, csCD180-negative CLL cases were characterised by a reduced surface expression of MD-1 co-receptor (Fig. 5e). To the best of our knowledge, this is the first report of downregulated MD-1 expression in CLL. Reduced csCD180 expression, could therefore be due to differential downregulation of MD-1. Ineffective packaging of the two molecules together could also prevent proper cell surface expression of the CD180/MD-1 complex. In support of this, we found that *gp96*, a gene which encodes for a chaperone protein of which CD180/MD-1 are clients [26], was downregulated in CLL (Fig. 5f). Further molecular evidence is needed to fortify these hypotheses within the context of CLL through silencing or upregulation of MD-1/ gp96 expression.

Interestingly, it was recently reported that the glycosylation status of MD-1 may also be a factor in defining CD180 expression since it was recently shown that the glycosylation of MD-1 at two specific sites (N96 and N196) determined the level of surface CD180 expression [27]. Ineffective glycosylation at these residues therefore prevents the formation of the MD-1/CD180 complex and prevents ineffective cell surface CD180 expression. Thus, the lack of surface expression of the MD1 co-receptor and/or differential expression of selectively glycosylated forms of MD-1, might lead to the aberrant deficiency of csCD180 assembly/expression or to conformational changes which result in the specific anti-CD180 mAbs failing to recognise the expressed CD180. Indeed, it was shown recently that the MHR73–11 clone of anti-CD180 preferentially detects a heavier form of CD180, the expression of which is determined by the selective glycosolation of MD-1 [27] and future studies should be conducted to determine the glycosylation status of MD-1 in CLL cells. Silencing of *MD-1* or knockout of the gene will also help to confirm the relative effect on ic/csCD180 expression by CLL cells.

Therefore, the differential expression of CD180 could affect its signalling behaviours alone and in conjunction with other receptors such as IgM [8], IgD [28] and CD150 [17]. Importantly, differential receptor expression and signalling may also translate into differences in the clinical behaviour of the disease [6]. Notably, it has been shown recently that increasing levels of IgM correspond with poor clinical features in CLL [29]. Thus, given that CD180 and IgM appear to be alternatively correlated M-CLL and U-CLL, measurement of these two receptors together may yield greater prognostic value.

5. Conclusions

We have previously shown that cells from CLL patients express reduced and highly heterogenous levels of CD180 compared to B cells from healthy controls. Importantly, CD180 may hold prognostic value since higher levels of CD180 expression by CLL cells in both the peripheral blood and lymph nodes are associated with a positive prognostic outlook. Here we show that reduced expression of csCD180 is not underpinned by reduced transcription of the gene, since CLL cells express comparable levels of *CD180* RNA to healthy B cells. We found that in csCD180-negative CLL cases, CD180 can be expressed intracellularly and propose that aberrantly low expression of csCD180 may be due to a downregulation in MD-1 expression, which results in defective export of the receptor to the cell surface. Our results therefore provide an explanation as to the causes of CD180 downregulation in CLL. We also report for the first time a downregulation in MD-1 expression in CLL. These results may also be applicable to other B cell malignancies where csCD810 expression is reduced or heterogenous. Moreover, our results may also be of therapeutic relevance in the future. Approaches which tackle the reconstitution of CD180 expression by CLL cells may be useful given that higher levels of CD180 expression is associated with an advantageous prognostic phenotype [6,30]. Moreover, improving CD180 expression would improve the potential for CD180 to become activated by its natural ligand, which may promote CLL cell apoptosis since we have previously shown that CD180 can rewire IgM-mediated pro--survival signalling in CLL [8].

Ethics approval and consent to participate

All samples from CLL patients and healthy control individuals were

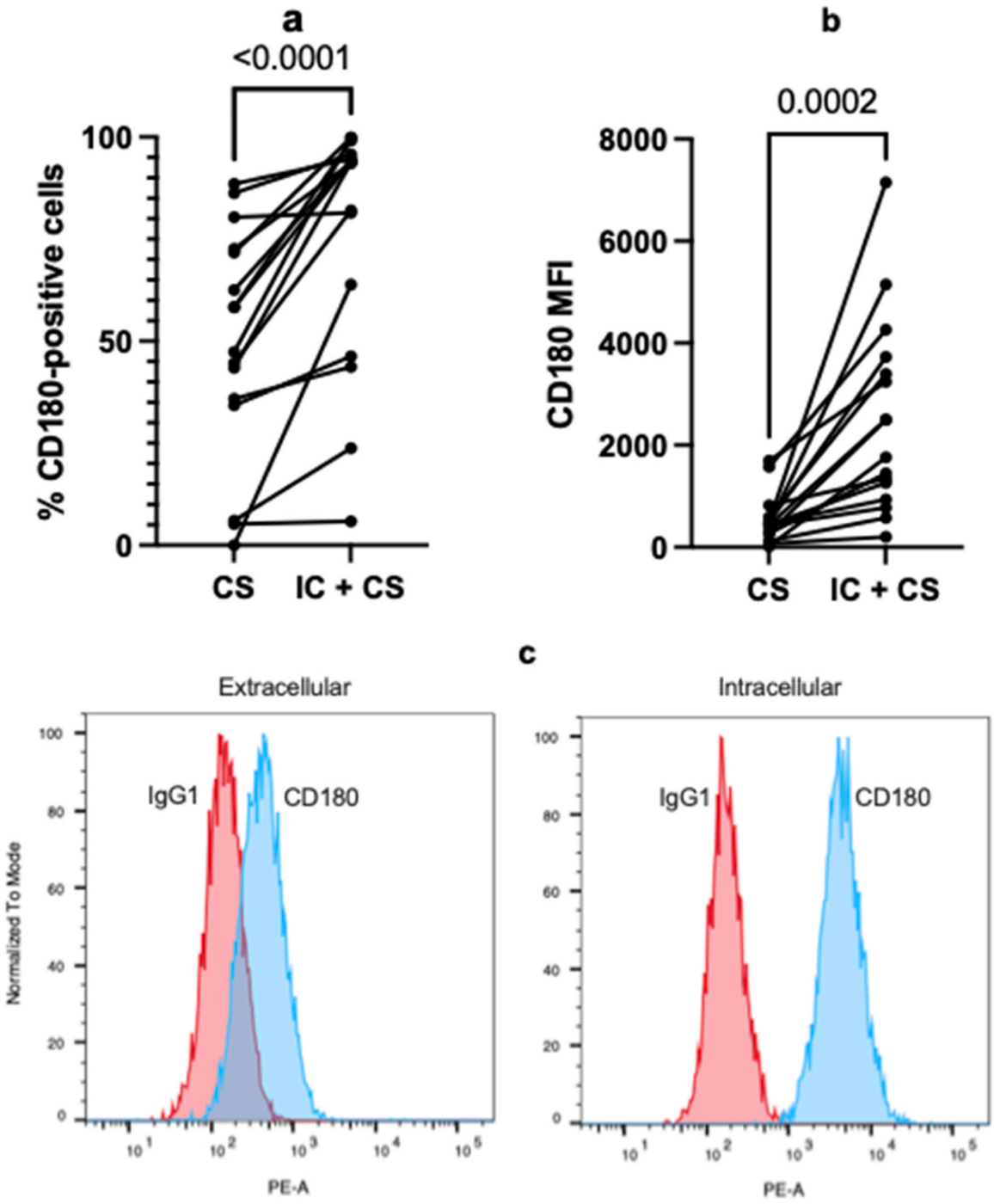

Fig. 3. Cell surface and intracellular expression patterns of CD180 by CLL cells. (**a & b**) The level of CD180 expression by CLL cells from 16 donors was measured with (CS + IC) or without permeabilisation (CS) on $CD19^{+}$ CLL cells was presented as the percentages of (**a**) CD180-positive CLL cells and as (**b**) CD180 mean fluorescent intensity. Comparison between unpermeablised and permeabilised samples was made by paired t-test. (**c**) representative histograms showing the increase in CD180 expression (blue) relative to the IgG1 isotype control (red) before and after permeabilisation. Histograms were generated by normalising the fluorescence values as a percentage of the modal fluorescence value for each PE conjugated antibody. CS – cell surface; IC – intracellular; MFI – Mean fluorescent intensity.

obtained after written informed consent in accordance with the Declaration of Helsinki and the UCL Ethical Committee.

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CRediT authorship contribution statement

Kurtis Edwards: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology,

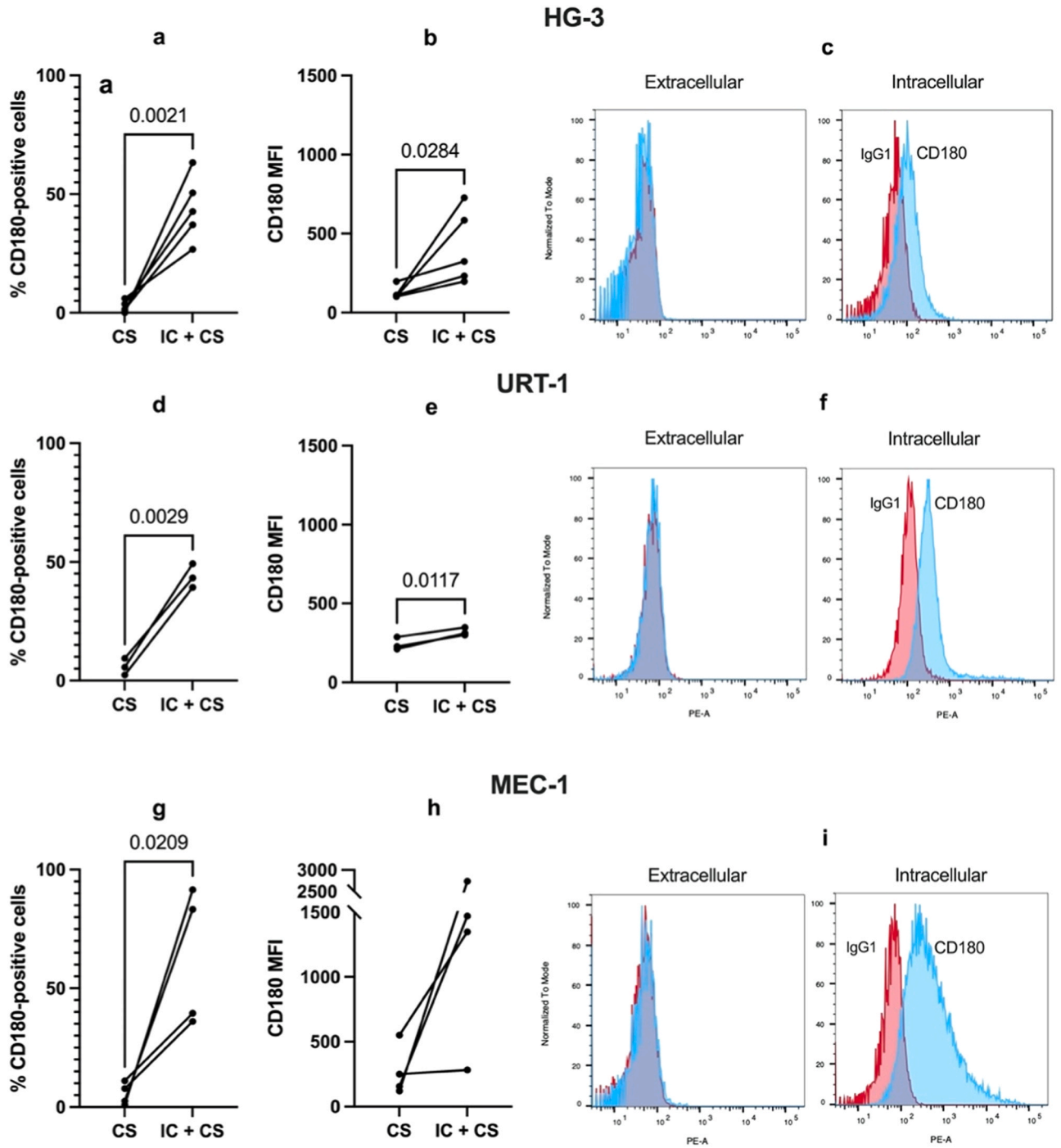

Fig. 4. Cell surface and intracellular expression patterns of CD180 by HG-3, U-RT1 and MEC-1 cells. HG-3 (**a-c**), URT-1 (**d-f**), and MEC-1 (**g-i**) cells lines were stained with anti-CD180 with (CS + IC) or without permeabilisation (CS) and analysed by flow cytometry. Data was presented as the percentages of CD180-positve CLL cells and CD180 MFI. Differences between CS and CS+IC was measured by paired t-test. Representative histograms (**c, f, i**) are also included to show the increase in CD180 expression (blue) relative to the IgG1 isotype control (red) before and after permeabilisation. Histograms were generated by normalising the fluorescence values as a percentage of the modal fluorescence value for each PE conjugated antibody. Data from 3 to 5 separate experiments was analysed by paired t-test. CS – cell surface; IC – intracellular; MFI – Mean fluorescent intensity.

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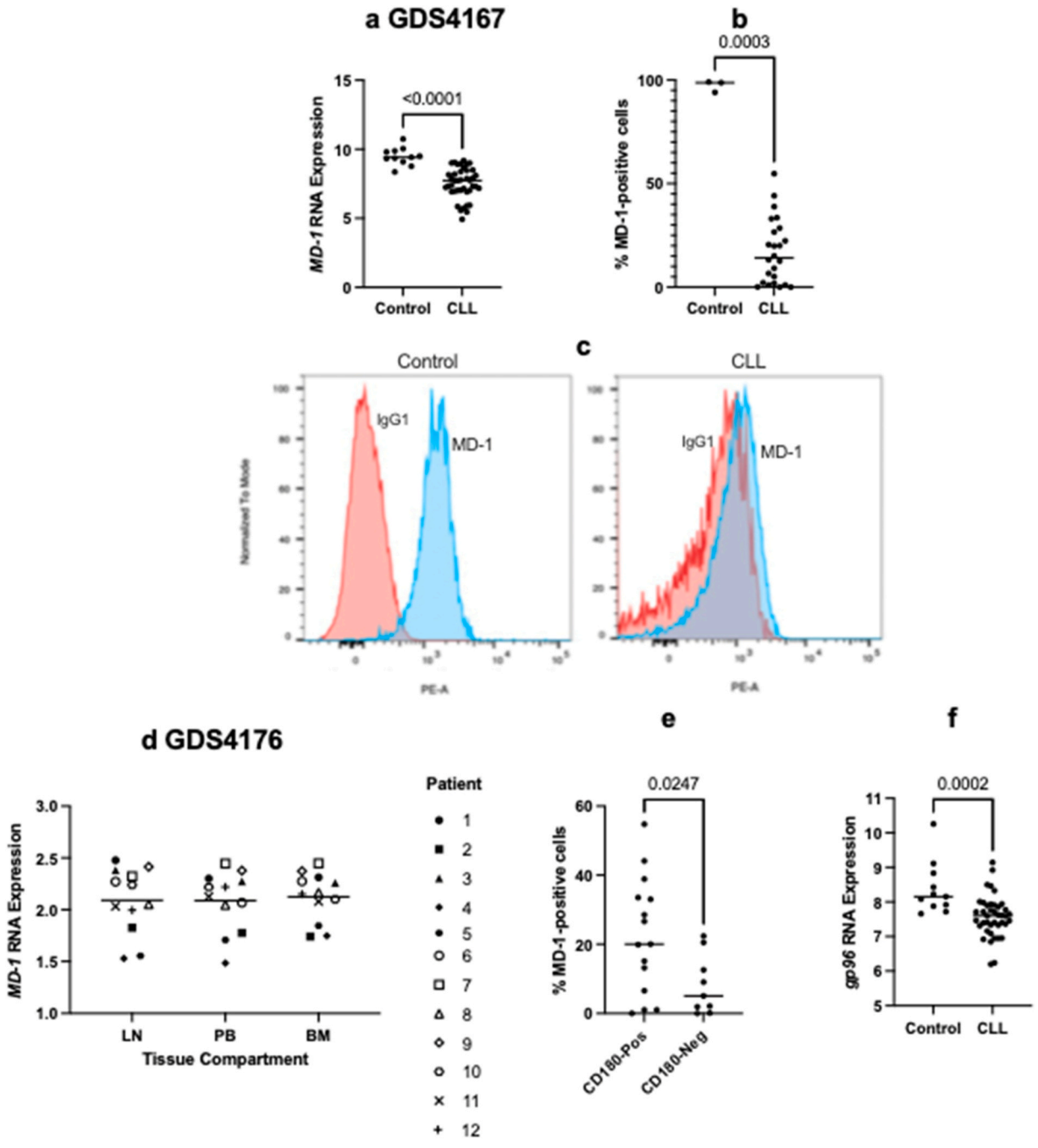

Fig. 5. The expression of MD-1 by CLL and control B cells. (**a**) Levels of *MD-1* RNA in CD19^{+} B cells from normal controls (n = 13) and CLL patients (n = 41; t-test). (**b**) Cell surface MD-1 expression by control B cells (n = 3) compared to CLL cells (n = 24; Mann-Whitney U test). (**c**) Representative histograms of cell surface MD-1 (blue) relative to the isotype control (red) in CLL and normal control B cells. Histograms were generated by normalising the fluorescence values as a percentage of the modal fluorescence value for each PE conjugated antibody. (**d**) Levels of *MD-1* RNA across different CLL tissue compartments in 12 patients with matched data extracted from the GEO under accession number GDS4176 (repeated measured ANOVA). (**e**) Levels of cell surface MD-1 protein in csCD180-positve (n = 15) and csCD180-negative (n = 9) CLL (Mann-Whitney U test). The negative threshold of CD180 expression was defined as cases where less than 35 % of the CLL cells were positive for CD180. (f) Decreased expression of *gp96* RNA in CD19^{+} B cells from normal controls (n = 13) and CLL patients (n = 41; t-test).

Declaration of Competing Interest

No declarations made by the authors.

Availability of data and material

The primary data generated in this study is available upon reasonable request to the corresponding author. The mRNA expression data analysed as a part of this study is publicly available from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) under accession numbers GDS4167 and GDS4176.

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Competing interests

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.leukres.2024.107540.

References

[1] N. Dubois, E. Crompot, N. Meuleman, et al., Importance of crosstalk between chronic lymphocytic leukemia cells and the stromal microenvironment: direct contact, soluble factors, and extracellular vesicles, Front Oncol. 10 (2020).

[2] S. Divanovic, A. Trompette, S.F. Atabani, et al., Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105 (https://doi.org/https://.doi.org/), Nat. Immunol. 6 (2005) 571–578, https://doi.org/10.1038/ni1198.

[3] Y. Miura, R. Shimazu, K. Miyake, et al., RP105 is associated with MD-1 and transmits an activation signal in human B cells, Blood 92 (1998) 2815–2822, https://doi.org/10.1006/geno.1996.0632.

[4] Y. Nagai, R. Shimazu, H. Ogata, et al., Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide, Blood 99 (2002) 1699–1705, https://doi.org/10.1182/blood.v99.5.1699.

[5] N. Porakishvili, N. Kulikova, A.P. Jewell, et al., Differential expression of CD180 and IgM by B-cell chronic lymphocytic leukaemia cells using mutated and unmutated immunoglobulin VH genes, Br. J. Haematol. 131 (2005) 313–319, https://doi.org/10.1111/j.1365-2141.2005.05775.x.

[6] K. Edwards, K. Zaitseva, U. Sayed, et al., Expression patterns of CD180 in the lymph nodes of patients with chronic lymphocytic leukaemia, Br. J. Haematol. N./a (2021), https://doi.org/10.1111/bjh.17680.

[7] N. Porakishvili, A. Memon, K. Vispute, et al., CD180 functions in activation, survival and cycling of B chronic lymphocytic leukaemia cells, Br. J. Haematol. 153 (2011) 486–498, https://doi.org/10.1111/j.1365-2141.2011.08605.x.

[8] N. Porakishvili, K. Vispute, A.J. Steele, et al., Rewiring of sIgM-Mediated Intracellular Signaling through the CD180 Toll-like Receptor, Mol. Med 21 (2015) 46–57, https://doi.org/10.2119/molmed.2014.00265.

[9] A. nbi Rosén, A.-C. Bergh, P. Gogok, et al., Lymphoblastoid cell line with B1 cell characteristics established from a chronic lymphocytic leukemia clone by in vitro EBV infection, Oncoimmunology 1 (2012) 18–27, https://doi.org/10.4161/onci.1.1.18400.

[10] A. Stacchini, M. Aragno, A. Vallario, et al., MEC1 and MEC2: two new cell lines derived from B-chronic lymphocytic leukaemia in prolymphocytoid transformation, Leuk. Res 23 (1999) 127–136, https://doi.org/10.1016/S0145-2126(98)00154-4.

[11] K.A. Zarember, P.J. Godowski, Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines, J. Immunol. 168 (2002) 554–561, https://doi.org/10.4049/jimmunol.168.2.554.

[12] Jr.A. Gutierrez, R.C. Tschumper, X. Wu, et al., LEF-1 is a prosurvival factor in chronic lymphocytic leukemia and is expressed in the preleukemic state of monoclonal B-cell lymphocytosis, Blood 116 (2010) 2975–2983, https://doi.org/10.1182/blood-2010-02-269878.

[13] Y. Herishanu, P. Pérez-Galán, D. Liu, et al., The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. Blood 117 (2011) 563–574, https://doi.org/10.1182/blood-2010-05-284984.

[14] R.N. Damle, T. Wasil, F. Fais, et al., Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia, Blood 94 (1999) 1840–1847.

[15] T.J. Hamblin, Z. Davis, A. Gardiner, et al., Unmutated Ig VH Genes Are Associated With a More Aggressive Form of Chronic Lymphocytic Leukemia, Blood 94 (1999) 1848–1854, https://doi.org/10.1182/blood.V94.6.1848.

[16] H. Antosz, J. Sajewicz, B. Marzec-Kotarska, et al., Different expression of CD180, CD284 and CD14 receptors on the CD19+ subpopulation of normal and B-CLL lymphocytes, Folia Histochem Cytobiol. 47 (2009) 593–598, https://doi.org/10.2478/v10042-009-0112-1.

[17] I. Gordiienko, L. Shlapatska, V. Kholodniuk, et al., The interplay of CD150 and CD180 receptor pathways contribute to the pathobiology of chronic lymphocytic leukemia B cells by selective inhibition of Akt and MAPK signaling, e0185940–e0185940, PLoS One 12 (2017), https://doi.org/10.1371/journal.pone.0185940.

[18] T. Schmid, J. Maier, M. Martin, et al., U-RT1 – A new model for Richter transformation, Neoplasia 23 (2021) 140–148, https://doi.org/10.1016/j.neo.2020.11.010.

[19] K. Miyake, R. Shimazu, J. Kondo, et al., Mouse MD-1, a molecule that is physically associated with RP105 and positively regulates its expression, J. Immunol. 161 (1998) 1348–1353, https://doi.org/10.4049/jimmunol.161.3.1348.

[20] Y. Yang, C. Wang, P. Cheng, et al., CD180 Ligation Inhibits TLR7- and TLR9-Mediated Activation of Macrophages and Dendritic Cells Through the Lyn-SHP-1/2 Axis in Murine Lupus, Front Immunol. 9 (2018) 2643, https://doi.org/10.3389/fimmu.2018.02643.

[21] K. Yamazaki, T. Yamazaki, S. Taki, et al., Potentiation of TLR9 responses for human naive B-cell growth through RP105 signaling, Clin. Immunol. 135 (2010) 125–136, https://doi.org/10.1016/j.clim.2009.12.013.

[22] A. Wolska, B. Cebula-Obrzut, P. Smolewski, T. Robak, Effects of Toll-like receptor 7 and Toll-like receptor 9 signaling stimulators and inhibitors on chronic lymphocytic leukemia cells ex vivo and their interactions with cladribine, Leuk. Lymphoma 54 (2013) 1268–1278, https://doi.org/10.3109/10428194.2012.741233.

[23] E. Kennedy, E. Coulter, E. Halliwell, et al., TLR9 expression in chronic lymphocytic leukemia identifies a promigratory subpopulation and novel therapeutic target, Blood 137 (2021) 3064–3078, https://doi.org/10.1182/blood.2020005964.

[24] E. Arvaniti, S. Ntoufa, N. Papakonstantinou, et al., Toll-like receptor signaling pathway in chronic lymphocytic leukemia: distinct gene expression profiles of potential pathogenic significance in specific subsets of patients, Haematol. Hematol. J. 96 (2011) 1644–1652, https://doi.org/10.3324/haematol.2011.044792.

[25] J. Abdi, T. Mutis, J. Garssen, F. Redegeld, Characterization of the Toll-like receptor expression profile in human multiple myeloma cells, e60671–e60671, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0060671.

[26] M.P. Weekes, R. Antrobus, S. Talbot, et al., Proteomic Plasma Membrane Profiling Reveals an Essential Role for gp96 in the Cell Surface Expression of LDLR Family Members, Including the LDL Receptor and LRP6, J. Proteome Res 11 (2012) 1475–1484, https://doi.org/10.1021/pr201135e.

[27] M. Biswas, T. Yamazaki, S. Tomono, et al., Cell surface expression of human RP105 depends on N-glycosylation of MD-1, FEBS Lett. N./a (2022), https://doi.org/10.1002/1873-3468.14452.

[28] N. Porakishvili, U. Sayed, K. Zaitseva, et al., Synergism between CD180 and IgD-mediated phosphorylation of p38MAPK and apoptosis of p38MAPK-signaller chronic lymphocytic leukaemia (CLL) cells, Leuk. Lymphoma 58 (2017) 134–135.

[29] G. Chiodin, S. Drennan, E.A. Martino, et al., High surface IgM levels associate with shorter response to ibrutinib and BTK bypass in CLL patients, Blood Adv. bloodadvances. 2021006659. (2022), https://doi.org/10.1182/bloodadvances.2021006659.

[30] N. Porakishvili, N. Kulikova, M. Manoussaka, et al., Differential expression of CD180 and sIgM by B chronic lymphocytic leukaemia cells (B-CLL) using mutated and unmutated IgVh genes, 768A, Blood 104 (2004), https://doi.org/10.1182/blood.V104.11.2807.2807.