

# Expression patterns of CD180 in the lymph nodes of patients with chronic lymphocytic leukaemia

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## Author Contributions

## Summary

Chronic lymphocytic leukaemia (CLL) cells can receive immunological cues via various receptors which modify tumour behaviour. One such receptor is CD180, a cell surface orphan toll-like receptor, which is heterogeneously expressed on peripheral blood (PB) CLL cells and is involved in the regulation of CLL cell signalling, proliferation and survival. Little is known however, about the expression of CD180 in the lymph nodes (LNs) of CLL patients. This is of particular interest since pathological pseudo-follicles, termed proliferation centres, found in the lymph nodes are thought to be the main sites for CLL cell expansion.

Using immunohistochemistry (IHC) on paraffin tissue sections and semi-quantitative analysis, here we show that whilst normal human tonsils express homogeneous levels of CD180, CD180 is heterogeneously expressed in the LNs of CLL patients, thus mirroring CD180 expression patterns in CLL PB cells. Of interest, analysis of RNA data obtained from a public database indicates that CD180 RNA is expressed at homogeneously high levels in all CLL tissue compartments– the PB, LNs and bone marrow (BM) – suggesting aberrant CD180 surface assembly in CLL cells. In the LNs, CD180 expression was associated with the expression of both Ki67 and TNFR1, implicating CD180 as one of the key receptors which contributes to CLL activation and proliferation within the proliferation centres.

Levels of CD180 RNA in CLL LN and BM, were strongly negatively correlated with CD38 RNA, a prognostic marker which negatively affects outcomes in CLL. In support of this data, we determined that CD180 positivity in the LNs was associated with superior overall survival. Taken together, these findings could suggest that CD180 plays a significant role in modifying the clinical behaviour of the disease.

## 1.0 Introduction

Chronic lymphocytic leukaemia (CLL) is the most common adult leukaemia in the western world and is characterised by a variable clinical course: some patients present with aggressive disease which requires treatment soon after diagnosis, whilst for others a “watch and wait” approach is more appropriate due to an indolent course (Schuh et al., 2018). CLL is characterised by the accumulation of malignant B cells in the bone marrow, lymph nodes (LN), secondary lymphoid tissues and the peripheral blood (PB), whereby the tumour microenvironment is indispensable for tumour growth, survival and evolution. CLL cells expand within the microenvironment of LN “proliferation centres” (PCs) in response to a range of immunological signals and through interactions with various accessory cells (Herreros et al., 2010).

The B cell receptor (BCR) is one of the main signalling receptors, both in normal and malignant B cells. BCR activation through unknown (auto)antigens is essential for the survival of CLL cells (Burger and Chiorazzi, 2013). In addition, chemoattractant molecules enable the recruitment of monocytes/macrophages, nurse-like cells and T cells which promotes a favourable tumour microenvironment (Ghia et al., 2002; Gandhi and Balakrishnan, 2012). Recently strong evidence has emerged implicating microenvironmental toll-like receptors (TLRs) in the pathobiology of CLL. Activation of TLRs in both PB and LNs can lead to the upregulation of costimulatory molecules, as well as the proliferation and survival of CLL cells (Mongini et al., 2015; Dadashian et al., 2019).

CD180 is an orphan toll-like receptor which is most closely related to TLR4 (Divanovic et al., 2005). We have previously shown that CD180 is heterogeneously expressed on PB CLL cells, with variable densities ranging from low/negative to high expression, although consistently below the expression levels on normal B cells (Porakishvili et al., 2005). Treatment of primary CLL cells with anti-CD180 monoclonal antibodies led to cell cycling and the upregulation of costimulatory molecules such as CD86 (Porakishvili et al., 2011). Our further studies of the signalling pathways utilised by CD180 in CLL cells revealed a unique pattern of phosphorylation of intracellular protein kinases. CLL cells were able to utilise two, often alternative signalling pathways mediated by AKT and p38MAPK which corresponded with proliferation and apoptosis, respectively. This contrasted normal B cells which simultaneously utilise both signalling axes (Porakishvili et al., 2015). Moreover, significant crosstalk exists between CD180 and sIgM since pre-treatment of CLL cells with anti-CD180 induced redirection of sIgM signalling from an AKT-mediated to a p38MAPK-mediated pathway (Porakishvili et al., 2015). Thus, CD180 can modify the fate and survival of CLL cells and may be significant in understanding CLL immunopathology.

Since the PCs are considered to be the main sites for CLL expansion and proliferation, it was important to study the expression patterns of CD180 in the LNs to (provide further insight into the CD180-mediated immunological pathways utilised by CLL cells and to) better understand how CLL cells behave within these pathological anatomical sites.

## 2.0 Materials and Methods

### Patients and controls

Formalin fixed paraffin embedded (FFPE) LN sections were studied from the total of 110 CLL patients aged 26 to 84 years, at Binet stages A, B or C of the disease, who attended St Bartholomew's Hospital, between 1980 and 2011. All samples were obtained with informed consent, in accordance with the Declaration of Helsinki and with ethical approval from the London Research Ethics Committee. Patients were grouped into two different cohorts according to whether they had undergone treatment or not. Fifty-five patients were untreated and 55 were treated using various therapies including steroids and cytotoxic chemotherapies. Clinical and phenotypic information was obtained from patient records. WBC counts ranged from  $3.2$  to  $126.2 \times 10^9/L$ . Immunophenotypic studies on ZAP-70 and CD38 were routinely conducted at St Bartholomew's hospital using flow cytometry on PB samples. Five normal tonsillar paraffin-embedded sections were used as controls.

### Immunohistochemistry

Formalin fixed paraffin embedded CLL LN or normal tonsillar tissue sections were cut from tissue microarray blocks at a thickness of  $3\mu m$ . The sections were affixed on glass slides and incubated at  $60^\circ C$  overnight to aid adhesion of the sample to the slide. Before immunostaining, slides were dehydrated in industrial methylated spirit (IMS) for 5 minutes. Endogenous peroxidase activity was blocked by placing slides in IMS with 2% (v/v)  $H_2O_2$ . Sections were then washed in IMS for 2 minutes and rinsed under tap water. Primary rabbit anti-human CD180 (Merk, UK) antibody titrations at concentrations of 1:150, 1:500, and 1:1000 in a specialised buffer (Cat # ZUC025-100, Zytomed Systems, Germany) were carried out on serial sections to determine optimum staining after 40 min. 1:500 was determined to be the optimum titration and was therefore used for subsequent immunostaining of the tissue sections.

Following a 40-minute incubation with the primary antibody, samples were washed in tris-buffered saline with Tween<sup>®</sup>20 (TBS-T) followed by a 20-minute incubation with the secondary antibody, goat anti-rabbit horseradish peroxidase (HRP, Abcam, UK). A ready-to-use enhancer (Biogenex, USA) was applied to the slides for 20 minutes, washed in tris-buffered saline with TBS-T, and a ready-to-use Super Sensitive<sup>™</sup> label reagent (Biogenex, USA) was added for 30 minutes before the slides were washed again in TBS-T. DAB (3,3'-Diaminobenzidine) substrate was prepared according to the manufacturer's instructions (Biogenex, USA) and applied to the slides for 10 minutes. The samples were placed under running tap water to stop the reaction before being counterstained with haematoxylin and then rinsed again in tap water. Slides were briefly dipped into acid alcohol solution before being placed into Scott's solution for 2 minutes. Slides were then rinsed with tap water, dehydrated in IMS and mounted using a DPX-based mounting agent.

### H-scores

The slides were evaluated using an Ariol automatic slide scanner (Leica, Germany). Acquisition of high quality brightfield images (20x) of the slides were captured by the Ariol scanner using dedicated Ariol software. The quality of the images was checked with CaseViewer

(Version 2.2.1) and scale bars were automatically generated by the software according to the respective digital magnification.

The amount of protein expressed was quantified through the calculation of a semi-quantitative histology-score (H-score). For each sample, a fixed field was analysed in triplicate. Using the Aiol software, individual cells were scored for staining intensity on a scale: 0 – negative; +1 – weak; +2 – moderate; +3 – intense. In order to generate an H-score, the percentages of positive cells were multiplied by their respective assigned intensity score, according to the formula below.

$$\text{H - Score}_{\text{CD180}} = (1 \times \% \text{ Cells}_{+1}) + (2 \times \% \text{ Cells}_{+2}) + (3 \times \% \text{ Cells}_{+3})$$

H-scores were averaged across the 3 fixed fields for each sample.

#### Measurement of Ki67 and TNFR1

The percentages of Ki67- or TNFR1-positive cells were determined through IHC. LN sections were stained with anti-TNFR1 (Abcam, USA), as above. Sections were then stripped and permeabilised before being stained with anti-Ki67 (MIB1 clone, Dako, USA), as above. Stained LN sections were analysed by the Aiol Automated Slide scanner which determines the total number of cells within the tissue section based on counts of nuclei. Cells positive for Ki67 or TNFR1 were also determined automatically and reported as a percentage of total cells within the LN tissue spot.

#### Analysis of RNA expression data

Data on LN, PB and BM samples from 24 patients, aged 30 to 74 years, at Rai stages 1-4, were obtained from the Gene Expression Omnibus (GEO) database under accession number GDS4176. CD180 RNA expression of twelve patients, for whom data were available across all the tissue compartments, were analysed and compared. RNA expression was measured by Affymetrix (U122 2.0) chips on CLL cells purified by CD19 positive selection.

#### Statistical Analysis

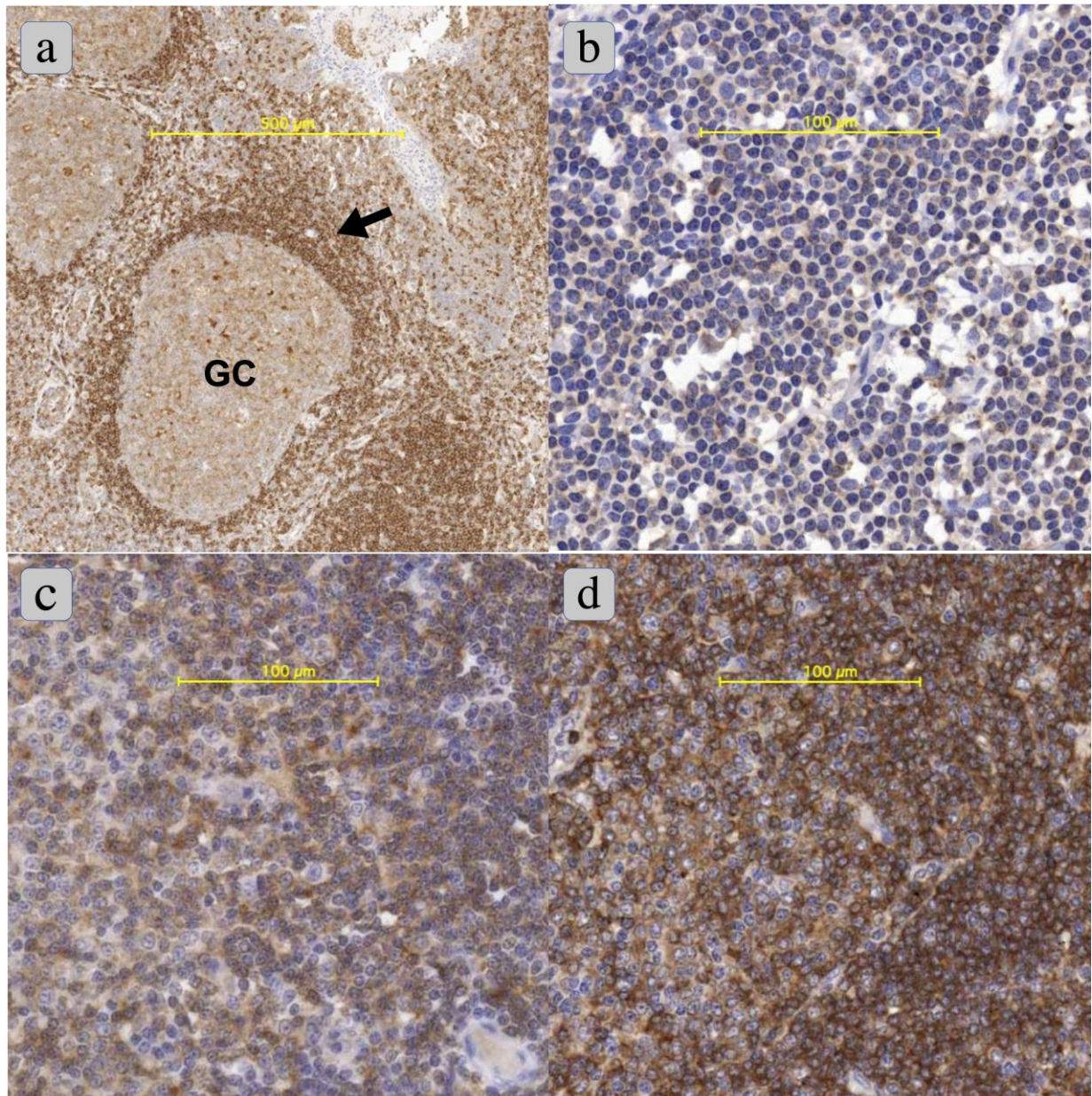
Non-parametric Mann-Whitney U test and Pearson's correlation were applied where appropriate. RNA expression was compared using an ANOVA and Tukey's test for multiple comparisons or using a matched ANOVA to compare the 3 tissue compartments. The Kaplan-Meier method was used to analyse survival data and curves were compared using the log-rank test. All analysis was completed using GraphPad Prism (version 8) and p values  $\leq 0.05$  were considered to be statistically significant.

## 3.0 Results

### CD180 is highly expressed in normal tonsillar lymph nodes

Immunostaining on tonsillar LN sections from healthy controls showed high expression of CD180. This aligns with previously reported high expression levels of CD180 in normal B cells (Porakishvili et al., 2005). In all 5 healthy control tonsillar samples, there was dense CD180 staining (Fig 1a). The respective CD180 H-scores for these control samples were also homogeneously high (Fig 2a). The median expression of CD180 in the lymph nodes of healthy controls was higher than that of CLL patients and showed a much narrower range ( $p = 0.0465$ ,  $n=55$ , Fig 2a). It should be noted however that a group of CLL patients (22, 40%) expressed CD180 H-scores within the normal range of healthy individuals (Fig 2a).





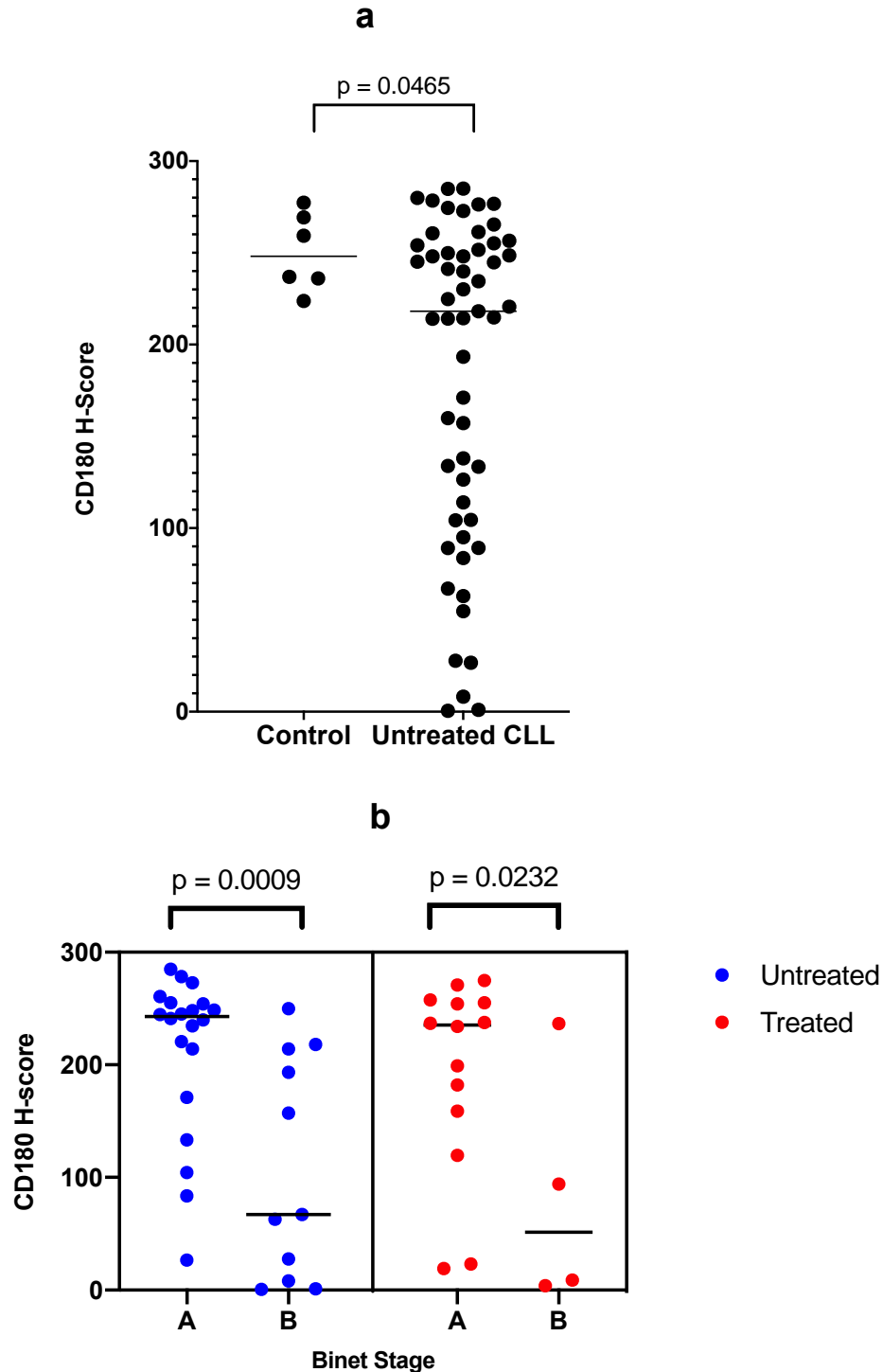
**Fig 1. Representative Immunohistochemistry of control tonsils and CLL LN sections.**

**(a)** Physiological distribution of CD180 expression in a representative control tonsillar lymph node section. Control tissue sections were stained with anti-CD180 antibodies at the optimum concentration of 1:500, as determined through antibody titration experiments in serial tonsillar tissue sections. CD180, indicated by brown staining, is densely expressed within the mantle B zone (black arrow) but lost within the germinal centres (GC). Tissue sections are visible at 5x magnification **(b-d)** Representative immunohistochemistry staining on LN sections of CLL patients. LN samples were stained with anti-CD180 antibodies at the optimum concentration of 1:500. Tissue sections are visible at 20x magnification. CD180 is expressed at low **(b)**, medium **(c)** and high densities **(d)** as determined by staining intensity. The CD180 H-score for these sections were determined to be 0.5, 104.25, and 284.85, respectively. The uniform expression of CD180 in tissue sections from CLL patients is consistent with widespread infiltration of malignant B cells throughout the LNs.

CD180 is heterogeneously expressed in the lymph nodes of untreated and treated CLL patients

CD180 was heterogeneously expressed in the LNs of untreated patients, as measured by H-score (Fig 2b). Three patterns of CD180 staining and H-scores could be observed: highly positive, intermediate, and low/negative (Figs 1b-d & 2b).

Interestingly, the median expression of CD180 was significantly elevated in the LN of Binet stage A patients compared to stage B ( $p = 0.0009$ , Fig 2b), whilst no relationship was found between CD180 H-score and the age of the patient ( $r = 0.07897$ ,  $p = 0.5666$ ;  $n = 55$ ). Moreover, CD180 H-score was not significantly different between males and females ( $p = 0.0745$ ;  $n = 55$ ). The distributions of CD180 H-scores when compared the LNs of treated (228.1, 3.95-288.4) and untreated (218.10, 0.5-285.00) patients were remarkably similar ( $p = 0.3384$ , Fig 2b). Within the treated cohort, the median CD180 H-score in stage A patients was significantly greater than in patients categorised as stage B ( $p = 0.0232$ , Fig 2b).



**Fig 2. CD180 H-scores in normal tonsillar and CLL LN sections. (a)** The CD180 H-scores in the LN of healthy controls (n = 6) and untreated CLL patients (n= 55). **(b)** The CD180 H-scores in the LN of untreated CLL patients stratified as Binet stage A (n = 20) or B (n = 11) and treated patients stratified as stage A and treated CLL patients stratified as Binet stage A (n = 14) and B (n = 4). Median CD180 H-Score in untreated stage A patients (243, 26.64 –284.80) was significantly higher than in stage B patients (67.00, 0.50 – 249.80,  $p = 0.0009$ ). Similarly, within the treated cohort, stage A patients (235.5, 19.23 – 274.0) demonstrated significantly higher CD180 H-scores compared to stage B patients (51.48, 3.945 -236.7,  $p = 0.0232$ ).

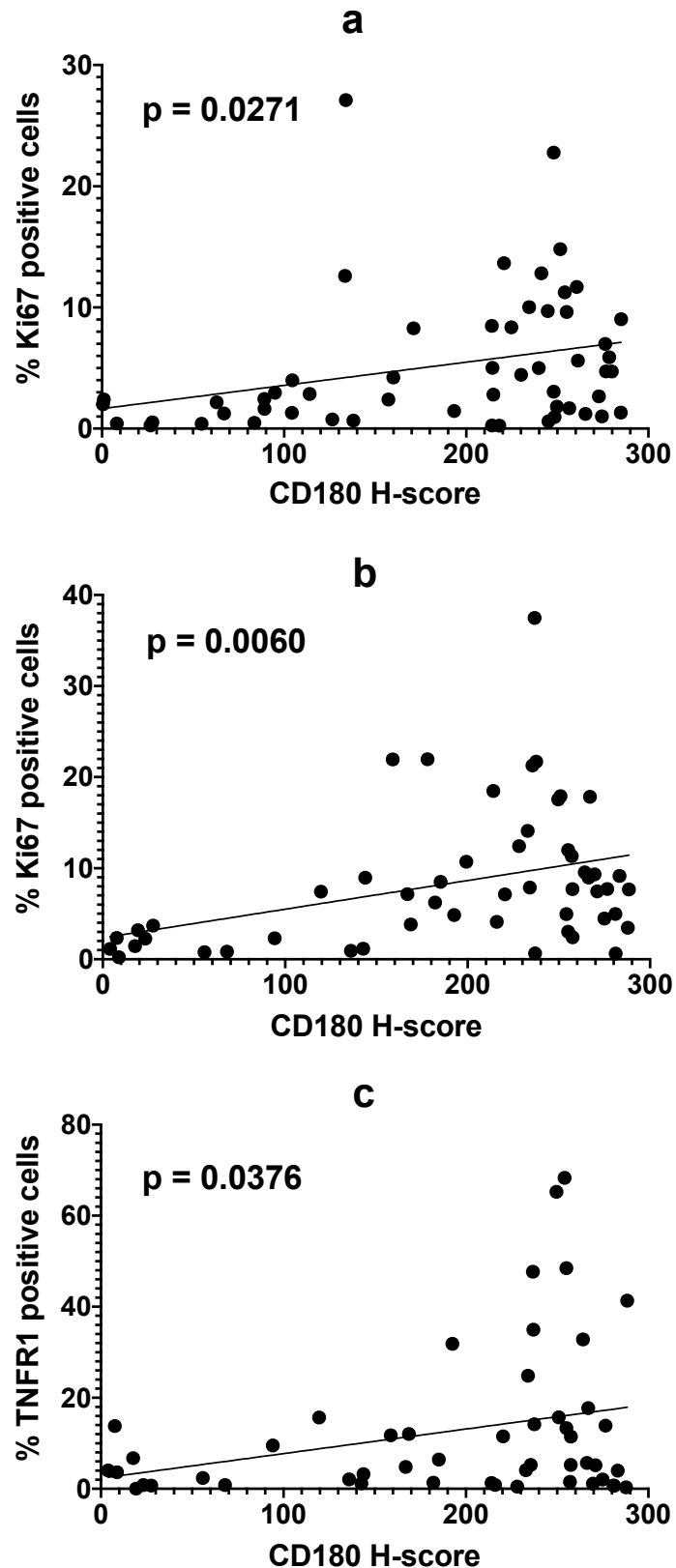


CD180 H-score correlates with the expression of Ki67 and TNFR1

PB and LN samples from the same cohort of patients were previously characterised for the expression of a range of biomarkers of prognostic and biological importance. PB immunophenotypic studies were previously used to stratify patients into CD38 and ZAP-70 subgroups based on established 30% (Damle et al., 1999) and 25% (Wiestner et al., 2003) cut-offs, respectively, which we applied here. However, no differences in the LN CD180 H-score were observed among the cohorts of untreated patients separated into ZAP-70 or CD38 positive or negative cases by their PB phenotype ( $p = 0.2872$  and  $p = 0.1825$ , respectively; data not shown).

No correlation was found between CD180 and the expression of the TNF $\alpha$  receptor – TNFR1, in the LNs of untreated patients ( $r = 0.1650$ ,  $p = 0.2333$ , data not shown). However, in the LNs, CD180 H-score significantly correlated with the percentages of Ki67-positive cells ( $r = 0.2980$ ,  $p = 0.0271$ , Fig 3a), an important marker of cellular proliferation (Miller et al., 2018; Sun and Kaufman, 2018).

Within the treated cohort, like with the untreated CLL patients, the CD180 H-Score and the percentages of Ki67+ cells in the LNs were also positively correlated ( $r = 0.3761$ ,  $p = 0.0060$ ; Concordantly, no significant differences were observed in CD180 H-scores when the treated cohort was stratified into ZAP-70 and CD38 subgroups by the PB profile ( $p = 0.1084$  &  $p = 0.3994$ , respectively; data not shown). Moreover, the correlation between CD180 and Ki67 appeared to be strengthened in treated LNs, compared with the untreated cohort (Figs 3a & b). Interestingly, a positive correlation was also found between CD180 H-score and TNFR1 expression in the LNs of treated patients ( $r = 0.2950$ ,  $p = 0.0376$ , Fig 3c). Concordantly, no significant differences were observed in CD180 H-scores when the treated cohort was stratified into ZAP-70 and CD38 subgroups by the PB profile ( $p = 0.1084$  and  $p = 0.3994$ , respectively; data not shown).



**Fig 3. The correlation between CD180 expression, Ki67 and TNFR1.** CD180 H-score in the LN of untreated CLL patients positively correlated with the percentages of Ki-67+ cells in the LN of untreated (a, n = 55) and treated CLL patients (b, n = 52). (c) CD180 H-score was positively correlated with the expression of TNFR1 in the LNs of treated CLL patients (n = 50).

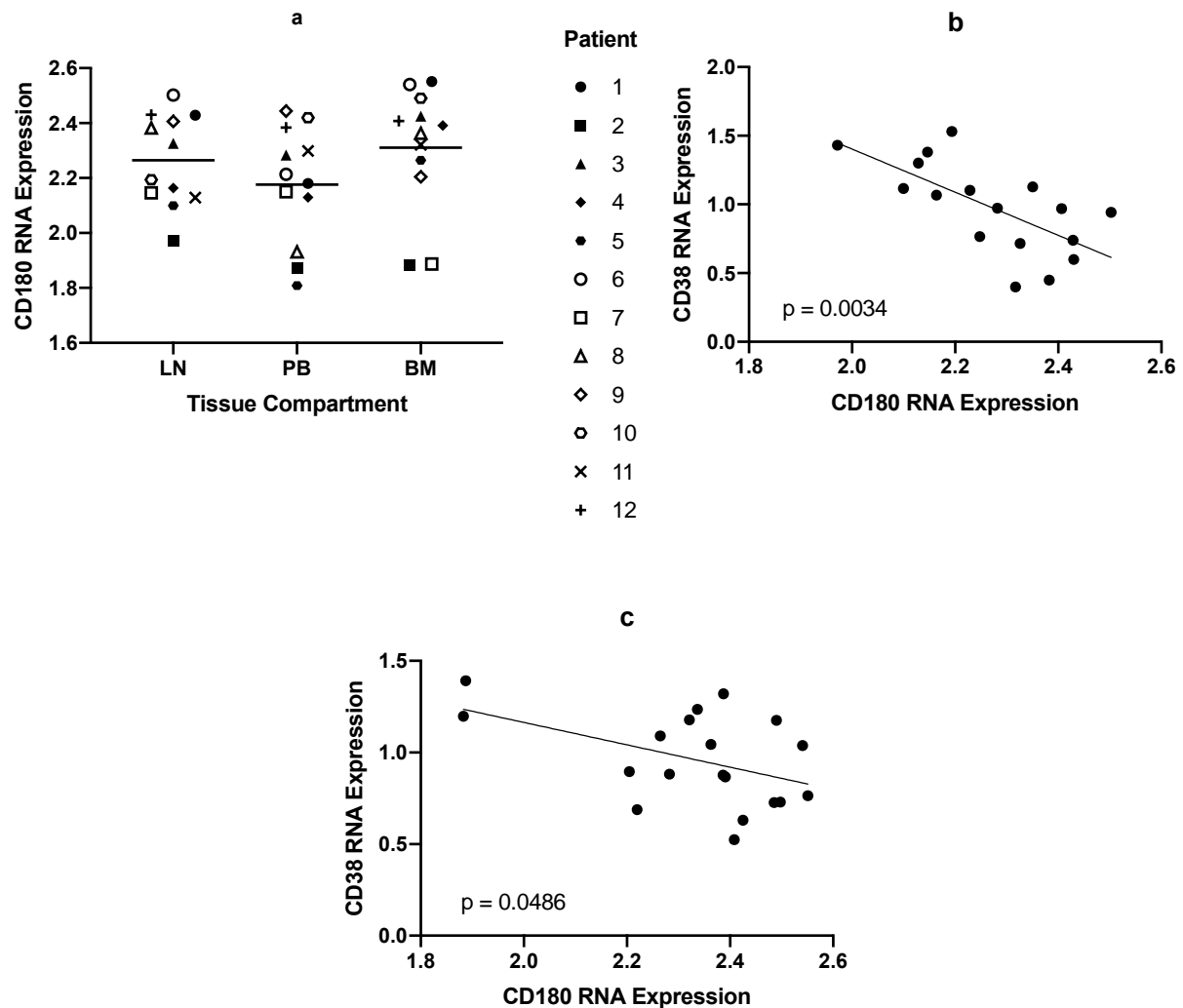
Levels of CD180 RNA transcripts are homogenously high across different tissue compartments in CLL.

In the present study, matched PB and LN samples were not available, and it was not therefore possible to compare CD180 protein expression on LN-resident CLL cells with those circulating in the matched PB. We therefore accessed a gene expression dataset which contained CD180 RNA transcription data as analysed by Affymetrix microarray on matched PB, LN and BM samples from untreated CLL patients. The dataset was obtained from the publicly available GEO under accession number GDS4176 (Herishanu et al. 2011).

We identified 12 patients where data were available for CD180 RNA expression across all tissue compartments. CD180 RNA expression was homogenously high in all of the 12 patients (Fig 4a). In order to illustrate this, we compared levels of RNA transcripts of CD180 with TLR4, which is known to be expressed at very low levels in both normal B and CLL cells (Hornung et al., 2002; Muzio et al., 2009) (Supplementary Fig 1). CD180 RNA expression appeared to be homogenous when analysed across the different tissue compartments and no significant difference in CD180 RNA transcripts between the LN, PB or BM were identified ( $p = 0.1031$ ; Fig 4a).

No significant differences in CD180 RNA transcript levels were identified between Rai stages within the PB ( $p = 0.7810$ ) or BM ( $p = 0.1205$ ) tissue compartments. However, within the LNs, the expression of CD180 RNA in patients stratified as stage 1 was lower (2.174; 1.972 -2.326) compared to those stratified as stage 2 (2.394; 2.317 – 2.430) ( $p=0.0422$ ; data not shown). No other significant differences in CD180 RNA expression in the LN were observed among multiple comparisons of other Rai staging groups (data not shown).

Next, we addressed the relationships between CD180 RNA transcripts and other markers of prognostic and biological significance which were previously investigated by us at the protein expression level. There was found to be a strong negative correlation between the RNA levels of CD180 and CD38 in both the LNs and the BM, but not the PB (Table 1; Figs 4b & c). No significant relationships could be identified between RNA expression of CD180 and ZAP-70 (Table 1). Further, no significant correlations could be determined between CD180 RNA transcripts and TNFR1 or Ki67 (Table 1).



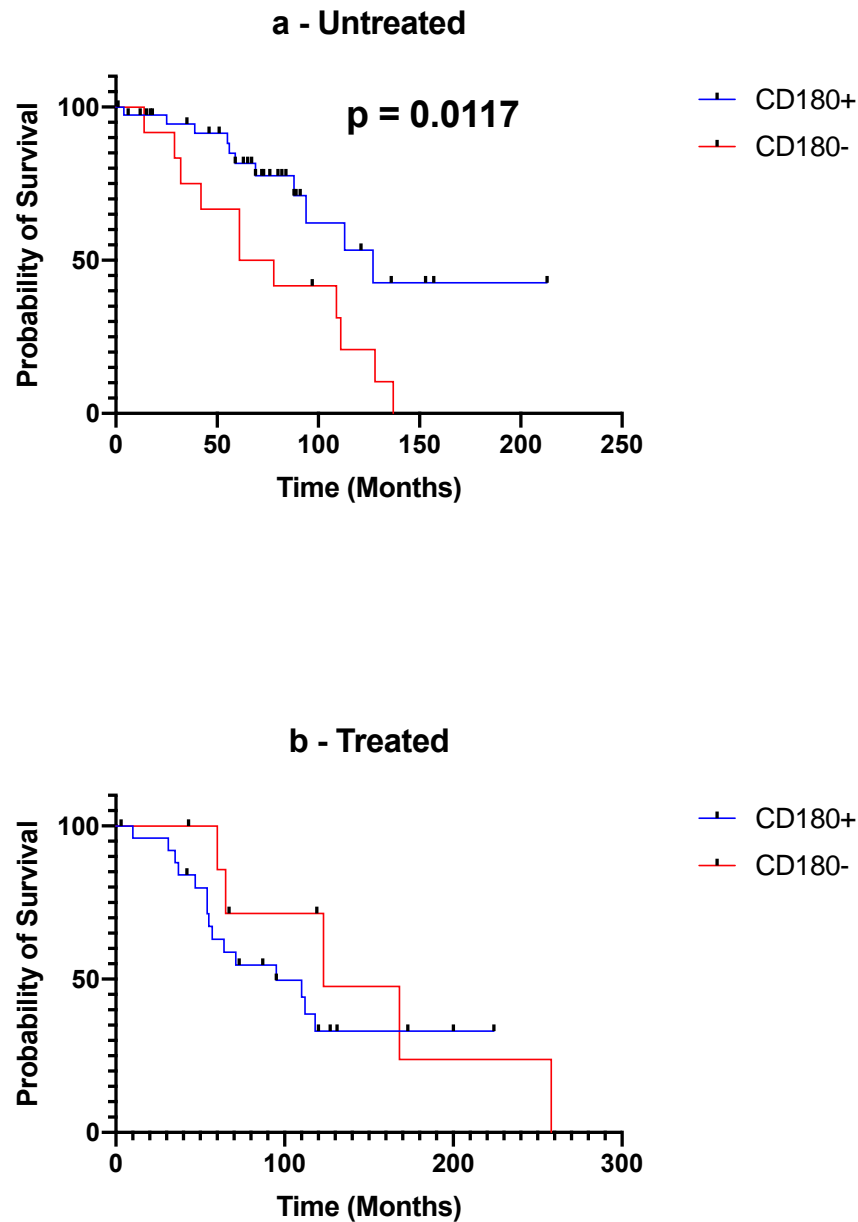
**Fig 4. CD180 RNA expression in the LN, PB and BM of CLL patients.** **(a)** Levels of CD180 expression across different tissue compartments in matched PB, LN and BM samples from untreated CLL patients (N = 12). Cross bars represent the medians. **(b)** The correlation between CD180 and CD38 RNA transcripts in the LNs (n = 17). **(c)** The correlation between CD180 and CD38 RNA transcripts in the BM (n = 18).

**Table 1. Correlations between RNA transcripts of CD180 and markers of biological and prognostic significance.** Using Pearson's correlation, RNA transcripts for CD38, ZAP-70, TNFR1 and Ki67 were analysed relative to CD180 in the corresponding tissue compartments. Significant results are highlighted in bold.

Tissue Compartment (n)	CD180 Vs			
	CD38	ZAP-70	TNFR1	Ki67
Lymph Nodes (17)	<b><math>r = -0.6680</math>, <math>p = 0.0034</math></b>	$r = -0.3485$ , $p = 0.1704$	$r = -0.128$ , $p = 0.6240$	$r = 0.183$ , $p = 0.4810$
Peripheral Blood (24)	$r = 0.0187$ , $p = 0.9308$	$r = -0.2081$ , $p = 0.3293$	$r = -0.189$ , $p = 0.3760$	$r = 0.3456$ , $p = 0.0981$
Bone Marrow (19)	<b><math>r = -0.4580</math>, <math>p = 0.0486</math></b>	$r = -0.1789$ , $p = 0.4637$	$r = -0.083$ , $p = 0.7360$	$r = -0.1778$ , $p = 0.4671$

## Disease progression/outcome

Data were collected on the disease outcome of both cohorts of treated and untreated patients. Based on the CD180 H-scores of 55 untreated patients a threshold for CD180 positivity was set as the mean CD180 H-score, less one SD (96.90, Porakishvili et al., 2005). Within the untreated cohort, CD180 negative cases were associated with a shorter overall survival (OS) compared to CD180 positive cases ( $p = 0.0117$ ; HR = 0.3598 (0.1395 – 0.9278), Fig5a). Survival did not differ significantly by CD180 expression within the treated cohort (CD180<sup>+</sup> = 26; CD180<sup>-</sup> = 8;  $p = 0.4219$ , Fig 5b). Correlation analysis demonstrated no significant relationships between CD180 H-score and time-to-treatment, -response or -relapse in both the treated and untreated cohorts (data not shown), despite of the fact that CD180 expression range and distribution was very similar in treated and untreated CLL patients (Fig 2b).



**Fig 5. The relationship between CD180 H-score and the survival of CLL patients.** Kaplan-Meier estimates for survival of treated (**a**; n=34) and untreated (**b**; n = 52) CLL patients. The log-rank test was used to determine significant differences between curves.



## 4.0 Discussion

The present study for the first time demonstrates that CD180 is heterogeneously expressed in the LNs of CLL patients as measured by IHC. We identified high, intermediate and low/negative expression which supports our earlier studies, where CD180 was shown to be heterogeneously expressed in PB of CLL patients, as measured by flow cytometry (Porakishvili et al., 2005). CD180 H-score was higher in tonsillar control samples where there was little heterogeneity in expression patterns compared to LNs of CLL patients, which is consistent with earlier PB immunophenotypic studies of control B cells (Porakishvili et al., 2005; Arvaniti et al., 2011).

Although no correlation of CD180 expression with the stage of the disease was found in PB CLL cells (Porakishvili et al., 2005), CD180 expression in LN, did vary depending on the Binet stage. In both the treated and untreated cohorts, the median expression of CD180 was reduced in Binet stage B compared to stage A (Figs 2b). This is consistent with the disease progression in the “proliferation centres” of lymph nodes, which is evidenced by the fact that these pseudo-follicles show an enriched expression of Ki67 (Herishanu et al., 2011) and NF- $\kappa$ B activation (Herreros et al., 2010). Expanded proliferation centres have also been associated with accelerated CLL (Giné et al., 2010). Indeed, in our hands CLL progression and morbidity was associated with the low expression of CD180 in the LNs, particularly those of untreated patients (Fig 5a). The growing organomegaly in CLL would confer increased CLL cell activation and proliferation which could account for a decreased density of expression of CD180. Downregulation of CD180 expression on activated B cells has been seen in systemic lupus erythematosus (Koarada et al., 1999; Koarada and Tada, 2012). Our earlier study demonstrated that the PB CLL cells from around 60% patients respond to CD180 ligation by increasing the surface expression of the activation marker CD86 as well as the proliferation marker Ki67 (Porakishvili et al., 2011). Herishanu et al. (2011) showed that Ki67 is upregulated in the LNs of CLL patients compared to the PB which strengthens the concept that CLL cells undergo proliferation within localised anatomical sites. In this study we have demonstrated that the expression of the proliferation marker Ki67 is positively correlated with the CD180 H-score in lymph nodes of CLL patients (Figs 3a & b) which is indicative of the involvement of CD180 in the proliferation and expansion of CLL cells within the LNs.

There was a remarkable similarity in distribution of the CD180 H-scores in the LNs of treated and untreated patients as well as the expression pattern (Fig 2a). This suggests that CD180 expression in LNs is unaffected by treatment and might represent a treatment-independent prognostic indicator for disease progression and morbidity. In order to identify any relationships between treatment modality and CD180 expression, we analysed available data within the treated cohort. CD180 expression was heterogenous across all prescribed treatments and no pattern could be identified (data not shown). However, in the present study it was not known if patients were treated with any class of small molecule inhibitors, such as ibrutinib or idelalisib. Therefore, further research is required to confirm that molecular therapies do not affect CD180 expression in the LNs or PB.

We have previously reported (Porakishvili et al., 2015) that CLL cells from a subset of patients are able to signal for survival via CD180 in an AKT-dependent manner. Therefore, the activation of this signalling axis could occur within the LNs and contribute to CLL cell survival. CD180 signalling can be triggered independently of CD40 and IL-4 pathways (Porakishvili et al., 2011), both of which are robust modalities for activating B cells thus suggesting that CLL

cells could concomitantly receive microenvironmental signals via various receptors in tandem. Moreover, IL-4 signalling is significantly upregulated in the LNs of CLL patients, compared to the PB, which contributes to an increased expression of sIgM (Aguilar-Hernandez et al., 2016). This could promote CLL cell proliferation through synergy between CD180 and the BCR. In contrast, a subset of CLL cases signal for apoptosis via p38MAPK (Porakishvili et al., 2015). Should this signalling capacity be preserved in the LNs and become activated, then CD180 may contribute to the apoptosis of CLL cells, and this could account for the favourable disease outcome for the patients with high CD180 H-score (Fig 5a). The diversity of outcomes which may result from CD180 signalling within both, the LN and the PB may therefore be significant for our understanding of the clinical heterogeneity observed among CLL cases. The hypothesis of a role for CD180 as a 'microenvironmental' sensor would strengthen the hypothesis that CLL cells are regulated by CD180 in the proliferation centres.

Apart from IL-4, tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) also plays an important role in CLL biology as an autocrine growth factor produced by CLL cells to promote their survival (Cordingley et al., 1988). Increasing levels of TNF $\alpha$  in the serum of CLL patients have been associated with disease progression, implicating the TNF $\alpha$  pathway in CLL expansion (Ferrajoli et al., 2002). TNF- $\alpha$  can be sensed by a family of tumour necrosis factor receptors (TNFR). In this study we were able to determine a positive correlation between the expression of CD180 and TNFR1 in the lymph nodes of treated CLL patients (Fig 3c). The expression of TNFR1 in the LNs might mean that CLL cells are prone to activation via the TNF $\alpha$  axis which promotes their maintenance (Dürr et al., 2018). Good, Avery and Tangye (2009) showed that memory B cells are characterised by an increased expression of members of the TNFR family and CD180.

Since matched LN and PB samples were not available for comparative IHC or flow cytometry studies of CD180 expression patterns, due to the logistical embargo on LN biopsies in CLL patients, we analysed publicly available RNA expression data. All tissue compartments displayed homogeneously high and comparable levels of CD180 RNA transcripts (Fig 4a), which is consistent with our own unpublished observations where we have also found homogeneously high levels of CD180 RNA in PB CLL cells, despite of a significant heterogeneity in the expression of CD180 at the protein level in the PB (Porakishvili et al., 2005, and our later observations) and LN in the present study.

We also found there to be higher levels of CD180 RNA in the LN of Rai stage 2 patients compared to stage 1, a pattern opposite to the one which was observed in our analysis of CD180 H-score among patients stratified by Binet stage (Fig 2b). The inconsistency between patterns of RNA and protein expression among the CLL stages therefore further indicates that RNA expression levels are not necessarily an accurate reflection of CD180 at the protein level.

Whilst the differences did not reach statistical significance, the levels of CD180 RNA transcripts were lower in the PB compared to the LN and BM (Fig 4a). When analysing levels of CD180 RNA transcripts on an individual basis, the majority of patients showed a decrease in RNA transcripts from the BM to PB (9/12, 75%) or from the LN to PB (10/12, 83.33%). Interestingly, all patients except one (Patient 11) showed either a consistent increase or decrease in levels of CD180 RNA between the LN/BM and the PB. The reduction of CD180 RNA levels in the LN and the BM compared to the PB is therefore consistent with the concept that CD180 expression is reduced in activated cells, given the proliferation centres found within the LNs and BM are rich in microenvironmental factors which support CLL cell activation.

The apparent discrepancy between the levels of RNA and protein expression means that CLL cells are able to readily transcribe CD180. However, in some cases CD180 cannot be effectively expressed on the cell surface. Defective or inhibited CD180 translation may prevent CD180 from being expressed in the cell surface in some instances which may account for the heterogeneous expression of CD180 at the protein level. Alternatively, if it is assumed that CD180 is successfully synthesised intracellularly, it is possible that post-translational events may induce heterogeneity in cell surface CD180 expression. This could occur either through defective protein export/trafficking mechanisms or through modulation by a negative signalling mechanism which becomes preferentially activated in some instances. Our preliminary microscopy data indicates that some of the CLL samples categorised as negative by the surface expression of CD180 on PB CLL cells, in fact express the protein intracellularly.

The regulatory mechanisms which govern the synthesis, export and expression of CD180, are yet to be fully elucidated however, parallels may be drawn with the systems which regulate the expression of other TLRs. The effective trafficking of the CD180 homolog TLR4 to the cell surface is dependent on the small satellite glycoprotein MD2 (Shimazu et al., 1999). Similarly, MD1 forms a complex with CD180 (Miyake et al., 1998; Ohto, Miyake and Shimizu, 2011) and is indispensable for cell surface expression of CD180 on murine and human B cells (Miura et al., 1998; Miyake et al., 1998; Nagai et al., 2002). The heterogeneity of CD180 expression at the protein level could therefore be largely dependent on the effective synthesis and availability of MD1 in CLL cells too.

A strong negative correlation between CD180 and CD38 RNA levels in the LN and BM, but not PB (Table 1), is consistent with our earlier PB immunophenotypic study where no significant correlation could be determined between CD180 and CD38 at the protein level (Porakishvili et al. 2005). The correlation between CD180 RNA transcripts in the LN and BM but not the PB could be accounted for by differences in the tissue compartments. It has been shown that CD38 is more readily expressed in the LN compared to the BM and the PB (Jaksic et al., 2004; Patten et al., 2008). Consistently, we also found that levels of CD38 RNA were significantly reduced in the PB compared to the LN ( $p = 0.0166$ , data not shown). These findings support the association between CD180 positivity in the LNs and improved overall survival given that CD38 expression negatively affects CLL prognosis.

Indeed, we have found that CD180 positivity in the LNs was associated with improved overall survival (Fig 5a). We have previously shown that CD180 expression is significantly higher in the PB M-CLL, which has a positive prognostic outlook (Porakishvili et al., 2005). This could begin to explain the positive associations between CD180 and survival. Furthermore, the possibility of the concomitant ligation of CD180 alongside other receptors *in vivo* must be considered. For example, in a large proportion of cases, CD180 ligation can cause CLL cells to rewire IgM mediated pro-survival signals to a pro-apoptotic pathway mediated by p38MAPK (Porakishvili et al., 2015). Moreover, CD180 and the IgD BCR isoform also tend to cooperate to induce the phosphorylation of p38MAPK and cause apoptosis in the majority of cases (Porakishvili et al., 2017). Therefore, CD180 could be activated together with IgD or IgM to modify CLL cell survival and reduce tumour burden thus resulting in improved overall survival.

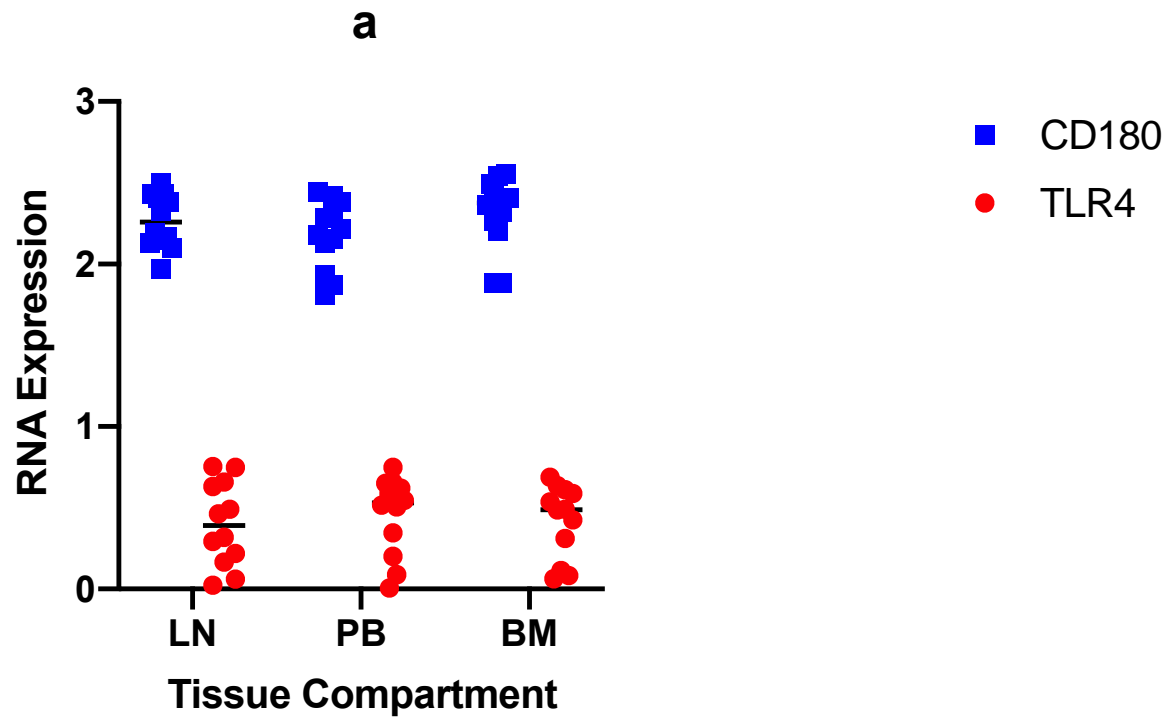
## Conclusions

We have demonstrated the heterogenous expression of CD180 in the LNs of CLL patients, with homogeneously high levels of CD180 expression in normal human tonsils. Whilst matched LN and PB samples were not available, the present IHC data together with previous peripheral immunophenotypic studies suggests that CD180 expression in the LN mirrors the PB, in both CLL patients and healthy individuals. However, analysis of RNA transcript data from matched LB, PB and BM samples from CLL patients indicate that CD180 RNA is homogeneously high in CLL cells, regardless of their anatomical residence and despite the significant heterogeneity observed at the protein level, in both the LN and PB. Understanding how translation of CD180 or its successful export/tracking to the cell surface becomes disrupted may therefore be key to understanding the heterogenous expression of CD180 in CLL.

CD180 expression in lymph nodes of CLL patients positively correlates with the expression of Ki-67 and TNFR1 which is concordant with our earlier studies on PB CLL samples. Together these findings indicated the involvement of CD180 in CLL cell activation, proliferation and expansion.

Our data indicates that higher densities of CD180 expression in the LNs are associated with the early stages of the disease and better overall survival indicating that CD180 plays a significant and heterogenous role in CLL pathobiology and the disease outcome.

## Supplementary Data



**Supplementary Fig 1.** Levels of CD180 and TLR4 RNA transcripts in matched CLL LN, PB, and BM samples (N = 12). TLR4 was highly negative across all tissue compartments relative to CD180 which was homogenously high.



## References

- Aguilar-Hernandez, M.M., Blunt, M.D., Dobson, R., Yeomans, A., Thirdborough, S., Larrayoz, M., Smith, L.D., Linley, A., Strefford, J.C., Davies, A., Johnson, P.M.W., Savelyeva, N., Cragg, M.S., Forconi, F., Packham, G., Stevenson, F.K. and Steele, A.J. (2016). IL-4 enhances expression and function of surface IgM in CLL cells. *Blood*, 127 (24), 3015–3025. Available from <https://doi.org/10.1182/blood-2015-11-682906>.
- Arvaniti, E., Ntoufa, S., Papakonstantinou, N., Touloumenidou, T., Laoutaris, N., Anagnostopoulos, A., Lamnissou, K., Caligaris-Cappio, F., Stamatopoulos, K., Ghia, P., Muzio, M. and Belessi, C. (2011). Toll-like receptor signaling pathway in chronic lymphocytic leukemia: distinct gene expression profiles of potential pathogenic significance in specific subsets of patients. *HAEMATOLOGICA-THE HEMATOLOGY JOURNAL*, 96 (11), 1644–1652. Available from <https://doi.org/10.3324/haematol.2011.044792>.
- Burger, J.A. and Chiorazzi, N. (2013). B cell receptor signaling in chronic lymphocytic leukemia. *Trends in immunology*, 34 (12), 592–601. Available from <https://doi.org/10.1016/j.it.2013.07.002>.
- Cordingley, F.T., Bianchi, A., Hoffbrand, A. V, Reittie, J.E., Heslop, H.E., Vyakarnam, A., Turner, M., Meager, A. and Brenner, M.K. (1988). Tumour necrosis factor as an autocrine tumour growth factor for chronic B-cell malignancies. *Lancet (London, England)*, 1 (8592), 969–971. Available from [https://doi.org/10.1016/s0140-6736\(88\)91782-5](https://doi.org/10.1016/s0140-6736(88)91782-5).
- Dadashian, E.L., McAuley, E.M., Liu, D., Shaffer 3rd, A.L., Young, R.M., Iyer, J.R., Kruhlak, M.J., Staudt, L.M., Wiestner, A. and Herman, S.E.M. (2019). TLR Signaling Is Activated in Lymph Node-Resident CLL Cells and Is Only Partially Inhibited by Ibrutinib. *Cancer research*, 79 (2), 360–371. Available from <https://doi.org/10.1158/0008-5472.CAN-18-0781>.
- Damle, R.N., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S.L., Buchbinder, A., Budman, D., Dittmar, K. and Kolitz, J. (1999). Ig V Gene Mutation Status and CD38 Expression As Novel Prognostic Indicators in Chronic Lymphocytic Leukemia: Presented in part at the 40th Annual Meeting of The American Society of Hematology, held in Miami Beach, FL, December 4-8, 1998. *Blood*, 94 (6), 1840–1847.
- Divanovic, S., Trompette, A., Atabani, S.F., Madan, R., Golenbock, D.T., Visintin, A., Finberg, R.W., Tarakhovsky, A., Vogel, S.N., Belkaid, Y., Kurt-Jones, E.A. and Karp, C.L. (2005). Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. *Nature immunology*, 6 (6), 571–578. Available from <https://doi.org/10.1038/ni1198> [doi].
- Dürr, C., Hanna, B.S., Schulz, A., Lucas, F., Zucknick, M., Benner, A., Clear, A., Ohl, S., Öztürk, S., Zenz, T., Stilgenbauer, S., Li-Weber, M., Krammer, P.H., Gribben, J.G., Lichter, P. and Seiffert, M. (2018). Tumor necrosis factor receptor signaling is a driver of chronic lymphocytic leukemia that can be therapeutically targeted by the flavonoid wogonin. *Haematologica*, 103 (4), 688–697. Available from <https://doi.org/10.3324/haematol.2017.177808>.
- Ferrajoli, A., Keating, M.J., Manshouri, T., Giles, F.J., Dey, A., Estrov, Z., Koller, C.A., Kurzrock,

- R., Thomas, D.A., Faderl, S., Lerner, S., O'Brien, S. and Albitar, M. (2002). The clinical significance of tumor necrosis factor- $\alpha$  plasma level in patients having chronic lymphocytic leukemia. *Blood*, 100 (4), 1215–1219.
- Gandhi, V. and Balakrishnan, K. (2012). CCL2 in chronic lymphocytic leukemia: a macro in microenvironment? *Leukemia & lymphoma*, 53 (10), 1849–1850. Available from <https://doi.org/10.3109/10428194.2012.688966>.
- Ghia, P., Strola, G., Granziero, L., Geuna, M., Guida, G., Sallusto, F., Ruffing, N., Montagna, L., Piccoli, P., Chilosi, M. and Caligaris-Cappio, F. (2002). Chronic lymphocytic leukemia B cells are endowed with the capacity to attract CD4<sup>+</sup>, CD40L<sup>+</sup> T cells by producing CCL22. *European journal of immunology*, 32 (5), 1403–1413. Available from [https://doi.org/10.1002/1521-4141\(200205\)32:5<1403::AID-IMMU1403>3.0.CO;2-Y](https://doi.org/10.1002/1521-4141(200205)32:5<1403::AID-IMMU1403>3.0.CO;2-Y).
- Giné, E., Martinez, A., Villamor, N., López-Guillermo, A., Camos, M., Martinez, D., Esteve, J., Calvo, X., Muntanola, A., Abrisqueta, P., Rozman, M., Rozman, C., Bosch, F., Campo, E. and Montserrat, E. (2010). Expanded and highly active proliferation centers identify a histological subtype of chronic lymphocytic leukemia ('accelerated' chronic lymphocytic leukemia) with aggressive clinical behavior. *Haematologica*, 95 (9), 1526–1533. Available from <https://doi.org/10.3324/haematol.2010.022277>.
- Good, K.L., Avery, D.T. and Tangye, S.G. (2009). Resting Human Memory B Cells Are Intrinsically Programmed for Enhanced Survival and Responsiveness to Diverse Stimuli Compared to Naïve B Cells. *JOURNAL OF IMMUNOLOGY*, 182 (2), 890–901. Available from <https://doi.org/10.4049/jimmunol.182.2.890>.
- Herishanu, Y., Pérez-Galán, P., Liu, D., Biancotto, A., Pittaluga, S., Vire, B., Gibellini, F., Njuguna, N., Lee, E., Stennett, L., Raghavachari, N., Liu, P., McCoy, J.P., Raffeld, M., Stetler-Stevenson, M., Yuan, C., Sherry, R., Arthur, D.C., Maric, I., White, T., Marti, G.E., Munson, P., Wilson, W.H. and Wiestner, A. (2011). The lymph node microenvironment promotes B-cell receptor signaling, NF- $\kappa$ B activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood*, 117 (2), 563–574. Available from <https://doi.org/10.1182/blood-2010-05-284984>.
- Herreros, B., Rodríguez-Pinilla, S.M., Pajares, R., Martínez-González, M.Á., Ramos, R., Munoz, I., Montes-Moreno, S., Lozano, M., Sánchez-Verde, L., Roncador, G., Sánchez-Beato, M., de Otazu, R.D., Pérez-Guillermo, M., Mestre, M.J., Bellas, C. and Piris, M.Á. (2010). Proliferation centers in chronic lymphocytic leukemia: the niche where NF- $\kappa$ B activation takes place. *Leukemia*, 24 (4), 872–876. Available from <https://doi.org/10.1038/leu.2009.285>.
- Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdörfer, B., Giese, T., Endres, S. and Hartmann, G. (2002). Quantitative Expression of Toll-Like Receptor 1–10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides. *The Journal of Immunology*, 168 (9), 4531 LP – 4537. Available from <https://doi.org/10.4049/jimmunol.168.9.4531>.
- Jaksic, O., Paro, M.M.K., Kardum Skelin, I., Kusec, R., Pejisa, V. and Jaksic, B. (2004). CD38 on B-cell chronic lymphocytic leukemia cells has higher expression in lymph nodes than in peripheral blood or bone marrow. *Blood*, 103 (5), 1968–1969. Available from <https://doi.org/10.1182/blood-2003-11-3890>.

- Koarada, S., Tada, Y., Ushiyama, O., Morito, F., Suzuki, N., Ohta, A., Miyake, K., Kimoto, M. and Nagasawa, K. (1999). B cells lacking RP105, a novel B cell antigen, in systemic lupus erythematosus. *Arthritis and rheumatism*, 42 (12), 2593–2600. Available from [https://doi.org/10.1002/1529-0131\(199912\)42:12<2593::AID-ANR12>3.0.CO;2-G](https://doi.org/10.1002/1529-0131(199912)42:12<2593::AID-ANR12>3.0.CO;2-G).
- Koarada, S. and Tada, Y. (2012). RP105-Negative B Cells in Systemic Lupus Erythematosus. *CLINICAL & DEVELOPMENTAL IMMUNOLOGY*. Available from <https://doi.org/10.1155/2012/259186>.
- Miller, I., Min, M., Yang, C., Tian, C., Gookin, S., Carter, D. and Spencer, S.L. (2018). Ki67 is a Graded Rather than a Binary Marker of Proliferation versus Quiescence. *Cell reports*, 24 (5), 1105–1112.e5. Available from <https://doi.org/10.1016/j.celrep.2018.06.110>.
- Miyake, K., Shimazu, R., Kondo, J., Niki, T., Akashi, S., Ogata, H., Yamashita, Y., Miura, Y. and Kimoto, M. (1998). Mouse MD-1, a molecule that is physically associated with RP105 and positively regulates its expression. *Journal of immunology (Baltimore, Md. : 1950)*, 161 (3), 1348–1353.
- Mongini, P.K.A., Gupta, R., Boyle, E., Nieto, J., Lee, H., Stein, J., Bandovic, J., Stankovic, T., Barrientos, J., Kolitz, J.E., Allen, S.L., Rai, K., Chu, C.C. and Chiorazzi, N. (2015). TLR-9 and IL-15 Synergy Promotes the In Vitro Clonal Expansion of Chronic Lymphocytic Leukemia B Cells. *Journal of immunology (Baltimore, Md. : 1950)*, 195 (3), 901–923. Available from <https://doi.org/10.4049/jimmunol.1403189>.
- Muzio, M., Scielzo, C., Bertilaccio, M.T.S., Frenquelli, M., Ghia, P. and Caligaris-Cappio, F. (2009). Expression and function of toll like receptors in chronic lymphocytic leukaemia cells. *British journal of haematology*, 144 (4), 507–516. Available from <https://doi.org/10.1111/j.1365-2141.2008.07475.x>.
- Nagai, Y., Shimazu, R., Ogata, H., Akashi, S., Sudo, K., Yamasaki, H., Hayashi, S.-I., Iwakura, Y., Kimoto, M. and Miyake, K. (2002). Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide. *Blood*, 99 (5), 1699–1705. Available from <https://doi.org/10.1182/blood.v99.5.1699>.
- Ohto, U., Miyake, K. and Shimizu, T. (2011). Crystal Structures of Mouse and Human RP105/MD-1 Complexes Reveal Unique Dimer Organization of the Toll-Like Receptor Family. *Journal of Molecular Biology*, 413 (4), 815–825. Available from <https://doi.org/10.1016/j.jmb.2011.09.020>.
- Patten, P.E.M., Buggins, A.G.S., Richards, J., Wotherspoon, A., Salisbury, J., Mufti, G.J., Hamblin, T.J. and Devereux, S. (2008). CD38 expression in chronic lymphocytic leukemia is regulated by the tumor microenvironment. *Blood*, 111 (10), 5173–5181. Available from <https://doi.org/10.1182/blood-2007-08-108605>.
- Porakishvili, N., Kulikova, N., Jewell, A.P., Youinou, P.Y., Yong, K., Nathwani, A., Heelan, B., Duke, V., Hamblin, T.J., Wallace, P., Ely, P., Clark, E.A. and Lydyard, P.M. (2005). Differential expression of CD180 and IgM by B-cell chronic lymphocytic leukaemia cells using mutated and unmutated immunoglobulin VH genes. *British Journal of Haematology*, 131 (3), 313–319. Available from <https://doi.org/10.1111/j.1365-2141.2005.05775.x>.
- Porakishvili, N., Memon, A., Vispute, K., Kulikova, N., Clark, E.A., Rai, K.R., Nathwani, A., Damle, R.N., Chiorazzi, N. and Lydyard, P.M. (2011). CD180 functions in activation,

survival and cycling of B chronic lymphocytic leukaemia cells. *British Journal of Haematology*, 153 (4), 486–498. Available from <https://doi.org/10.1111/j.1365-2141.2011.08605.x>.

Porakishvili, N., Vispute, K., Steele, A.J., Rajakaruna, N., Kulikova, N., Tsertsvadze, T., Nathwani, A., Damle, R.N., Clark, E.A., Rai, K.R., Chiorazzi, N. and Lydyard, P.M. (2015). Rewiring of sIgM-Mediated Intracellular Signaling through the CD180 Toll-like Receptor. *Molecular medicine (Cambridge, Mass.)*, 21 (1), 46–57. Available from <https://doi.org/10.2119/molmed.2014.00265>.

Porakishvili, N., Sayed, U., Zaitseva, K., Rajakaruna, N., Tsertsvadze, T., Mitskevich, N., Nathwani, A., Damle, R.N., Clark, E.A., Rai, K.R., Chiorazzi, N., Murphy, J. and Lydyard, P.M. (2017). Synergism between CD180 and IgD-mediated phosphorylation of p38MAPK and apoptosis of p38MAPK-signaller chronic lymphocytic leukaemia (CLL) cells. *LEUKEMIA & LYMPHOMA*, 58 (1), 134–135.

Schuh, A.H., Parry-Jones, N., Appleby, N., Bloor, A., Dearden, C.E., Fegan, C., Follows, G., Fox, C.P., Iyengar, S., Kennedy, B., McCarthy, H., Parry, H.M., Patten, P., Pettitt, A.R., Ringshausen, I., Walewska, R. and Hillmen, P. (2018). Guideline for the treatment of chronic lymphocytic leukaemia. *British Journal of Haematology*, 182 (3), 344–359. Available from <https://doi.org/10.1111/bjh.15460>.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *The Journal of experimental medicine*, 189 (11), 1777–1782. Available from <https://doi.org/10.1084/jem.189.11.1777>.

Sun, X. and Kaufman, P.D. (2018). Ki-67: more than a proliferation marker. *Chromosoma*, 127 (2), 175–186. Available from <https://doi.org/10.1007/s00412-018-0659-8>.

Wiestner, A., Rosenwald, A., Barry, T.S., Wright, G., Davis, R.E., Henrickson, S.E., Zhao, H., Ibbotson, R.E., Orchard, J.A., Davis, Z., Stetler-Stevenson, M., Raffeld, M., Arthur, D.C., Marti, G.E., Wilson, W.H., Hamblin, T.J., Oscier, D.G. and Staudt, L.M. (2003). ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood*, 101 (12), 4944–4951. Available from <https://doi.org/10.1182/blood-2002-10-3306>.