



Research paper

Expression and signaling patterns of CD180/MD-1 and CD150 in Chronic Lymphocytic Leukaemia (CLL) cells

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ABSTRACT

Chronic lymphocytic Leukaemia (CLL) is a B-cell malignancy marked by biological heterogeneity, variable progression and survival outcomes. Orphan toll-like receptor CD180 (RP105) complexed with the accessory protein MD-1 and CD150, which belongs to the signalling lymphocyte activation molecule family (SLAMF1), have emerged as an important modulators of CLL cell signaling and fate. However, their functional interplay, remains insufficiently understood.

This study investigated the surface and intracellular expression of CD180, CD150, and MD-1 in primary CLL cells and the MEC-1 cell line, alongside their downstream signaling responses. Flow cytometric analyses revealed that MD-1 expression is tightly associated with CD180 and CD150 positivity, which may further indicate a role for MD-1 in stabilizing CD180 surface localization.

Stimulation of MD-1⁺CD180⁺ CLL cells revealed four distinct signaling phenotypes: AKT signalers, p38MAPK signalers, dual signalers, and non-responders. CD150^{low} cells preferentially activated AKT, whereas CD150^{high} cells favored p38MAPK signaling, indicating context-dependent modulation of downstream pathways. Notably, stimulation via CD150 alone induced phosphorylation of both AKT and p38MAPK in specific subsets, confirming its independent signaling capacity.

In synchronized MEC-1 cells, CD180 and MD-1 expression peaked at 48 h, coinciding with enhanced AKT phosphorylation upon combined CD180/CD150 stimulation. These data suggest that the temporal dynamics of CD180 expression modulate the outcome of CD150-driven signaling, indicating a tightly regulated and inter-dependent interaction.

Collectively, these findings define a CD180/MD-1/CD150 signaling axis that underlies the intracellular signaling heterogeneity in CLL. The relative expression balance between CD180 and CD150 may steer cells toward pro-survival or stress-associated signaling, providing novel mechanistic insight into CLL biology and identifying candidate biomarkers or therapeutic targets.

1. Introduction

Chronic lymphocytic Leukaemia (CLL), the most common form of B cell leukaemia in adults, is a complex disease characterized by considerable molecular and clinical [1]. Despite significant advances in

understanding of the molecular drivers of CLL, its variable clinical outcomes from indolent forms to aggressive subtypes remain incompletely understood. Important regulators of CLL cell fate and survival include receptors CD180 (RP105) and CD150 (SLAMF1), which influence signaling pathways critical for the disease progression [2–8].

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CD180, an orphan Toll-like receptor (TLR) has been shown to influence CLL cell signaling and survival, with elevated expression correlating with favorable prognostic indicators, including early Binet stages and extended overall survival (OS) [2–4,7,15]. CD150, a member of the signaling lymphocyte activation molecule (SLAM) family, has been implicated in promoting B-cell survival and proliferation, yet its specific contribution to CLL pathogenesis remains underexplored [5,6,9]. Both receptors co-localize with CD19 on the CLL cell surface, suggesting potential cooperative interactions in modulating downstream signaling events [5].

A key regulator of CD180 is MD-1, an accessory protein that stabilizes its surface expression and influences intracellular signaling, particularly through the AKT and p38MAPK pathways [10–12]. While CD180/MD-1 interaction is well characterized in normal B cells, its role in CLL remains unclear [8,13,16]. Evidence suggests that MD-1 not only regulates CD180 surface expression but also modulates signaling cascades governing CLL cell survival and apoptosis [8].

This study aimed to expand our understanding of the functional interplay between CD180, CD150, and MD-1 in CLL cells by characterizing the expression patterns of CD180, CD150, and MD-1 at both, surface and intracellular levels and identifying molecular interactions between these receptors and their downstream signaling pathways. We hypothesize that CD180/MD-1 and CD150 form a coordinated signaling network that dictates CLL cell fate by influencing the balance between pro-survival (AKT) and pro-apoptotic (p38MAPK) signaling as suggested in our earlier publications [2,3]. We propose that MD-1 enhances CD180 surface expression and works with CD150 to activate distinct signaling subsets that may contribute to CLL heterogeneity. Furthermore, we suggest that the expression levels of CD150 may delineate distinct subpopulations of CLL cells with differential survival capacities.

2. Material and methods

2.1. Patients

Sample collection was approved by the Medical-Biological Research Ethics Committee of the National Center for Disease Control and Public Health (NCDC), Georgia (approval number #2024–056). Patient samples were collected under the supervision of Dr. Mikheil Gadabadze at the Medinvest Hematology and Transfusiology Institute and Dr. Irina Datikashvili-David at the Aversi Clinic.

The study enrolled 56 patients with CLL, aged 52–82 years (mean age: 73). All patients provided written informed consent. None had autoimmune diseases, and none had received treatment for at least four months prior to the study. White blood cell counts ranged from 30 to $200 \times 10^6/\text{mL}$.

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from CLL patients using density gradient centrifugation as previously described [3–5]. The cells were resuspended in RPMI-1640 containing 10 % FBS at a concentration of 2×10^6 cells/mL and were further adjusted to the appropriate concentration for subsequent experiments.

2.3. MEC-1 cell line

MEC-1 cells were obtained from the American Type Culture Collection (ATCC) and were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (Sigma-Aldrich). The cells were incubated at 37°C in 5 % CO₂ and the medium were changed every 4 days.

MEC1 cells were synchronized by serum starvation to facilitate accurate cell cycle analysis. Briefly, cells were cultured in RPMI-1640 medium supplemented with 0.5 % FBS for 24 h to induce G0/G1 arrest. After starvation, cells were released into complete medium (10 %

FBS) and harvested at 0, 24, 48, and 72 h for analysis. Cell viability was assessed using 0.4 % Trypan Blue (Thermo Fisher), with 10 µL of the dye mixed with 10 µL of the cell suspension. Cell counts were conducted using a Thermo Fisher Countess® II FL Automated Cell Counter. Ethidium bromide staining was employed to determine cell cycle distribution.

2.4. Immunophenotyping

2.4.1. PBMCs

For cell surface expression (CS) 2×10^6 PBMCs were stained with the following monoclonal antibodies (mAbs): FITC anti-CD19 (BD, clone HIB19, catalog # 555412) and PE-Cy7 anti-CD5 (BD, Clone UCHT2, catalog # 571696), APC-Cy7 anti-CD180 (Abcam, clone G28–8, catalog ab253257), PE anti-MD-1 (antibodies-online, clone AA21162, catalog ABIN5565815), and APC anti-CD150 (Abcam, clone SLAMF4, #catalog ab270641), PE anti-CD38 (eBioscience™, clone HIT2, #catalog 12–0389–42) and Corresponding isotype controls were used for each antibody (BD isotypes for BD antibodies, Abcam/antibodies-online isotypes for APC-Cy7, APC, PE-conjugated antibodies, eBioscience isotype for PE anti-CD38).

For intracellular expression (IC), cells were stained with FITC anti-CD19, PE-Cy7 anti-CD5 and APC anti-CD150, fixed and permeabilized using Fixation/Permeabilization Solution (BD Biosciences, catalog #554714) for 20 min at room temperature (RT) in the dark. Cells were washed with BD Perm/Wash™ buffer and stained with APC-Cy7 anti-CD180, PE anti-MD-1 or PE anti-ZAP70 (eBioscience™, clone 1E7.2, catalog # 12–6695–42), with corresponding isotype controls as above.

The samples were analyzed by flow cytometry using a NovoCyte 2060 (ACEA Biosciences, San Diego, CA, USA). Results were calculated as percentages of positive cells in the CD19⁺CD5⁺ population for CS or IC+CS expression.

2.4.2. MEC-1 cells

Synchronized MEC-1 cells were assessed for surface expression of CD180, CD150, and MD-1 at time points 0h–72h. Cells were incubated with anti-CD180, anti-MD-1, and anti-CD150 mAbs as above. Intracellular expression of CD180, MD-1 and CD150 was assessed as above for PBMC.

Flow cytometry analysis was performed as above. Results were calculated as percentages of positive cells for CS or IC+CS expression.

2.4.3. Flow cytometry gating and positivity determination

Positivity thresholds for surface and intracellular markers were determined using unstained and isotype control samples acquired in each experiment. Cells with fluorescence intensity exceeding background levels were classified as positive. Specifically, cut-off values were calculated as the mean fluorescence intensity (MFI) of the isotype control plus two standard deviations ($\text{Mean} \pm 2 \times \text{SD}$), ensuring an objective discrimination between negative and positive populations. In addition to the percentage of positive cells, MFI values were quantified to validate expression patterns and ensure consistency across CLL patient samples and MEC-1 cells.

2.4.4. Cell stimulation and phosphorylation of intracellular protein kinases

2×10^6 cells of CD180⁺MD-1⁺ CLL samples were selected and stimulated with anti-CD180 mAb (eBioscience™, clone G28–8, catalog # MA1–10125) for 30 min at 37°C and 5 % CO₂. CD150⁺ CLL samples were selected and stimulated with anti-CD150 mAb (eBioscience™, clone IPO-3, catalog # MA1–7626), whilst double-positive CD180⁺CD150⁺ CLL samples were treated with a combination of anti-CD180 and anti-CD150 mAbs under the same conditions. Unstimulated cells were used as controls. Following stimulation, cells were collected, washed as above and stained with FITC anti-CD19 and PE-Cy7 anti-CD5 mAbs, washed, fixed, and permeabilized using the Fix&Perm Kit (BD Biosciences). Subsequently, cells were stained with one of the

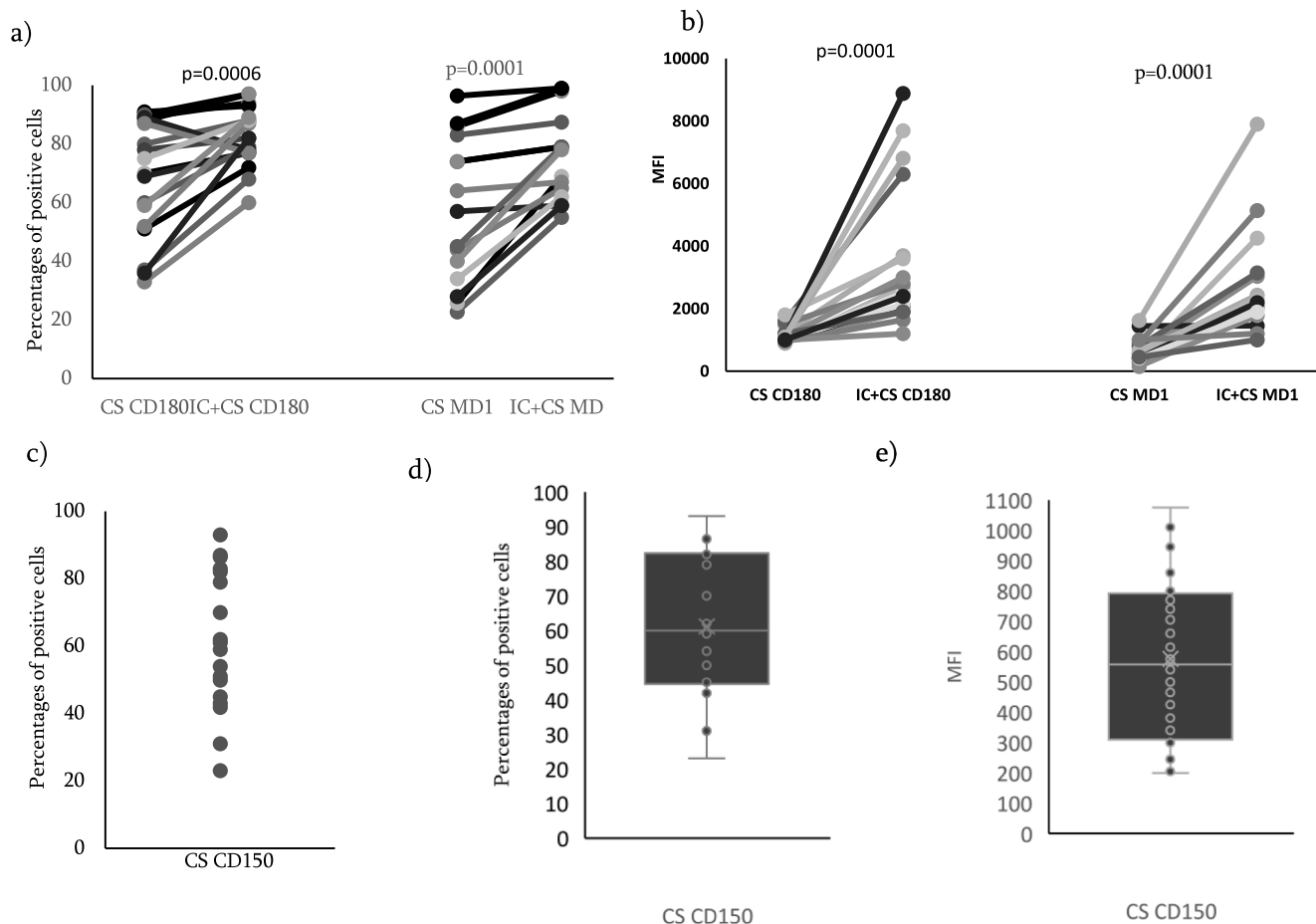


Fig. 1. Cell surface and intracellular expression of CD180, MD-1, and CD150 in CLL cells. a) Cell surface (CS) and intracellular (IC + CS) expression of CD180 and MD-1 in CLL cells (CS CD180: $68.5 \pm 10.0\%$, $n = 18$; IC+CS CD180: $82.5 \pm 5.5\%$, $n = 18$, $p = 0.0006$; CS MD-1: $59.4 \pm 9.8\%$, $n = 10$; IC+CS MD-1: $76.5 \pm 11.8\%$, $n = 15$, $p = 0.0001$). b) Cell surface (CS) and intracellular (IC + CS) expression of CD180 and MD-1 in CLL cells (CS CD180 MFI: 1139 ± 251 , $n = 18$; IC+CS CD180 MFI: 3356 ± 2410 , $n = 18$, $p = 0.00013$; CS MD-1 MFI: 694 ± 412 , $n = 15$; IC+CS MD-1 MFI: 2610 ± 1930 , $n = 15$, $p = 0.0001$). c) and b) Cell surface (CS) expression of CD150 in CLL cells (CS CD150: $61.1 \pm 10.0\%$, $n = 18$). e) Cell surface (CS) expression of CD150 in CLL cells (CS CD150: 552 ± 210 , $n = 18$). Differences were determined by paired *t*-test. PBMCs – peripheral blood mononuclear cells; CS – cell surface; IC – intracellular; MFI – mean fluorescence intensity.

following antibodies: PE anti-pAKT (BD Phosflow™, clone pS473, catalog # 560378) and APC anti-p-p38MAPK (eBioscience™, clone Thr180, Tyr182, catalog # 17-9078-42) at 4°C for 30 min, followed by flow cytometry analysis. The results were expressed as the percentages of positive cells in the CD19⁺CD5⁺ cell population.

2×10^6 MEC-1 cells were cultured for 24 or 48 h prior to stimulation. These time points were selected based on the immunophenotyping data (not shown), which indicated peak CD180 expression between 24–48 h. Cells were stimulated as described above (anti-CD180, anti-CD150, or both antibodies in combination) for 30 min at 37°C and 5% CO₂. Unstimulated cells were used as controls.

After stimulation, MEC-1 cells were fixed, permeabilized and stained with either PE anti-pAKT or APC anti-p-p38MAPK mAbs. Flow cytometry analysis was performed, and the data were expressed as percentages of positive cells.

2.5. Statistical analysis

Where appropriate, data were analyzed using the Mann–Whitney *U* test for unpaired data, paired *t*-test for matched samples, and one-way ANOVA for multiple comparisons. Data are presented as mean $\pm$ standard deviation (SD) for population samples to illustrate variability, and mean $\pm$ standard error of the mean (SEM) for cell line experiments to

indicate measurement precision. Statistical significance was considered at $p < 0.05$. All analyses were performed using Microsoft Excel and GraphPad Prism 10.

3. Results

CD180 and MD-1 expression in CLL samples was assessed as cell surface (CS) or intracellular and cell surface (IC+CS). The CS expression of both receptors was found to be heterogeneous, as expected and reported by us earlier (Fig. 1a, b) [8]. Positivity thresholds were defined according to the flow cytometry gating strategy described in the Methods section (Mean $\pm 2 \times$ SD of isotype control; see Supplementary Figure 1). Importantly, permeabilization significantly increased the percentages of cells positive for CD180 (IC+CS) across cell samples, including those with high surface levels ($p = 0.0006$; Fig. 1a) thus indicating that there is a substantial intracellular CD180 expression. A similar increase was observed for MD-1 ($p = 0.0001$; Fig. 1a), likewise pointing to a significant intracellular component. CD150 expression was assessed only on the cell surface and was also heterogeneous across CLL samples (Fig. 1b), showing a pattern broadly comparable to CD180 and MD-1 CS expression. This was further demonstrated by a positive correlation between CD150 and CD180 ($r = 0.7484$, $p = 0.0079$; Fig. 2a). We also confirmed this correlation in a different group of patients at a

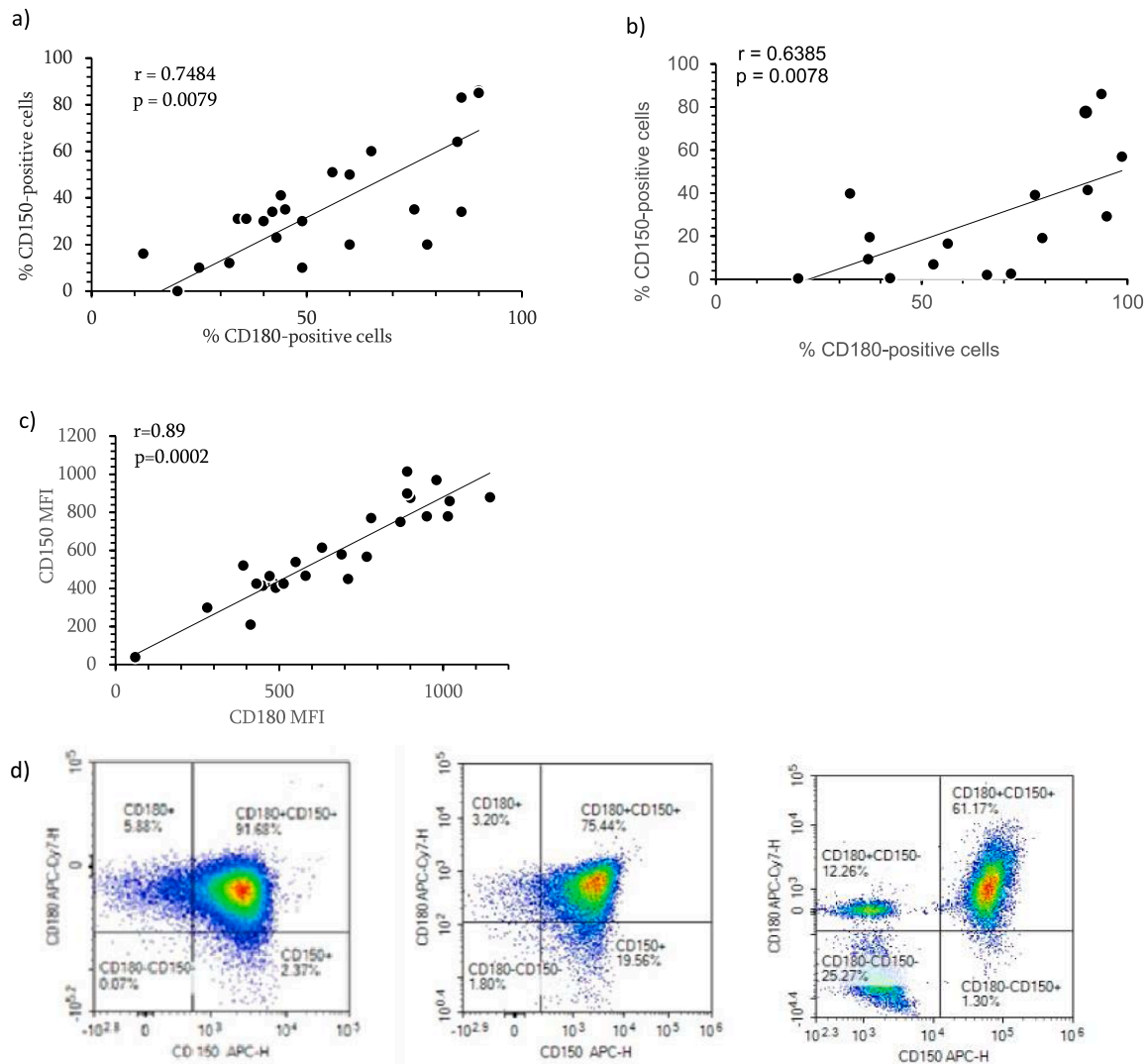


Fig. 2. The correlation between CD180 and CD150 expression in CLL cells. a) A positive correlation between CD150 and CD180 expression was observed in PBMCs from CLL patients ($n = 24$; $r = 0.7484$, $p = 0.0079$). b) This correlation was confirmed in an independent cohort of CLL patients from a different centre in the UK ($n = 16$; $r = 0.6385$, $p = 0.0078$). c) Correlation between the mean fluorescence intensities (MFIs) of CD150 and CD180 in the first cohort ($n = 24$; $r = 0.89$, $p = 0.0002$) indicates that these molecules are often co-expressed within individual CLL cells. d) Representative flow cytometry dot plots from individual patients show double positive, double negative, and single positive populations, illustrating coordinated expression across CLL cells. PBMCs, peripheral blood mononuclear cells; mAbs, monoclonal antibodies; CS, cell surface; IC, intracellular; MFI, mean fluorescence intensity.

different centre in the UK ($r = 0.6385$, $p = 0.0078$; Fig. 2b). As shown in Fig. 2a–d, CD150 and CD180 expression is positively correlated within individual CLL cells.

This is supported by MFI correlations ($r = 0.89$, $p = 0.0002$; Fig. 2c) and representative dot plots illustrating double positive, double negative, and single positive populations (Fig. 2d), highlighting coordinated expression across CLL cells.

PBMCs were stained with anti-CD19 and anti-CD5, and either anti-CD180 or anti-CD150 PE mAbs. The expression of each receptor was measured using flow cytometry relative to the isotype control on CD19⁺CD5⁺ populations and presented as the percentages of positive cells. Correlation between the two markers was assessed based on both the percentages of positive cells and parallel changes in MFI, as reflected in the distribution of double positive and double negative populations.

Due to the heterogeneity of CD180 and MD-1 CS expression, we next assessed whether CD180^{high} and CD180^{low} CLL cells expressed different levels of MD-1. Based on the FC profiles, the threshold for high/low levels was set at 60 % of positive CLL cells for CD180 and CD150 (Fig. 3). This cutoff was chosen as it reflects the mean expression level in our dataset and allows balanced grouping of samples. As expected, CS and

IC+CS levels of MD-1 were significantly higher on CD180^{high} compared to CD180^{low} subset (Fig. 4). Interestingly, CS MD-1 and IC+CS MD-1 expression was significantly elevated in CD150^{high} compared to CD150^{low} subset (Fig. 5), suggesting a direct correlation between the expression of MD-1 with both CD180 and CD150.

To further explore the heterogeneity of CD180 and CD150 expression in CLL, we stratified patient samples according to CD38 and ZAP70 status and assessed both the percentage of positive cells and mean fluorescence intensity (MFI).

To examine these dynamics in more detail, we analyzed the cell surface and intracellular expression of CD180 and MD-1 in MEC-1 cells over time following serum starvation (Fig. 7)

CS CD180 significantly increased after 24 h ($p = 0.009$) and 48 h ($p = 0.006$), compared to 0 h. At 72 h, CS CD180 expression returned to levels similar to those at 0 h ($p = 0.15$). Intracellular and surface CD180 (IC+CS CD180) expression followed a comparable pattern but increased more gradually, peaking at 48 h ($p = 0.008$). At 72 h, IC+CS CD180 levels declined ($p = 0.003$) yet remained significantly higher than CS CD180 at the same time point ($p = 0.03$) (Fig. 7a).

CS MD-1 expression also significantly increased at 24 h ($p = 0.001$),

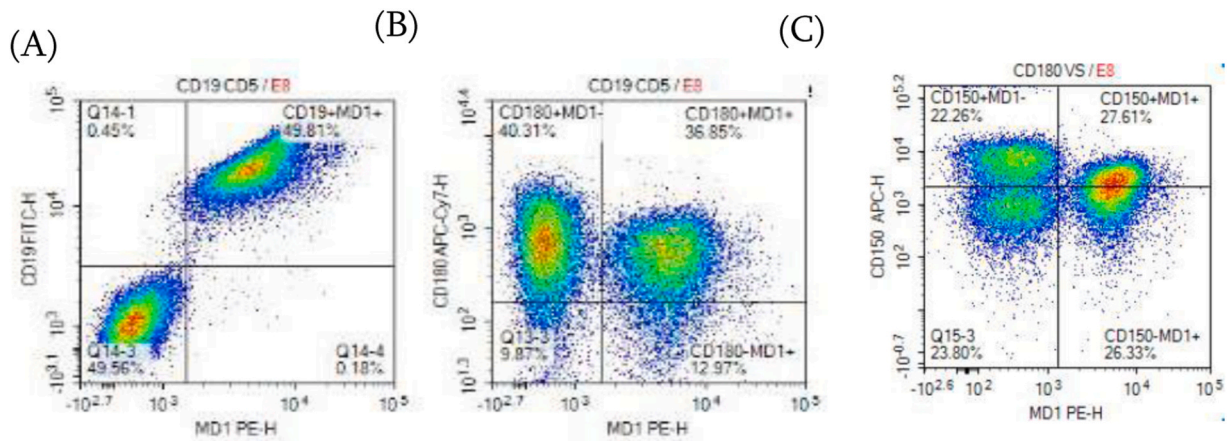


Fig. 3. Flow cytometry analysis of MD-1 expression in CD19⁺CD5⁺B cell subpopulations: (A) Representative dot plot showing the gating strategy used to define MD-1⁺ and MD-1⁻ subsets within the CD19⁺CD5⁺ population, based on PE-conjugated anti-MD-1 antibody. MD-1 gating was based on comparison with isotype control staining and consistent fluorescence thresholds across samples. (B) CD180 vs MD-1 co-expression. CD180 expression exhibited a unimodal but heterogeneous distribution with a distinct brighter shoulder, suggesting a subset of CD180^{high} cells. This gating approach was consistently applied across samples. (C) CD150 vs MD-1 expression. CD150 displayed a bimodal distribution, enabling direct gating into CD150^{high} and CD150^{low} subsets. These populations were used in subsequent signaling and phosphorylation analysis.

but subsequently decreased at 48 h ($p = 0.003$) and further at 72 h ($p = 0.0004$). IC+CS MD-1 expression followed a similar trend, rising more sharply at 24 h ($p = 0.003$), then gradually declining at 48 h ($p = 0.02$) and 72 h ($p = 0.017$), relative to the 24-hour peak, indicating that MD-1 expression precedes CD180 expression in actively cycling MEC-1 cells. Notably, IC+CS MD-1 levels remained significantly higher than CS MD-1 at both 24 h ($p = 0.007$) and 72 h ($p = 0.003$) (Fig. 7a).

In contrast, cell surface CD150 expression was high at all time points in actively cycling MEC-1 cells, slightly decreasing at 48 h compared to the 24-hour cultures ($p = 0.05$) and 72 h ($p = 0.03$) (Fig. 7b).

Stimulation of CD180⁺MD-1⁺ CLL samples with anti-CD180 revealed distinct signaling subsets, including AKT-signalers (AKT-S), p38MAPK-signalers (p38MAPK-S), non-signalers (NS), and dual AKT/p38MAPK signalers (DS) as reported by us before (Fig. 8a and b).

Notably, all cases identified as AKT-S were CD180^{high}/CD150^{low}, underscoring a potential link between low CD150 expression and preferred AKT signaling (Fig. 9).

Interestingly, combined stimulation of CD180^{high}MD-1^{high}CD150^{high}CLL cells with anti-CD180 and anti-CD150 mAbs did not lead to any gain of function expressed by the enhanced phosphorylation of AKT or p38MAPK compared to the stimulation with sole antibodies (Fig. 11a), as confirmed by one-way ANOVA ($p = 0.05$ for AKT and $p = 0.06$ for p38MAPK).

However, analysis of mean fluorescence intensity (MFI) revealed that the combined stimulation significantly increased AKT phosphorylation ($p = 0.002$) and p38MAPK MFI ($p = 0.009$) (Fig. 11b).

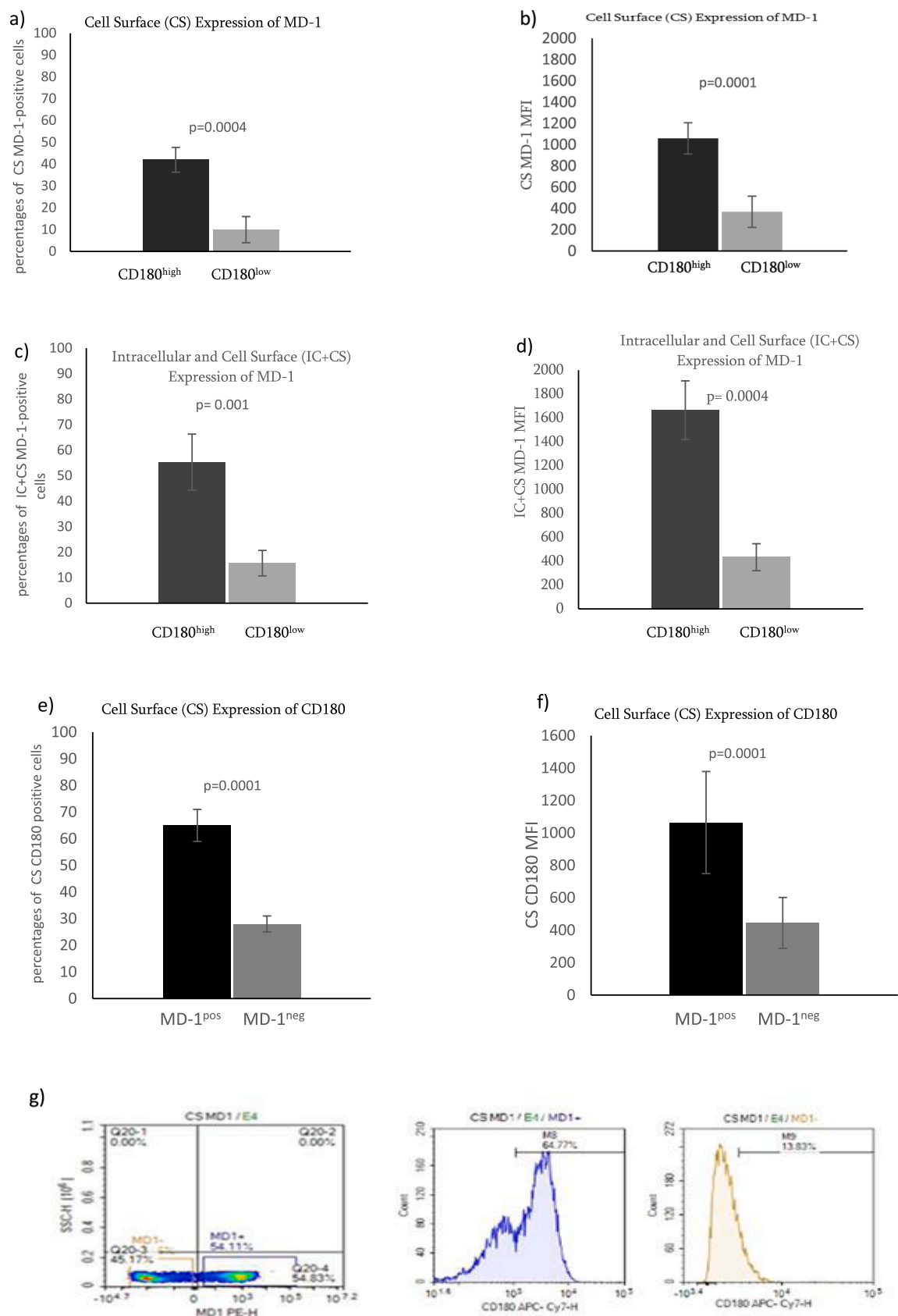
Despite substantial expression of CS CD180/CS MD-1 (Fig. 7a) and CS CD150 (Fig. 7b) at the 24 h time point of synchronized MEC-1 cells, stimulation with anti-CD180, anti-CD150, or their combination failed to induce phosphorylation of AKT or p38 MAPK, as demonstrated by both the percentages of positive cells and the corresponding MFI values (Fig. 12a, b).

At 48 h, stimulation with anti-CD150 ($p = 0.04$) or combined anti-CD180 + anti-CD150 ($p = 0.017$) significantly decreased p-p38 MAPK expression, reflected by reduced percentages of positive cells and lower MFI values compared with unstimulated controls (Fig. 12c, d). Conversely, at the same time point, stimulation with anti-CD150 ($p = 0.005$) or combined anti-CD180 + anti-CD150 ($p = 0.002$) markedly increased both the proportion and MFI of pAKT⁺ cells relative to controls (Fig. 12c, d).

4. Discussion

Our results demonstrate heterogeneous expression of CD180, MD-1, and CD150 in CLL cells and the dynamic modulation of these molecules in actively cycling MEC-1 cells. The significant increase in intracellular CD180 expression following permeabilization ($p = 0.0006$; Fig. 1a), together with a higher mean fluorescence intensity (MFI) ($p = 0.0001$; Fig. 1b), highlights that a substantial portion of CD180 is localized within the cytoplasm. This finding is consistent with our previous reports indicating that CD180 undergoes intracellular trafficking, which may play a crucial role in its surface expression and signaling capacity [8]. A similar significant increase was observed for MD-1 ($p = 0.0001$; Fig. 1a), with elevated MFI ($p = 0.0001$; Fig. 1b). The high intracellular presence of MD-1 supports its role as a chaperone molecule facilitating CD180 transport to the plasma membrane, suggesting that intracellular storage and trafficking of both CD180 and MD-1 are critical for the surface expression of CD180. CD150 expression in CLL samples was heterogeneous (Fig. 1c, d, e), highlighting phenotypic diversity of these cells.

The elevated CS MD-1 and IC+CS MD-1 expression in CD180^{high} and CD150^{high} populations compared to the CD180^{low} and CD150^{low} populations (Figs. 3, 4 and 5) further indicates a strong association between MD-1 and CD180/CD150 co-expression. It should be noted that the bimodal distribution of CD150 surface levels is predominantly observed in MD-1^{neg} cells, whereas MD-1^{pos} cells show a more uniform distribution, suggesting that MD-1 stabilizes CD150 expression (Fig. 3C). The threshold used to define the high-fluorescence subpopulation of CD180^{high} cells was based on fluorescence intensity relative to isotype controls (approximately 60 % of positive cells) and applied consistently across all patients. This finding may imply that MD-1 plays a pivotal role in the stability and surface presentation of CD180, consistent with previous studies demonstrating that MD-1 is required for CD180 surface expression [10,11,14]. Moreover, the markedly increased MFI of MD-1 in CD180^{high} and CD150^{high} subpopulations reflects not only a greater fraction of protein-positive cells but also an elevated per-cell protein density, which may potentiate receptor-mediated signaling (Fig. 4b, d and Fig. 5b, d). Notably, the positive co-correlation between the expression of CD180 and CD150 (Fig. 2a-c) suggests a potential crosstalk between these receptors, which could modulate intracellular signaling pathways [5]. The preferential expression of MD-1 in CD180^{high} and CD150^{high} cells suggests that MD-1 may act as a molecular bridge facilitating the co-localization and functional interplay between CD180



(caption on next page)

Fig. 4. Cell surface and intracellular expression of MD-1 in CD180^{high} and CD180^{low} CLL cells and CD180 expression in MD-1^{pos} and MD-1^{neg} CLL cells. a) Cell surface expression of MD-1 in CD180^{high} (42.0 ± 10.0 %, $n = 18$) and CD180^{low} (10.7 ± 9.3 %, $n = 18$) CLL cells ($p = 0.001$). b) Cell surface expression of MD-1 in CD180^{high} (1060.1 ± 53 , $n = 18$) and CD180^{low} (369 ± 137 , $n = 18$) CLL cells ($p = 0.0001$), MFI. c) Intracellular and cell surface expression of MD-1 in CD180^{high} (55.3 ± 11.0 %, $n = 18$) and CD180^{low} (14.0 ± 4.9 %, $n = 18$) CLL cells ($p = 0.0003$). d) Intracellular and cell surface expression of MD-1 in CD180^{high} (1664.1 ± 248 , $n = 18$) vs CD180^{low} (433.9 ± 103 , $n = 18$), MFI ($p = 0.0004$). e) Expression of CS CD180 in MD-1^{pos} (65.0 ± 6.0 %, $n = 18$) and MD-1^{neg} (28.0 ± 3.0 %, $n = 18$) CLL cells ($p = 0.0001$). f) Expression of CS CD180 in MD-1^{pos} (MFI: 1064 ± 315 , $n = 18$) and MD-1^{neg} (MFI: 445 ± 157 , $n = 18$) CLL cells ($p = 0.0001$). g) Representative dot plot and histograms showing gating of MD-1^{pos} and MD-1^{neg} subsets within CD19⁺CD5⁺CLL cells and the corresponding CD180 expression in each subset. The gating threshold for MD-1⁺ cells was defined based on isotype control fluorescence and applied consistently across all samples. Differences were determined by paired *t*-test. CS – cell surface; IC – intracellular; MFI – mean fluorescence intensity.

and CD150 in CLL cells.

To further explore the heterogeneity of CD180 and CD150 expression in CLL, patient samples were stratified according to CD38 and ZAP70 status. Both the percentage of positive cells and MFI were assessed (Fig. 6). CD38^{pos} and ZAP70^{pos} CLL cells exhibited lower CD180 and CD150 expression compared to CD38^{neg} and ZAP70^{neg} cells, respectively (Fig. 6a–d). Similarly, MFI analysis confirmed reduced per-cell CD180 and CD150 abundance in CD38^{pos} and ZAP70^{pos} subsets (Fig. 6e–h). These data suggest that clinical-biological markers such as CD38 and ZAP70 are associated with differential surface expression of CD180 and CD150 in CLL cells. Importantly, this confirms our previous observations that CD180 expression on CLL cells is associated with favourable prognostic indicator M-CLL [2] and with improved overall survival [7].

The time-course experiments in MEC-1 cells revealed dynamic changes in CD180 and MD-1 expression over 72 h after serum starvation. The significant increase in CS CD180 and IC+CS CD180 expression at 24 h and 48 h, followed by a decline at 72 h (Fig. 7a), suggests a transient upregulation of CD180 during cell cycling. Similarly, CS MD-1 and IC+CS MD-1 expression peaked at 24 h and declined at later time points (Fig. 7a), supporting the idea that MD-1 expression is tightly regulated during cell proliferation. Notably, MD-1 expression peaks at 24 h in a cycling MEC-1 cell line, and precedes that of CD180 at 48 h once more indicating the importance of MD-1 in CD180 surface expression. It is also plausible that the decline in MD-1 expression precedes and causally contributes to the decrease in CD180 expression (Fig. 7a). These findings highlight the dynamic nature of CD180 and MD-1 expression in actively cycling CLL cells, which may reflect a co-ordinated regulation of these molecules during the cell cycle. Interestingly, CD150 surface expression remained consistently high across all time points, with only minor reductions at 48 h (Fig. 7b). Although CD150 surface expression is consistently high in the MEC-1 cell line, its expression across primary CLL samples is markedly heterogeneous (Fig. 1c–d) [6], likely contributing to inter-patient variability in CD150-mediated signaling. In contrast, CD180 displays temporal stability, maintaining consistent surface expression over a 24-month period [2]. This sustained expression suggests that CD180 may act as a stable signaling scaffold, whose functional output is modulated by the presence or absence of co-receptors such as CD150 and MD-1. Therefore, in CLL, the interplay between the stable CD180 and the variable CD150 may represent a mechanism through which differential signaling responses are regulated across patients.

CD180 stimulation revealed distinct signaling subsets, including AKT-signalers (AKT-S), p38MAPK-signalers (p38MAPK-S), non-signalers (NS), and dual AKT/p38MAPK signalers (DS) (Fig. 8a–d) as reported earlier [4]. The predominance of AKT signaling in CD180^{high}/CD150^{low} cells (Fig. 9) suggests that CD150 expression may alter the signaling balance towards AKT activation. This observation aligns with the hypothesis that CD150 provides additional regulatory signals that fine-tune downstream signaling pathways [5]. AKT signaling is often associated with pro-survival pathways in CLL, while p-p38MAPK activation has been linked to apoptosis [4]. Therefore, the differential signaling profiles of CD180^{high}CD150^{low} and

CD180^{high}CD150^{high} cells may have important implications for CLL cell survival and disease progression.

To determine whether CD150 itself could modulate signaling responses, we also analyzed the effect of stimulation with anti-CD150 (Fig. 10a–d). Similar to CD180 stimulation, AKT-S, p38MAPK-S, NS, and DS subsets were observed. This confirms that CD150 stimulation alone is also capable of inducing differential signaling responses, further supporting its functional role in shaping CLL signaling networks [5].

This strong correlation (Fig. 2a–c) suggests potential coordinated regulation between CD180 and CD150, implying that these receptors may function together in leukemic B cell biology. Notably, this association was independently validated using an external dataset [10], underscoring the biological robustness and reproducibility of our findings.

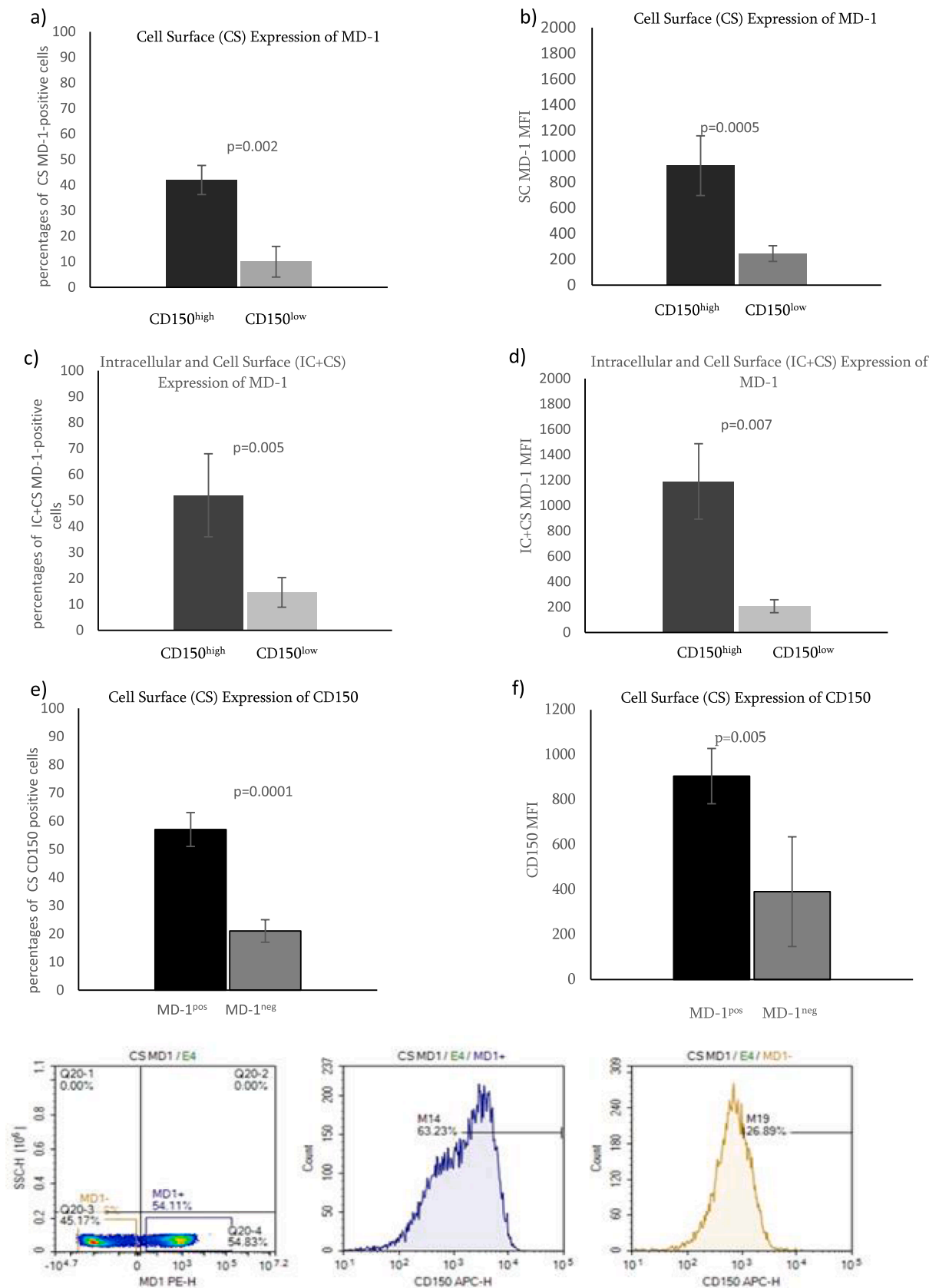
Both individual and combined stimulation of CD150 and CD180 induced moderate activation of pAKT and p38MAPK pathways in CLL cells (Fig. 11). However, no significant enhancement was observed with dual stimulation compared to individual stimulations, suggesting that CD150 and CD180 may act through partially overlapping or non-synergistic pathways in CLL cells. This finding is consistent with previous studies [5], which showed that co-engagement of surface receptors in B-cell malignancies promotes cell survival and proliferation. Activation of these signaling pathways may play an important role in CLL progression.

Importantly, analysis of mean fluorescence intensity (MFI) revealed that combined stimulation with anti-CD150 and anti-CD180 significantly increased p-AKT ($p = 0.002$) and p-p38MAPK MFI ($p = 0.009$) in CD180^{high}MD-1^{high}CD150^{high} CLL cells (Fig. 11b). These results indicate that, although the overall percentages of positive cells do not substantially change, the signaling intensity within individual cells is significantly enhanced, rather than an increase in the proportion of cells activated, potentially promoting CLL cell survival and progression.

Stratification of CLL cells according to CD38 and ZAP70 status revealed that cells positive for these prognostic markers consistently exhibited lower CD180 and CD150 expression and MFI (Fig. 6). This suggests that the clinical-biological context may influence the availability of these receptors and potentially modulate downstream signaling pathways, contributing to inter-patient variability in CLL.

Importantly, stimulation by anti-CD180, anti-CD150 or both did not induce appreciable phosphorylation of AKT or p38MAPK after 24 h of stimulation in MEC-1 cells, as reflected by both the percentages of positive cells and MFI (Fig. 12a, b). However, at 48 h, CD150 and combined CD180/CD150 stimulation significantly increased pAKT and dramatically decreased p-p38MAPK levels, as measured by both the percentages of positive cells and MFI (Fig. 12c, d). This signaling response is aligned with high CD180 expression at the 48 h time-point of MEC-1 cell culture supporting the hypothesis that CD180 particularly enhances AKT-mediated signaling and its high expression is essential for AKT phosphorylation, despite of the consistently high level of CD150 expression in MEC-1 cell line (Fig. 7b).

Differences in CD150-mediated signaling between primary CLL cells and MEC-1 cells may be attributable to differential CD180 expression



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Fig. 5. Cell surface and intracellular expression of MD-1 in CD150^{high} and CD150^{low} CLL cells; and CD150 expression in MD-1^{pos} and MD-1^{neg} CLL cells. a) Cell surface expression of MD-1 in CD150^{high} (39.0 ± 13.0 %, n = 18) and CD150^{low} (7.2 ± 4.1 %, n = 18) CLL cells (p = 0.002). b) Cell surface expression of MD-1 in CD150^{high} (MFI: 928 ± 232, n = 18) and CD150^{low} (MFI: 245 ± 61, n = 18) CLL cells (p = 0.0005). c) Intracellular and cell surface expression of MD-1 in CD150^{high} (51.3 ± 16.0 %, n = 18) vs CD150^{low} CLL cells (14.6 ± 5.7 %, n = 18, p = 0.005). d) Intracellular and cell surface expression of MD-1 in CD150^{high} (MFI: 1190 ± 297, n = 18) vs CD150^{low} (MFI: 207 ± 51, n = 18) CLL cells (p = 0.007). e) Expression of CD150 in MD-1^{pos} (57.0 ± 6 %, n = 18) and MD-1^{neg} (21.0 ± 4 %, n = 18) CLL cells (p = 0.0001). f) Expression of CD150 in MD-1^{pos} (MFI: 905 ± 123, n = 18) and MD-1^{neg} (MFI: 391 ± 244, n = 18) CLL cells. g) Representative dot plot and histograms showing gating of MD-1^{pos} and MD-1^{neg} subsets within CD19⁺CD5⁺ CLL cells and the corresponding CD150 expression in each subset. The gating threshold for MD-1⁺ cells was defined based on isotype control fluorescence and applied consistently across all samples. Differences were determined by paired t-test. CS – cell surface; IC – intracellular; MFI – mean fluorescence intensity.

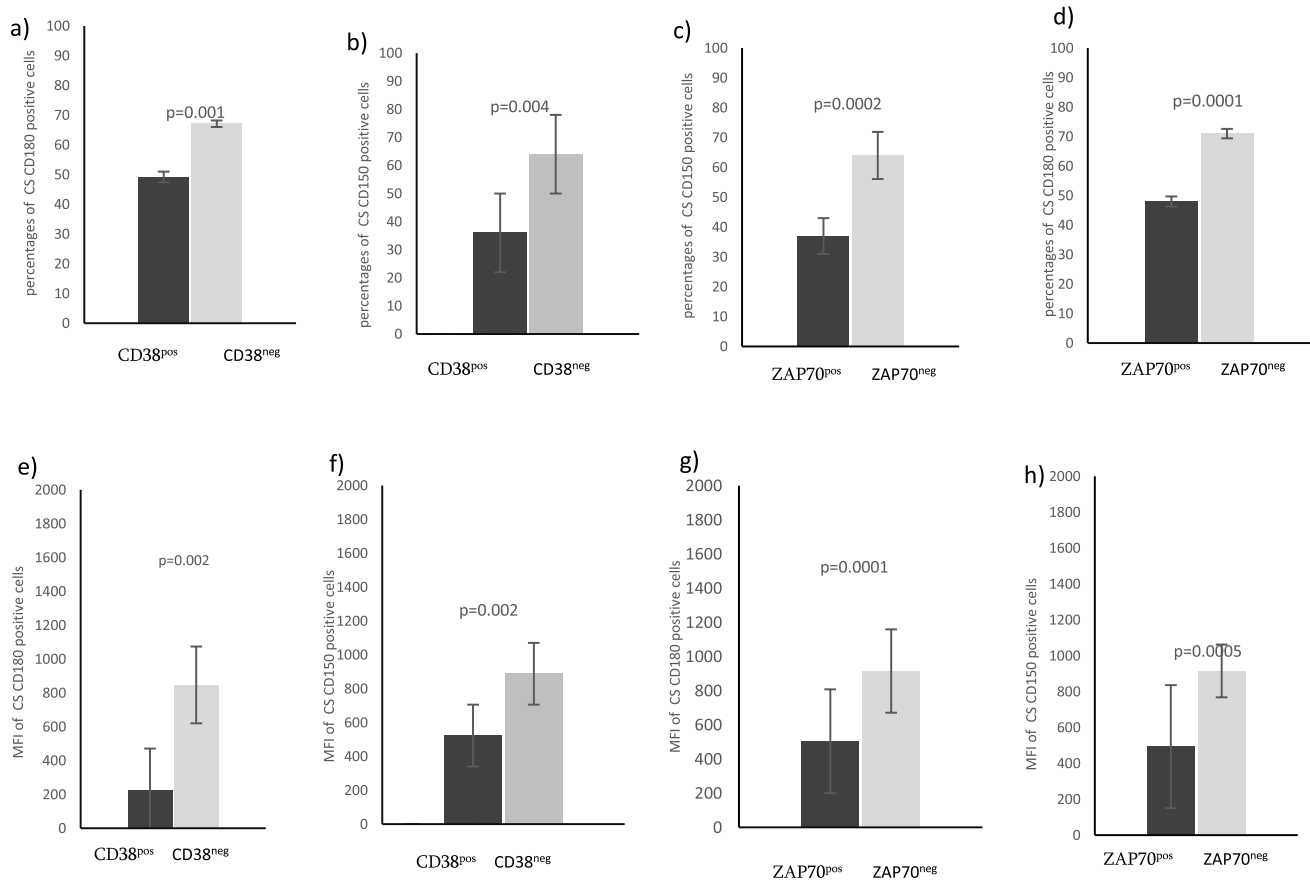


Fig. 6. Surface cell expression of CD180 and CD150 in CLL cells stratified by CD38 and ZAP70 status. (a, b) Percentages of CD180⁺ and CD150⁺ cells in CD38^{pos} versus CD38^{neg} CLL samples. CD38^{pos} cells show lower CD180 expression compared to CD38^{neg} cells (49.2 ± 1.8 % vs. 67.1 ± 1.1 %, n = 15 and 37, respectively) and reduced CD150 expression (36 ± 6 % vs. 64 ± 7 %, n = 9 and 15, respectively). (c, d) Percentages of CD180⁺ and CD150⁺ cells in ZAP70^{pos} versus ZAP70^{neg} CLL samples. ZAP70^{pos} cells display lower CD180 (48 ± 1.7 % vs. 71 ± 1.6 %, n = 19 and 33, respectively) and CD150 expression (37 ± 6 % vs. 64 ± 7.9 %, n = 8 and 16, respectively). (e, f) MFI of CD180 (e) and CD150 (f) in CD38^{pos} versus CD38^{neg} cells. CD38^{pos} cells show lower CD180 and CD150 MFI compared to CD38^{neg} cells (CD180: 227 ± 244 vs. 847 ± 227, n = 15 and 37; CD150: 523 ± 6 vs. 888 ± 7, n = 9 and 15). (g, h) MFI of CD180 (g) and CD150 (h) in ZAP70^{pos} versus ZAP70^{neg} cells. ZAP70^{pos} cells exhibit reduced CD180 and CD150 MFI compared to ZAP70^{neg} cells (CD180: 504 ± 304 vs. 915 ± 244, n = 19 and 33; CD150: 493 ± 343 vs. 915 ± 147, n = 8 and 16). Data are presented as mean ± SEM. Statistical significance between groups was determined using unpaired t-test (p-values indicated on graphs). CLL, chronic lymphocytic leukemia; MFI, mean fluorescence intensity; SEM, standard error of the mean; pos, positive; neg, negative; mAb, monoclonal antibody.

levels and broader cellular context [4,8].

Dual stimulation experiments further support a modulatory role for CD180 in CD150-mediated signaling: co-engagement of CD180 and CD150 results in enhanced AKT phosphorylation without a corresponding increase in p38MAPK activation compared to CD150 stimulation alone. These findings suggest that the level of CD180 expression may critically influence CD150-driven intracellular signaling dynamics, potentially shifting the balance between survival and stress/apoptosis pathways in CLL cells.

Overall, our findings highlight the intricate regulation of CD180, MD-1 and CD150 in CLL cells and suggest that CD180/CD150 co-expression may have important implications for intracellular signaling and disease progression. The differential signaling responses observed in CLL cells suggest that CD180/MD-1 and CD150 may play distinct yet complementary roles in modulating cell survival and apoptosis. Further studies are warranted to elucidate the functional significance of these molecules in CLL pathophysiology and their potential as therapeutic targets.

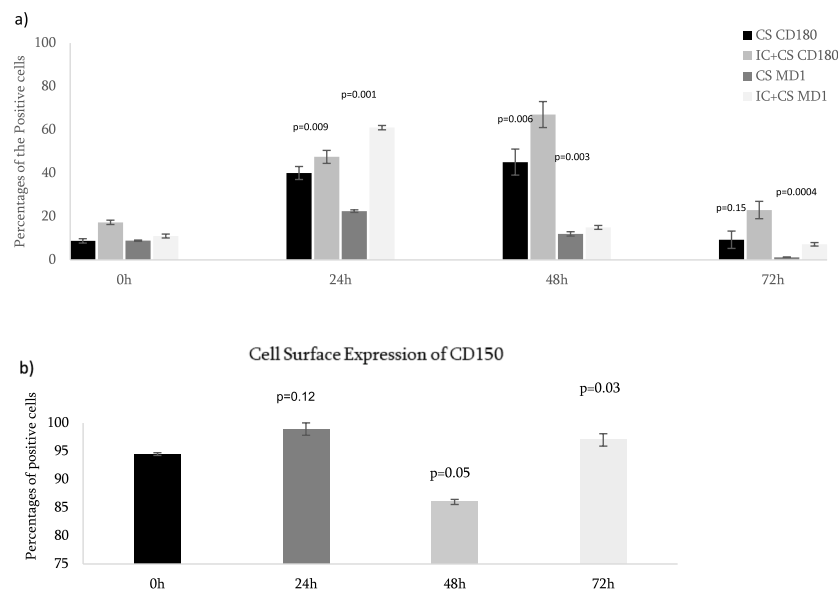
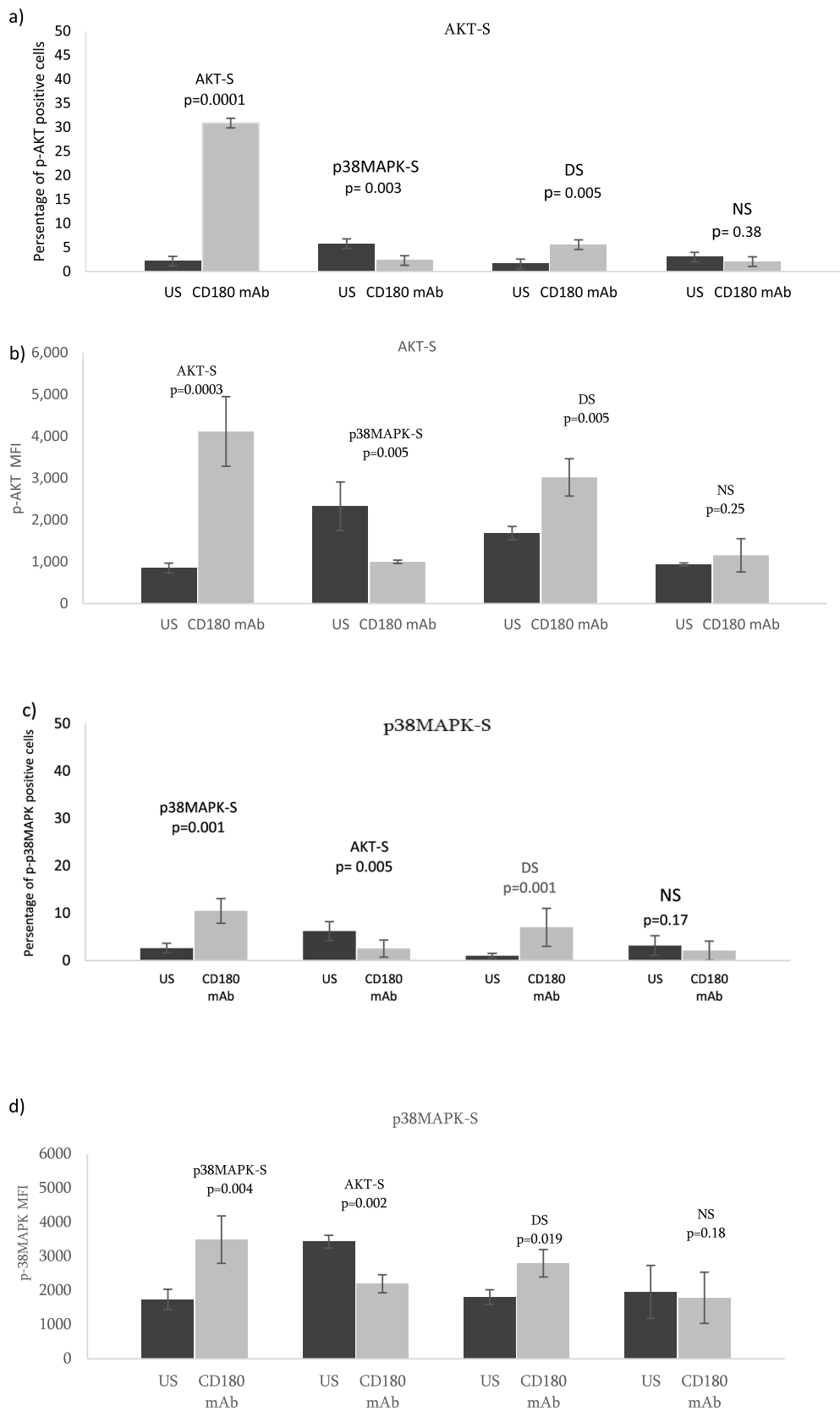


Fig. 7. Time-dependent expression of CD180, MD-1, and CD150 in MEC-1 cells significance (n = 8). a) Surface (CS) and intracellular and surface (IC+CS) expression of CD180 and MD-1 at 0 h, 24 h, 48 h, and 72 h of actively cycling MEC-1 cell culture. Statistical analysis was performed using ANOVA, followed by paired two-tailed *t*-test for comparisons between individual time points. b) Cell surface expression of CD150 at 0 h, 24 h, 48 h, and 72 h of actively cycling MEC-1 cell culture. CS – cell surface; IC – intracellular; MFI – mean fluorescence intensity.



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Fig. 8. Phosphorylation of AKT and p38MAPK in CLL cells following anti-CD180 stimulation. (a) AKT phosphorylation in CLL cells after anti-CD180 stimulation, shown for AKT signalers (AKT-S, $n = 9$), p38MAPK signalers (p38MAPK-S, $n = 10$), non-signalers (NS, $n = 11$), and dual AKT/p38MAPK signalers (DS, $n = 10$). (c) p38MAPK phosphorylation in CLL cells after anti-CD180 stimulation, shown for p38MAPK signalers (p38MAPK-S, $n = 10$), AKT signalers (AKT-S, $n = 9$), dual AKT/p38MAPK signalers (DS, $n = 10$), and non-signalers (NS, $n = 11$). Statistical differences were assessed using paired t -test. (b, d) Corresponding mean fluorescence intensity (MFI) values for AKT (c) and p38MAPK (d) support the phosphorylation patterns observed in (a) and (c). MFI values were analyzed using paired t -test. (b, d) Corresponding mean fluorescence intensity (MFI) values for AKT (b) and p38MAPK (d) support the phosphorylation patterns observed in (a) and (c) and were analyzed using paired t -test. MFI, mean fluorescence intensity; US, unstimulated; AKT-S, AKT signalers; p38MAPK-S, p38MAPK signalers; DS, dual AKT/p38MAPK signalers; NS, non-signalers.

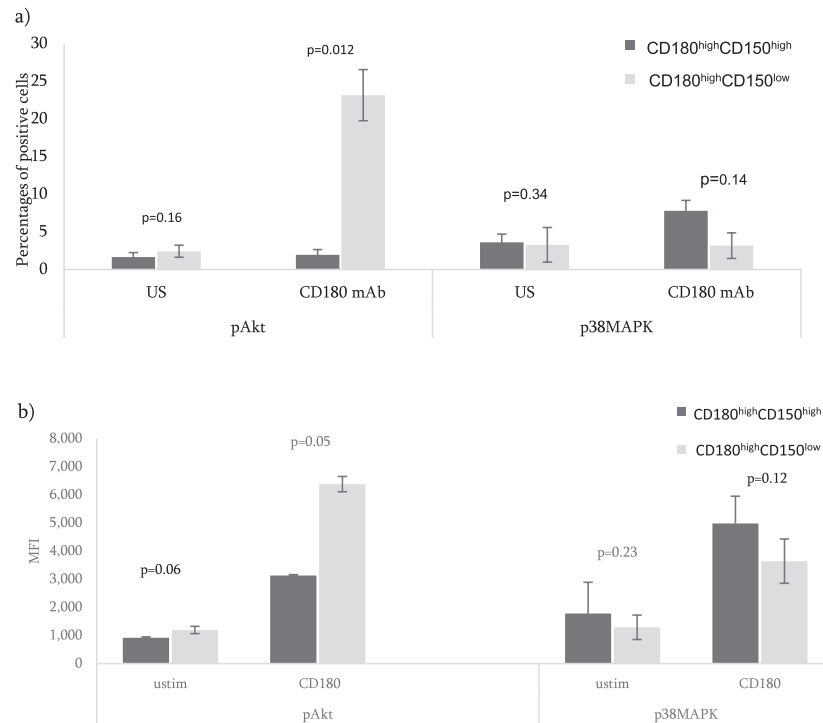


Fig. 9. Anti-CD180 induced phosphorylation of AKT and p38MAPK in CD180^{high}CD150^{high} and CD180^{high}CD150^{low} CLL subsets. a) CD180^{high}CD150^{high} subset: AKT (US 2.6 ± 0.6 %, CD180 mAb 1.9 ± 0.7 %, $n = 16$); p38MAPK (US 2.6 ± 1.4 %, CD180 mAb 7.8 ± 0.9 %, $n = 16$). CD180^{high}CD150^{low} subset: AKT (US 2.4 ± 1.6 %, CD180 mAb 23.2 ± 6.7 %, $n = 11$); p38MAPK (US 4.3 ± 1.4 %, CD180 mAb 2.1 ± 1.8 %, $n = 11$). b) CD180^{high}CD150^{high} subset: AKT MFI (US 920 ± 30 , CD180 mAb 3133 ± 622 , $n = 16$); p38MAPK MFI (US 1783 ± 1106 , CD180 mAb 4984 ± 3641 , $n = 16$). CD180^{high}CD150^{low} subset: AKT MFI (US 1195 ± 272 , CD180 mAb 6381 ± 1902 , $n = 11$); p38MAPK MFI (US 1289 ± 434 , CD180 mAb 3641 ± 788 , $n = 11$). Statistical differences were determined using paired t -test. Positive cells were defined relative to isotype controls (mean $\pm 2 \times$ SD). US – unstimulated; MFI – mean fluorescence intensity.

5. Conclusions

Downregulation of surface and cytoplasmic expression of MD-1 is associated with a reduction in CD180 surface expression in CLL cells, which could impair CD180-mediated signaling and thus contribute to disease progression. This finding supports the hypothesis that MD-1 plays a pivotal role in regulating CD180 availability and signaling capacity, particularly in the context of CLL.

The inverse relationship between CD150 expression and CD180-mediated AKT signaling in CLL highlights the complex regulatory mechanisms involved in CLL cell survival and proliferation. Our results suggest that CD150 expression may modulate the signaling pathways activated by CD180, influencing the heterogeneity of CLL cell responses.

Importantly, CD150 stimulation alone was also capable of inducing distinct signaling profiles. This suggests that CD150 functions as a co-receptor and an independent modulator of intracellular signaling. CD150-mediated signaling may depend on the expression levels of

CD180, further highlighting the dynamic interplay between these receptors.

These findings are consistent with previous research on the CD180/CD150/MD-1 axis and offer further validation of the importance of these molecules in CLL pathophysiology.

CCRediT authorship contribution statement

Tamar Tsertsvadze: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kurtis Edwards:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Sarah John-Olabode:** Writing – review & editing, Formal analysis. **Irina Datikashvili-David:** Writing – review & editing, Resources, Methodology, Funding acquisition, Data curation. **Ani Bilanishvili:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data

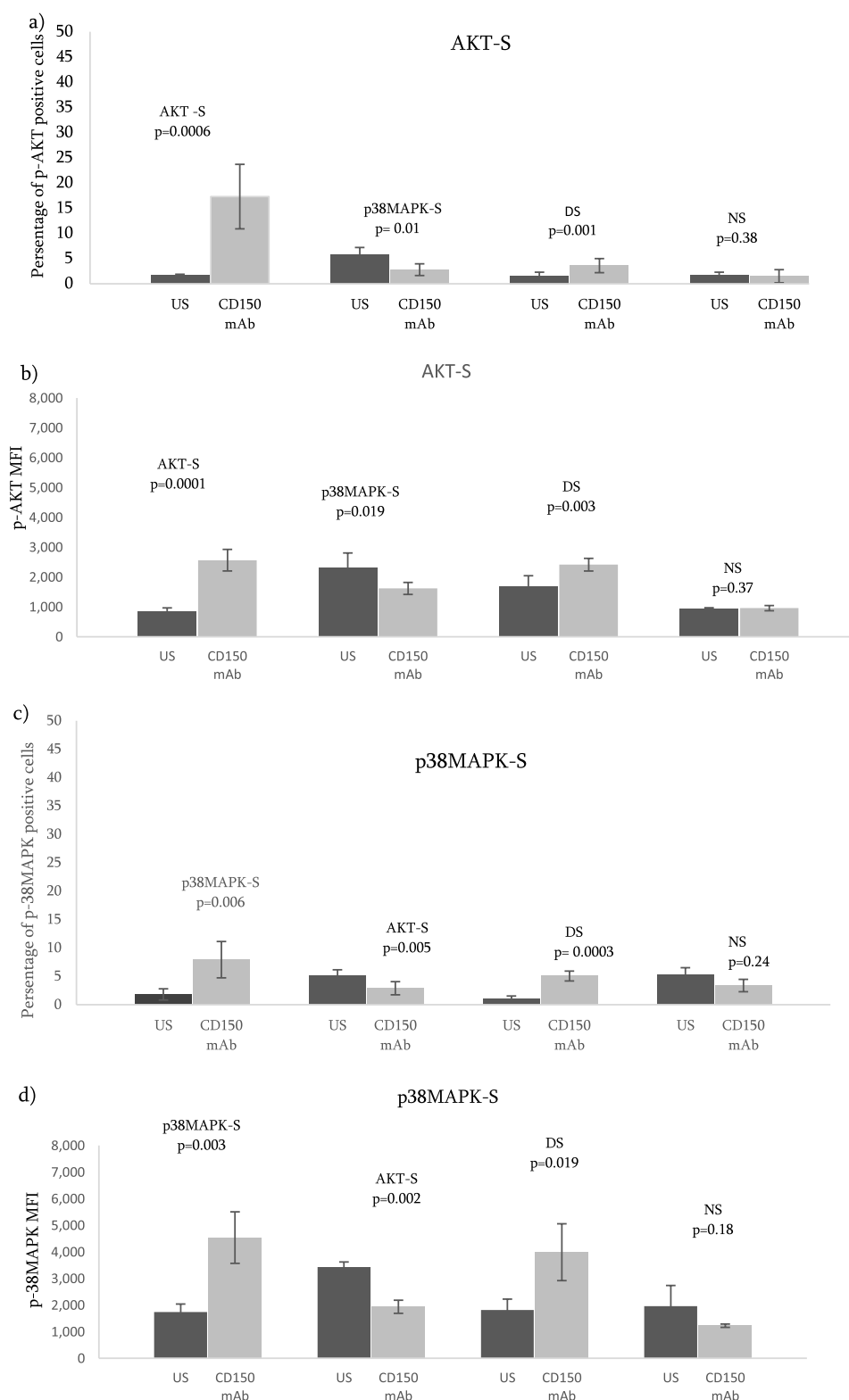


Fig. 10. Phosphorylation responses of AKT and p38MAPK in CLL cells following anti-CD150 stimulation. a) AKT phosphorylation in CLL cells after anti-CD150 stimulation, shown for AKT signalers (AKT-S, n = 8), p38MAPK signalers (p38MAPK-S, n = 9), non-signalers (NS, n = 10) and double AKT/p38MAPK signalers (DS, n = 9). c) p38MAPK phosphorylation in CLL cells after anti-CD150 stimulation, shown for p38MAPK signalers (p38MAPK-S, n = 9), AKT signalers (AKT-S, n = 8), dual AKT/p38MAPK signalers (DS, n = 9), and non-signalers (NS, n = 10). Statistical comparisons were made using paired *t*-test. (b, d) Corresponding mean fluorescence intensity (MFI) values for AKT (c) and p38MAPK (d) support the phosphorylation patterns observed in (a) and (c) and were analyzed using paired *t*-test. MFI, mean fluorescence intensity; US, unstimulated; AKT-S, AKT signalers; p38MAPK-S, p38MAPK signalers; DS, dual AKT/p38MAPK signalers; NS, non-signalers.

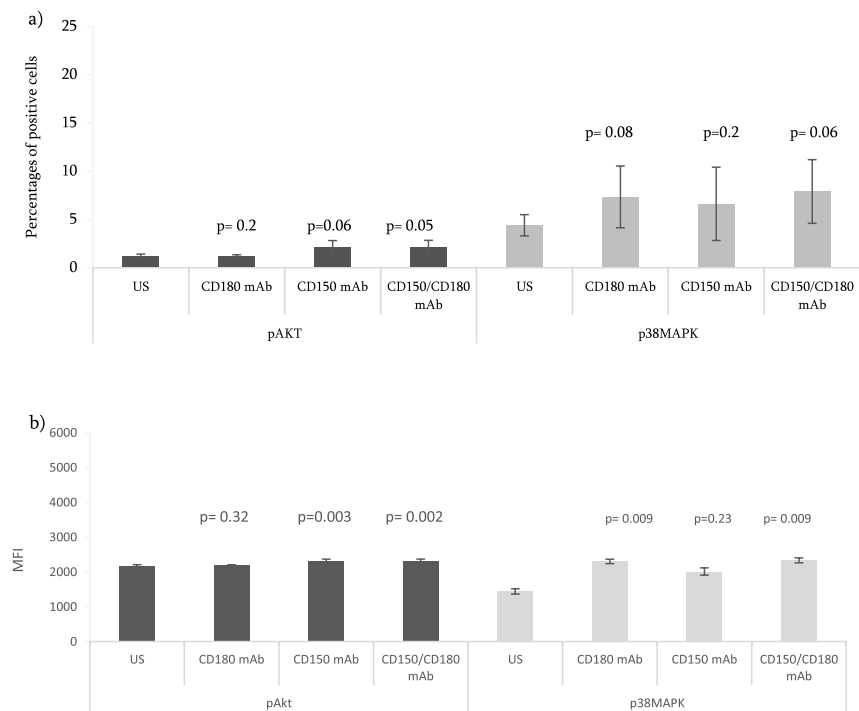


Fig. 11. Phosphorylation of AKT and p38MAPK in CD180^{high}MD-1^{high}CD150^{high} CLL cells following single and combined stimulation. a) Percentages of AKT and p38MAPK phosphorylation levels in CD180^{high}MD-1^{high}CD150^{high}CLL cells following single and combined stimulation. b) MFI of AKT and p38MAPK phosphorylation levels in CD180^{high}MD-1^{high}CD150^{high}CLL cells following single and combined stimulation. Positive cells were defined using isotype controls (mean $\pm$ 2 $\times$ SD). Statistical significance was assessed by one-way ANOVA followed by pairwise *t*-test; p-values are indicated in the main text. MFI values illustrate the observed phosphorylation patterns. MFI, mean fluorescence intensity; US, unstimulated; mAb, monoclonal antibody.

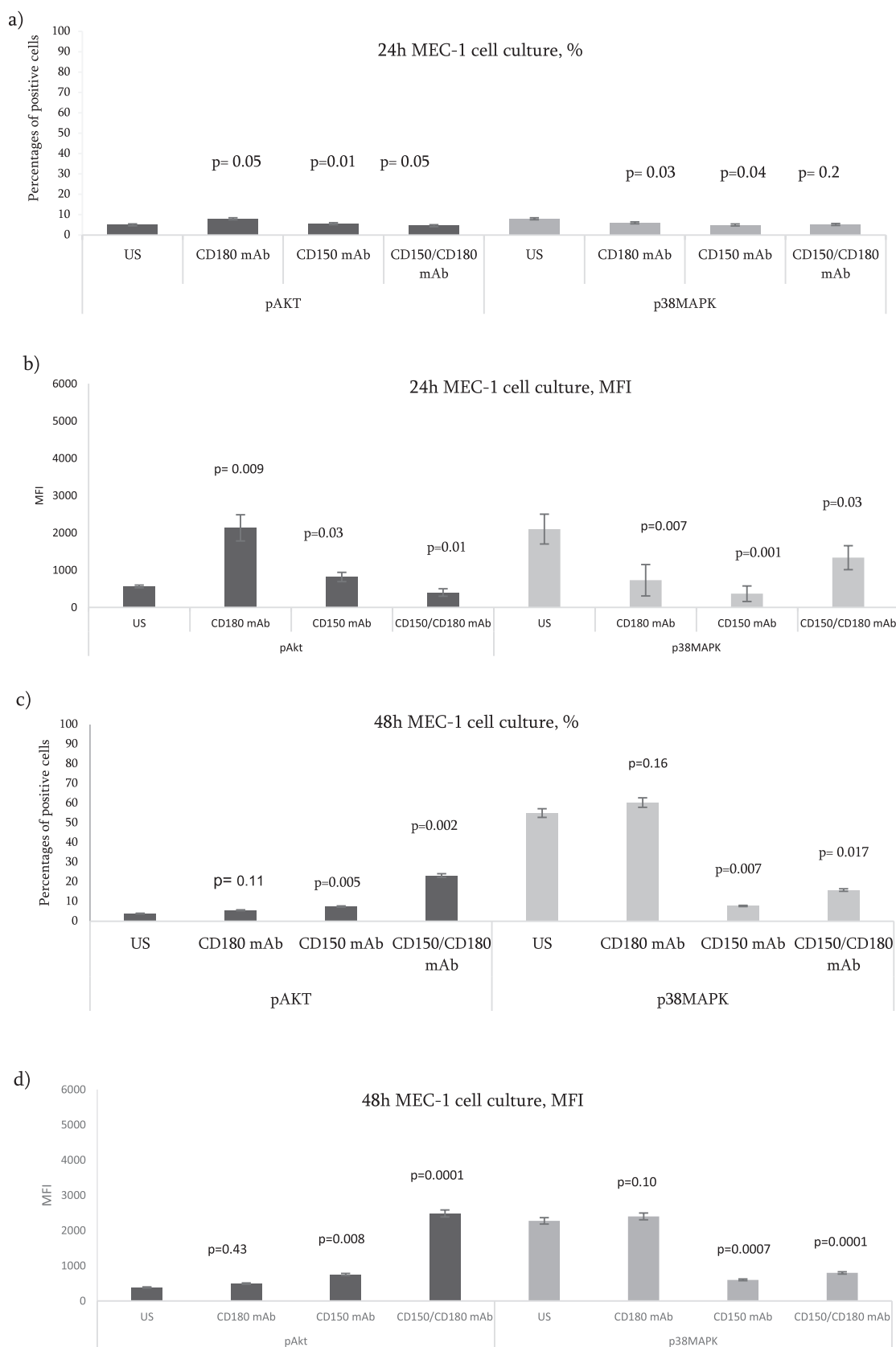


Fig. 12. Phosphorylation of AKT and p38MAPK in MEC-1 cells, expressed as both the percentages of positive cells and mean fluorescence intensity (MFI), in unstimulated (US) cultures and following stimulation with anti-CD180, anti-CD150, or both antibodies together. (a, c) Percentages of pAKT and p-p38MAPK cells at 24 h and 48 h; (b, d) Corresponding MFI at the same time points. Statistical comparisons were made using the Mann–Whitney *U* test. Positive cells were defined using isotype controls (mean $\pm$ 2 $\times$ SD). MFI, mean fluorescence intensity; US, unstimulated; mAb, monoclonal antibody.

curation. **John Murphy:** Writing – review & editing, Formal analysis. **Emanuela V Volpi:** Writing – review & editing, Formal analysis. **Peter M Lydyard:** Writing – review & editing, Formal analysis, Conceptualization. **Nina Porakishvili:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Author contributions

Study conception and design: TT, IDD, AB, PML, NP. Sample collection governance: TT, IDD, NP. Experiments: TT, KE, AB. Data analysis and presentation: TT, AB, KE. Preparation of the first draft of the manuscript: TT. Interpretation of the data, critical revision of the manuscript and approval of the final submitted manuscript: TT, KE, SJO, IDD, AB, JM, EVV, PML, NP.

Consent to participate

Written informed consent was obtained from all participants included in this study.

Consent to publish

Not applicable to this research since no individual data (e.g images or videos) are being published.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University of Georgia and University of Westminster.

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Declaration of Competing Interest

The authors declare that they have no competing interests or any other interests that could be perceived to influence the results and discussion reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.leukres.2026.108169](https://doi.org/10.1016/j.leukres.2026.108169).

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