Sialylation regulates migration in chronic lymphocytic leukemia

Alessandro Natoni,[1*] Marina Cerreto,[1*] Maria Stefania De Propris,[1] Ilaria Del Giudice,[1] Roberta Soscia,[1] Nadia Peragine,[1] Stefania Intoppa,[1] Maria Laura Milani,[1] Anna Guarini[2] and Robin Foà[1]

[1]Hematology, Department of Translational and Precision Medicine and [2]Department of Molecular Medicine, Sapienza University, Rome, Italy

AN and MC contributed equally as co-first authors.

Correspondence: A. Natoni
alessandro.natoni@uniroma1.it

Received: August 25, 2022.
Accepted: January 27, 2023.
Early view: February 9, 2023.

https://doi.org/10.3324/haematol.2022.281999

Abstract

Sialylation is the terminal addition of sialic acid to underlying glycans. It plays a prominent role in cell adhesion and immune regulation. Sialylated structures found on adhesion molecules, such as CD49d, mediate the interactions between cancer cells and the microenvironment, facilitating metastatic seeding in target organs. Chronic lymphocytic leukemia (CLL) is a clonal B-cell malignancy characterized by the accumulation of CD5-positive B cells in the peripheral blood, bone marrow and lymph nodes. CLL cells proliferate mainly in the lymph node "proliferation centers", where the microenvironment provides pro-survival signals. Thus, migration and homing into these protective niches play a crucial role in CLL biology. In recent years, therapeutic strategies aimed at inducing the egress of CLL cells from the lymph nodes and bone marrow into the circulation have been highly successful. In this study, the sialylation status of 79 untreated and 24 ibrutinib-treated CLL patients was characterized by flow cytometry. Moreover, the effect of sialic acid removal on migration was tested by a transwell assay. Finally, we examined the sialylation status of CD49d by Western blot analysis. We found that CLL cells are highly sialylated, particularly those characterized by an "activated" immune phenotype. Notably, sialylation regulates CLL migration through the post-translational modification of CD49d. Finally, we showed that therapeutic agents that induce CLL mobilization from their protective niches, such as ibrutinib, modulate sialic acid levels. We propose that sialylation is an important regulator of CLL trafficking and may represent a novel target to further improve CLL therapy.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of clonal, CD5-positive mature B cells in the peripheral blood (PB), bone marrow (BM) and lymph nodes (LN).[1] Since circulating PB CLL cells are mainly arrested in the G0/G1 phase of the cell cycle, CLL proliferation essentially occurs in the BM and in the LN.[2] In the latter, CLL cells proliferate in a specific compartment called "proliferation centers" (PC) or pseudofollicles.[3] Not only does the microenvironment support CLL proliferation, but it also provides malignant cells with survival factors that induce resistance to otherwise extremely effective therapeutic agents.[4] Given their dependency on the microenvironment, CLL cells continue to circulate between the PB, BM and LN. CLL homing to the BM and LN is mediated by key molecules including chemokine [C-X-C motif] receptor 4 (CXCR4) and CD49d.[5,6] CXCR4 represents the receptor for the stromal cell-derived factor 1α (SDF1α), an essential chemokine that mediates CLL chemotaxis and transendothelial migration.[7,8] Different levels of CXCR4 and CD5 expression have been associated with specific PB CLL immune phenotypes, proliferation, persistence in the PB, and survival.[9,10] CD49d is the α4 integrin subunit that, in association with the β1 subunit, forms the very late antigen 4 (VLA4). VLA4 promotes proper localization and recirculation of CLL cells into protective niches by binding to the vascular cell adhesion molecule 1 (VCAM1) and fibronectin (FN) present on endothelial and BM stromal cells, respectively.[11] The importance of CD49d in CLL is shown by its clinical application as a prognostic marker.[12-16]

Novel therapeutic agents, including the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib, aim at targeting pro-survival and proliferative signals that originate from the

microenvironment.[5,6] A direct outcome of such treatments is the downregulation of key adhesion molecules that induces mobilization of CLL cells from the BM and LN to the PB, resulting in spontaneous apoptosis and re-sensitization to chemotherapeutic agents.[17-19]

Sialylation represents a post-translational modification of proteins and lipids catalyzed by a class of enzymes, which reside in the Golgi apparatus, known as sialyltransferases (Sts).[20] These enzymes mediate the attachment of sialic acids via different glycosidic linkages (α2-3, α2-6, or α2-8) to the underlying glycan chain. Aberrant sialylation contributes to tumor immune evasion, dissemination and metastasis.[21] Indeed, hypersialylation impairs natural killer (NK) cell-mediated cytotoxicity in multiple myeloma (MM)[22] and CLL[23] by promoting the binding to sialic acid-binding immunoglobulin-like lectins (Siglecs), a family of inhibitory receptors that are predominantly expressed by immune cells. Moreover, hypersialylation promotes tumor dissemination and metastasis by modulating the function of different integrins.[24] For example, inhibiting sialylation in MM impairs interactions between tumor cells, VCAM1 and mucosal vascular addressin cell adhesion molecule 1 (MADCAM1) by altering the maturation of CD49d.[25] Sialylation also participates in the generation of selectin ligands. The α2-3 linked sialic acid is an essential component of the tetrasaccharide structure known as Sialyl Lewis$^{a/x}$ (SLe$^{a/x}$), a key determinant of selectin recognition and binding. SLe$^{a/x}$ characterizes tumors with a metastatic phenotype and its expression negatively correlates with patient survival.[26] In acute myeloid leukemia (AML), blocking the interactions between E-selectin and its ligands by the small molecule GMI1271/Uproleselan effectively inhibits niche-mediated pro-survival signaling, dampens AML blast regeneration, and strongly synergizes with chemotherapy.[27]

In this study, we investigated the sialylation profile of CLL cells isolated from the PB of untreated and ibrutinib-treated patients. We observed that CLL cells constitutively express high levels of sialic acids that can be modulated by therapeutic agents such as ibrutinib. Importantly, sialylation seems to regulate VCAM1- and FN-dependent CLL migration through the modification of the integrin subunit CD49d.

Methods

Chronic lymphocytic leukemia patients

Peripheral blood samples were collected after informed consent from 79 untreated CLL patients and from 24 CLL patients treated with ibrutinib. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Sapienza University. Patients' clinical features are listed in *Online Supplementary Tables S1* and *S2*.

Flow cytometry analysis

Leukocytes from PB of CLL patients were isolated using an ammonium chloride-based red blood lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.2 mM ethylenediaminetetraacetic acid [EDTA] tetrasodium salt, all from Merck; Rahway, NJ, USA). To determine the levels of α2-3 and α2-6 linked sialic acids, leukocytes ($2x10^6$ per tube) were incubated for 15 minutes (min) at room temperature (RT) with gentle rocking with *Maackia amurensis* lectin II and *Sambucus nigra* lectin (1:20000; Vector Laboratories; Newark, CA, USA), which preferentially bind the α2-3 and α2-6 linked sialic acids, respectively. After incubation, cells were washed, stained with APC-conjugated streptavidin beads (1:400; BD Bioscience, Franklin Lakes, NJ, USA), FITC-conjugated anti-CD5 and PE-conjugated anti-CD19 antibodies (both from Immunological Science, Rome, Italy), AlexaFluor 647-conjugated cutaneous lymphocyte antigen antibody (clone Heca452; BD Bioscience) and incubated for 30 min at RT with gentle rocking. After incubation, cells were washed, resuspended in 500 µL staining buffer supplemented with 7-aminoactinomycin D (7-AAD, 1:80; Immunological Science) to exclude dead cells. Cells were acquired on a BD FACS Canto I (BD Biosciences). Data were analyzed using the Infinicyt software v 2.0.5.b.007 (Cytognos; Salamanca, Spain).

Migration assay

For the migration assay, only samples containing more than 85% CLL cells were used. Moreover, we selected samples with CD49d-positive CLL cells. CLL cells were isolated by Ficoll gradient centrifugation and seeded in the upper chamber of a transwell (5 µm pore size; Sarstedt; Hildesheim, Germany) at $2x10^6$ in 100 µL of serum-free RPMI 1640. The transwells had been previously coated with human FN (10 µg/cm^2, Bio-Techne; Minneapolis, MN, USA), recombinant human VCAM1-Fc Chimera (1 µg/cm^2, Biolegend; San Diego, CA, USA), or bovine serum albumin (BSA, 1% [v/v] in PBS; Merck) as control. Lower chambers were filled with either 600 µL of serum-free RPMI 1640 medium or serum free RPMI 1640 medium supplemented with recombinant human SDF1α (200 ng/mL, Peprotech; London, UK). After 5 hours (h) at 37°C, migrated cells in the lower chambers were collected and mixed with 25 µL of CountBright absolute counting beads (ThermoFisher Scientific; Waltham, MA, USA) together with 5 µL of 7AAD to exclude dead cells. Samples were acquired on a BD FACS Canto I. Acquisition was stopped after collecting 2,000 events in the gate drawn around the beads. The cell number was estimated using the following equation: absolute count (cells/µL) = (cell count x counting bead volume) / (counting bead count x cell volume) x counting bead concentration (beads/µL).

Statistical analysis

The Mann-Whitney test or the two-way ANOVA followed by Sidak's multiple comparison *post-hoc* testing were used to determine significance, using $P<0.05$ as the cut-off. *$P<0.05$; **$P<0.01$; ***$P<0.001$. GraphPad Prism 6.02 software (La Jolla, CA, USA) was used to compute all statistical calculations.

Results

Chronic lymphocytic leukemia cells express elevated levels of α2-3 linked sialic acid and α2-6 linked sialic acid and low levels of Sialyl Lewis$^{a/x}$

To characterize the sialylation status of CLL cells, we used flow cytometry to measure the expression levels of the α2-3 and α2-6 linked sialic acids (α2-3 Sia, α2-6 Sia) in CLL cells isolated from the PB of 79 untreated patients, using the *Maackia amurensis* lectin II (MALII) and the *Sambucus nigra* lectin (SNA). We also included in our analysis the Heca452 antibody, which recognizes the tetrasaccharide SLe$^{a/x}$, an important determinant in selectin recognition and binding. All CLL samples analyzed displayed a high proportion of cells positive for MALII and SNA, indicating that the majority of CLL cells express both α2-3 Sia and α2-6 Sia (Figure 1A). In contrast, CLL cells were negative or weakly positive to the Heca452 antibody, suggesting low levels of SLe$^{a/x}$ expression (Figure 1A). The distribution of the median fluorescence intensity (MFI) for all three markers was rather heterogeneous, particularly for α2-6 Sia, indicating a high degree of inter-patient variability (Figure 1B, C and D). There was no difference in the proportion of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ positive cells between CLL cells with either favorable or adverse prognostic indicators including the *IGVH* mutational status, CD38 and CD49d positivity (*Online Supplementary Figures S1-S3*). With regard to MFI, we only found a decrease in the SLe$^{a/x}$ MFI of the CD38-positive compared to the CD38-negative CLL cells (*Online Supplementary Figure S2F)*; the difference between these two CLL populations was, however, small and its biological significance is, therefore, unclear. In conclusion, PB CLL cells express α2-3 Sia, α2-6 Sia and, to a lesser extent, SLe$^{a/x}$.

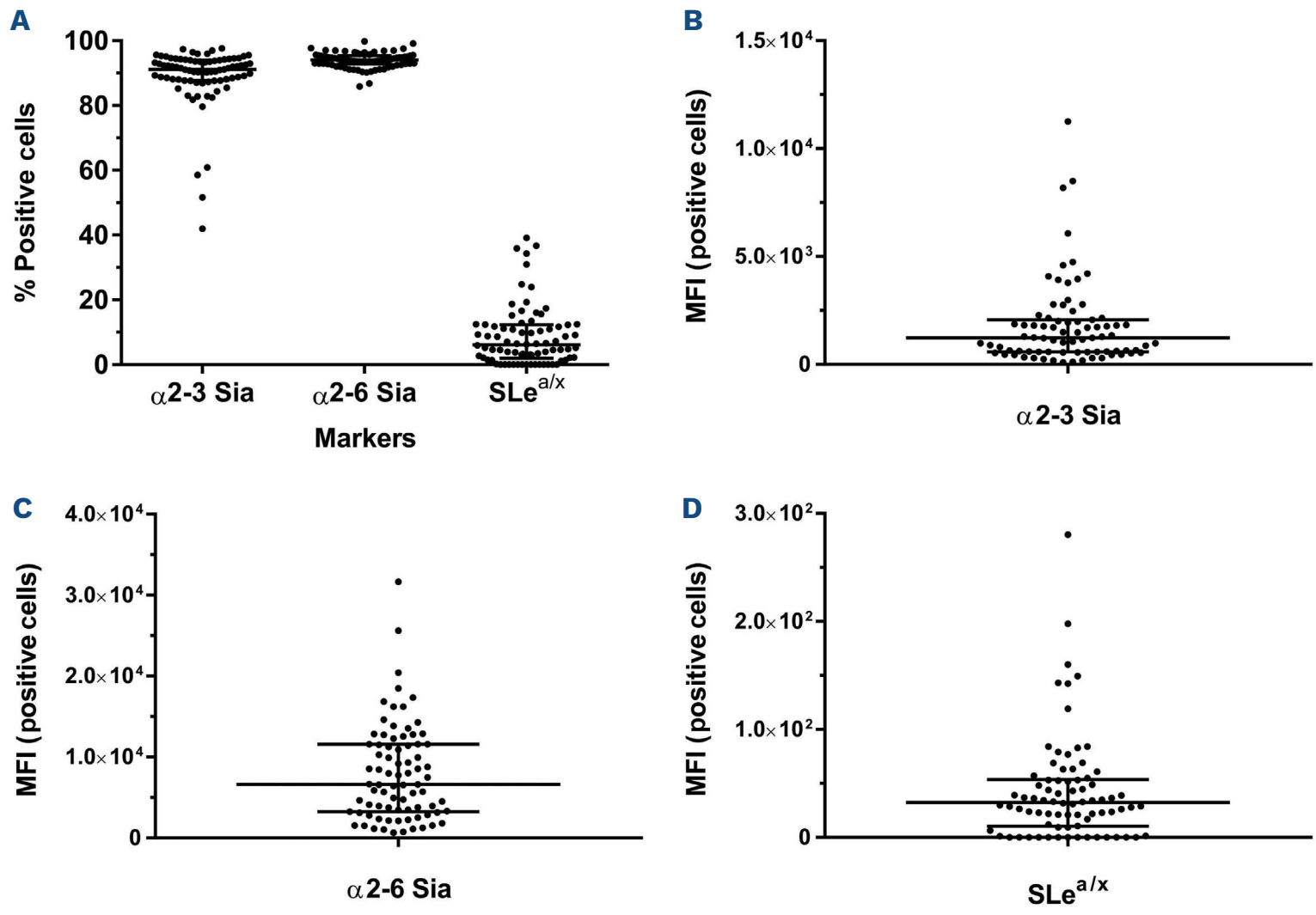

Figure 1. Expression of α2-3 linked sialic acid, α2-6 linked sialic acid and Sialyl Lewis$^{a/x}$ in chronic lymphocytic leukemia cells. Peripheral blood (PB) collected from 79 untreated chronic lymphocytic leukemia (CLL) patients were lysed and stained for flow cytometry. CLL cells were identified by the expression of CD5 and CD19. Dead cells were excluded using the 7-aminoactinomycin D (7AAD). The levels of α2-3 linked sialic acid (α2-3 Sia), α2-6 linked sialic acid (α2-6 Sia), and Sialyl Lewis$^{a/x}$ (SLe$^{a/x}$) were determined using *Maackia amurensis* lectin II (MALII) and *Sambucus nigra* (SNA) lectins and the Heca452 antibody, respectively. At least 30,000 events were acquired in the CD5 CD19 gated population. Graphs display the percentages of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ positive cells (A), and the median fluorescence intensity (MFI) of the α2-3 Sia (B), α2-6 Sia (C), and SLe$^{a/x}$ (D) positive cells. Dots represent individual measurements. Horizontal lines depict median and interquartile range.

α2-3 linked sialic acid and Sialyl Lewis$^{a/x}$ are present at higher levels in chronic lymphocytic leukemia cells with an "activated" phenotype

We then investigated whether sialic acids could be differentially expressed in distinct CLL subclones. To this end, we focused on two surface markers, CD5 and CXCR4, whose combined expression discriminates between CLL subclones enriched in recently divided cells that have left a solid lymphoid tissue (CXCR4^{dim} CD5^{bright}) and those that have been circulating in the periphery longer and are characterized by a resting phenotype (CXCR4^{bright} CD5^{dim}).[9] Using a similar gating strategy to that used in previous studies (Figure 2A),[9,10] we identified CXCR4^{dim} CD5^{bright} and CXCR4^{bright} CD5^{dim} cells in 28 CLL samples from untreated patients and examined the expression levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$. We observed that CXCR4^{dim} CD5^{bright} cells displayed higher expression levels of the α2-3 Sia and of SLe$^{a/x}$ compared to CXCR4^{bright} CD5^{dim} cells (Figure 2B and D). These data suggest that the α2-3 Sia, and in particular SLe$^{a/x}$, may be expressed at higher levels in CLL cells with an "activated" phenotype.

Removal of sialic acids on the cell surface by neuraminidase treatment inhibits VCAM1- and fibronectin-dependent chronic lymphocytic leukemia migration

Sialylation is a post-translational modification that occurs on proteins and lipids expressed predominantly on the cell surface. Therefore, one of its major roles is the modulation of cell-cell and cell-environment interaction.[28] Indeed, sialylation seems to enhance the metastatic potential of several tumors by promoting migration and invasion.[21] Since CLL cells continuously migrate in and out of the LN and BM, which represent essential niches for CLL cell sur-

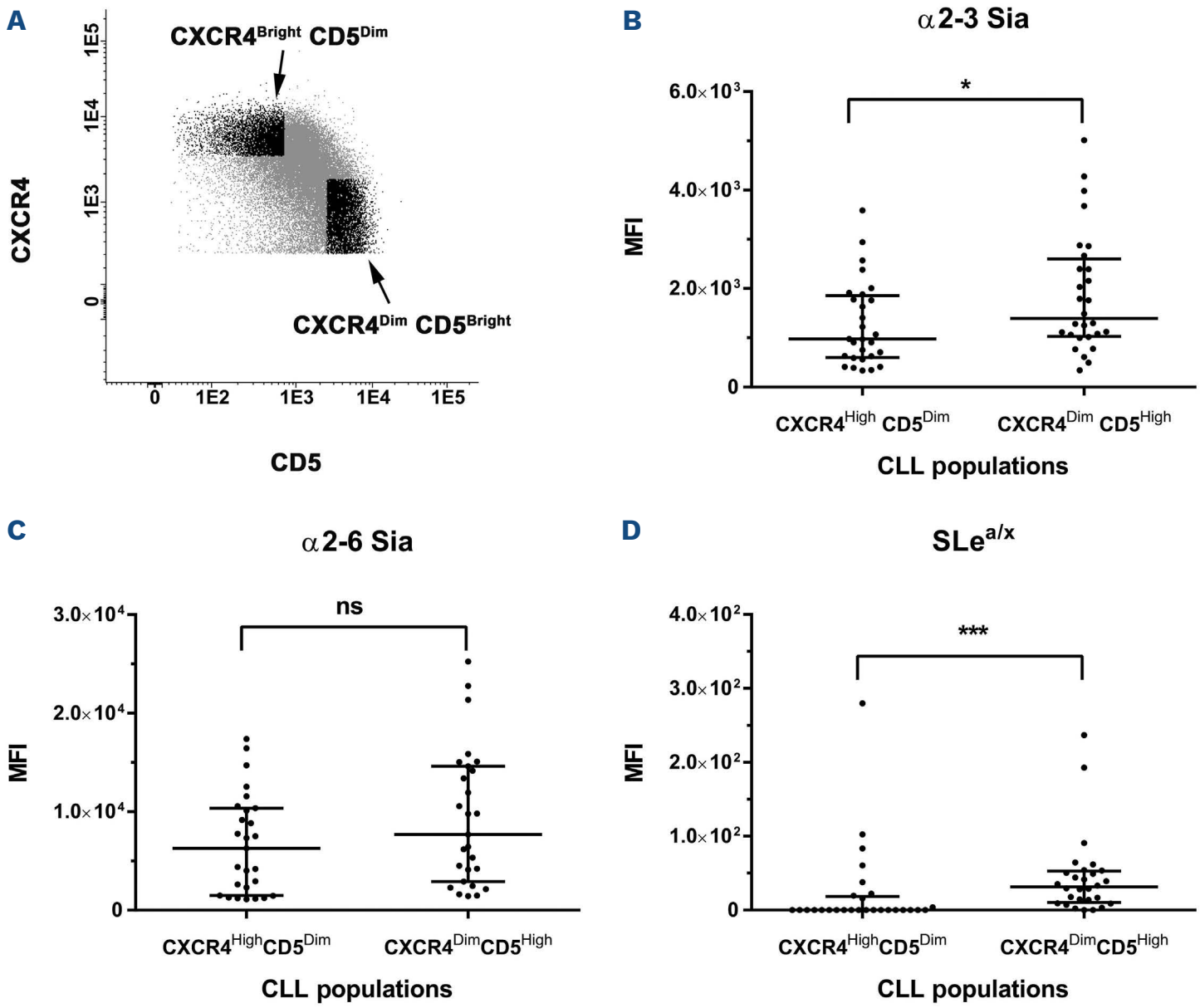

Figure 2. Expression of α2-3 linked sialic acid, α2-6 linked sialic acid and Sialyl Lewis$^{a/x}$ in CXCR4^{dim} CD5^{bright} and CXCR4^{bright} CD5^{dim} chronic lymphocytic leukemia cells. Peripheral blood (PB) collected from chronic lymphocytic leukemia (CLL) patients were lysed and stained for flow cytometry as described above. CLL cells with chemokine [C-X-C motif] receptor 4^{dim} (CXCR4^{dim}) CD5^{bright} and CXCR4^{bright} CD5^{dim} phenotypes were identified by the expression of CD5 and CXCR4 markers within the CD5 CD19 positive population. (A) Example of the gating strategy used to determine CXCR4^{dim} CD5^{bright} and CXCR4^{bright} CD5^{dim} populations. α2-3 linked sialic acid (α2-3 Sia) (B), α2-6 linked sialic acid (α2-6 Sia) (C), and Sialyl Lewis$^{a/x}$ (SLe$^{a/x}$) (D) median fluorescence intensity (MFI) of the CXCR4^{dim} CD5^{bright} and CXCR4^{bright} CD5^{dim} CLL cells. At least 30,000 events were acquired in the CD5 CD19 population. Dots represent individual measurements. Horizontal lines depict median and interquartile range. Mann-Whitney test: *P<0.05; ***P<0.001; ns: not significant.

vival and proliferation, we asked whether sialylation could regulate CLL migration. Given the clinical significance of CD49d (α4) in CLL,[12-16] we examined the involvement of sialylation in VCAM1- and FN-dependent migration, which is primarily mediated by CD49d/β1. Indeed, it has been shown that only CLL cells expressing CD49d display strong migration into these protective niches, a process that is dependent on the expression of VCAM1 on targeted endothelium.[29,30] We thus selected CD49d-positive CLL cells and tested them in a transwell assay under conditions in which sialic acids were removed from the cell surface by treatment with neuraminidase from *Vibro Cholerae*. Transwells were coated with BSA, VCAM1 and FN, and migration was stimulated by SDF1α. CLL cells from different patients displayed a considerable variation in their ability to migrate in response to SDF1α (*Online Supplementary Figure S4*), which could be explained only in part by CXCR4 expression (*Online Supplementary Figure S5A*). Migration did not correlate with the levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ (*data not shown*). VCAM1 and FN exhibited variable effects on migration of CLL cells. Indeed, VCAM1 and FN inhibited or enhanced migration in response to SDF1α, suggesting a high degree of inter-patient variability (*Online Supplementary Figure S4*). Moreover, FN-dependent but not VCAM1-dependent migration correlated with the expression levels of CXCR4 (*Online Supplementary Figure S5B* and *C*), highlighting the importance of this receptor in FN-mediated migration. VCAM1-dependent migration did not correlate with the degree of CD49d positivity (*data not shown*), suggesting that additional factors besides CXCR4 and CD49d modulate this process. Neuraminidase treatment had no effects on migration stimulated by SDF1α (Figure 3). However, in all CLL samples analyzed, neuraminidase treatment inhibited VCAM1- and FN-dependent migration (Figure 3). Importantly, we observed that neuraminidase from *Vibro Cholerae* almost exclusively removed the α2-3 Sia (*Online Supplementary Figure S6*), indicating that this form of sialic acid is predominantly involved in modulating VCAM1- and FN-mediated migration. Since neuraminidase treatment does not specifically remove sialic acids from CD49d but rather induces a global desialylation, we performed the transwell assay on CD49d-negative CLL cells obtained from 4 patients to test whether migration of these cells was also impaired by the treatment. We could observe that CLL cells treated with neuraminidase showed a decrease in the number of migrating cells compared to untreated control (*Online Supplementary Figure S7*). Although they did not reach statistical significance, these data suggest that removal of sialic acids could affect migration independently of CD49d, an observation that requires further study. Importantly, the presence of VCAM1 and FN did not stimulate migration (*Online Supplementary Figure S7*), highlighting the importance of CD49d in VCAM1- and FN-dependent migration.

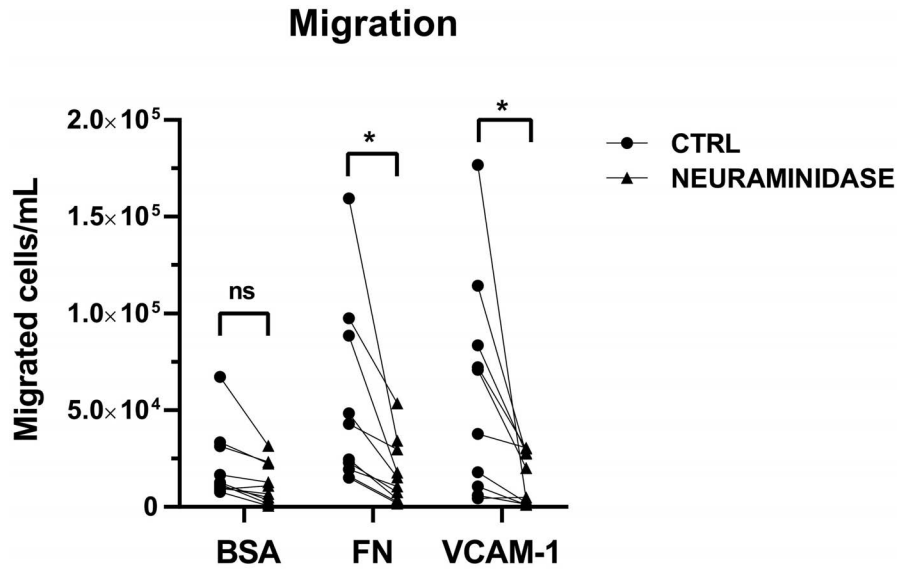

Figure 3. Inhibition of VCAM1- and fibronectin-dependent chronic lymphocytic leukemia migration by neuraminidase treatment. Chronic lymphocytic leukemia (CLL) cells were treated/mock treated with neuraminidase from *Vibro Cholerae* (0.1 U/mL) for 45 minutes and then seeded on top of transwells coated overnight with bovine serum albumin (BSA), vascular cell adhesion molecule 1 (VCAM1)-Fc chimera and fibronectin (FN). The bottom chambers of the transwells were filled with serum free media supplemented with stromal cell-derived factor 1α (SDF1α [200 ng/mL]). Cells were allowed to migrate for 5 hours at 37°C. After incubation, cells in the lower chambers were collected and mixed with 25 µL of counting beads. Migrated CLL cells were counted using a BD FACS Canto I flow cytometer by gating on the counting beads and acquiring, in this gate, 2000 events. Two-way ANOVA followed by Sidak's multiple comparison *post-hoc* testing: *P<0.05; ns: not significant.

CD49d is post-translationally sialylated in chronic lymphocytic leukemia

Since VCAM1 and FN represent the main counter-receptors for the integrin α4/β1, we sought to investigate whether sialylation represents a post-translational modification of the α4 and the β1 integrin subunits. To this end, we examined the effects of neuraminidase treatment on the mobility of these integrin subunits on a sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) by Western blot analysis. Indeed, we observed that neuraminidase treatment consistently induced a shift in the mobility of the α4 subunit in CLL samples collected from 4 patients (Figure 4), indicating that CD49d is post-translationally sialylated. The β1 subunit became apparent only after a long exposure and was not affected by neuraminidase treatment (Figure 4). Taken together, these data indicate that sialylation of CD49d regulates VCAM1- and FN-dependent migration.

Ibrutinib alters the levels of α2-3 linked sialic acid, α2-6 linked sialic acid and Sialyl Lewis$^{a/x}$

We next explored whether therapeutic agents that have an impact on the interactions between CLL and the microenvironment could modify the levels of sialic acids. We thus compared the sialylation profile between CLL cells from the untreated patient cohort with that of cells iso-

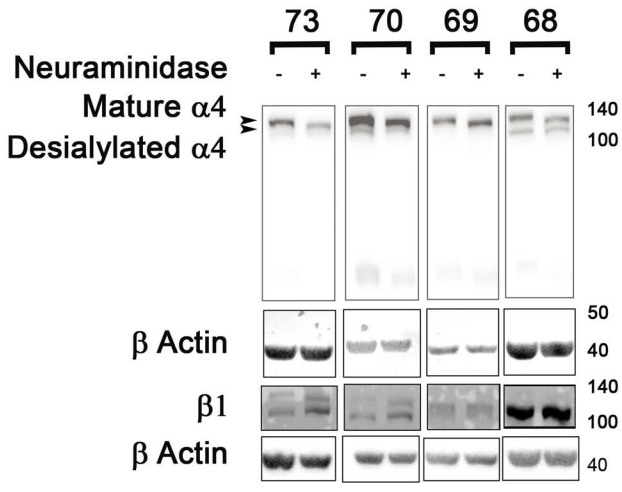

Figure 4. Neuraminidase treatment alters the post-translational modification of CD49d. Chronic lymphocytic leukemia (CLL) cells collected from 4 patients were treated/mock treated with neuraminidase from *Vibro Cholerae* as described above. After treatment, whole cell extracts were prepared and subjected to sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane and blotted for integrin α4, β1 and β-actin as loading control. Numbers above the blots represent patient number. Upper and lower arrows indicate mature and desialylated α4, respectively. Numbers on right-hand side represent molecular weight marker.

lated from a cohort of patients treated with ibrutinib. Although there was no difference between the proportion of CLL cells positive for α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ in both cohorts of patients (Figure 5A, C and E), CLL cells from the ibrutinib-treated cohort exhibited a lower α2-3 Sia and α2-6 Sia MFI compared to that from the untreated cohort (Figure 5B and D), indicating that ibrutinib treatment did have an impact on sialylation. Interestingly, we observed that the SLe$^{a/x}$ MFI was higher in CLL cells derived from ibrutinib-treated patients compared to that of cells derived from untreated patients (Figure 5F). We were able to examine the levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ in cryopreserved CLL cells taken from the ibrutinib-treated patient cohort prior to ibrutinib treatment and to compare them with those from matched samples after ibrutinib treatment. We observed a decrease in the α2-3 Sia, α2-6 Sia MFI and an increase in the SLe$^{a/x}$ MFI after ibrutinib treatment, thus confirming our previous observation (*Online Supplementary Figure S8*).

Discussion

In this study, we carried out a sialylation profile analysis of CLL cells by measuring the levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ expression on the cell surface. We observed that all CLL cells examined invariably expressed α2-3 Sia and α2-6 Sia, although the levels of expression differed considerably between patients. The levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ expression did not correlate with the clinical markers examined in this study, namely the *IGVH* mutational status, and CD38 and CD49d expression. Therefore, sialylation seems to be a general feature of CLL. However, we cannot exclude the possibility that individual and not global glycoconjugates may be differentially sialylated according to the clinical markers examined in this study. A difference in CLL sialylation has been reported in association with resistance to rituximab (RTX)-mediated complement-dependent cytotoxicity (CDC).[31] Treatment of resistant CLL cells with neuraminidase could revert this resistance and increase RTX-mediated CDC in sensitive CLL cells. High expression of sialoglycans has been also found in the context of Siglec 7 ligands in CLL.[23] Siglec 7 is a member of immunomodulatory receptors expressed on cells of the immune system, which, upon binding to sialic acids, triggers inhibitory signals that suppress the immune response.[32] CLL cells express high levels of the disialyl-T antigen, a Siglec 7 ligand, which is synthesized by ST6GalNAc-IV and blocked by core 2 GlcNAc transferase.[23] Importantly, the expression pattern of these two genes, that is predictive of high disialyl-T antigen expression (*ST6GalNAC4^{High}* and *GCNT1^{Low}*), is associated with a poor prognosis.[23] Taken together, these results indicate that sialylation of CLL cells serves as a means to protect them from the cytotoxic activity of the immune system. Moreover, NK response in CLL patients is markedly impaired,[33-36] most likely due to low levels of activating ligands expressed on CLL cells,[37] together with low expression of activating receptors on NK cells.[38,39] Therefore, sialylation may co-operate with all these mechanisms in generating an immune suppressive phenotype that promotes CLL immune escape.

In the present study, we found that sialylation of CLL cells is also involved in modulating VCAM1- and FN-dependent migration. CLL cells rely on the LN and BM microenvironment for their survival and proliferation; therefore, they constantly circulate between PB, BM and LN. The relevance of CLL recirculation between these different anatomical compartments is proven by the prognostic significance of CD49d, one of the strongest prognostic markers in CLL, predicting OS at diagnosis and shorter time to treatment.[13-16] Functionally, CD49d is the α4 subunit of the VLA4 integrin that binds to adhesion molecules such as VCAM1 and FN. Binding to these adhesion molecules stimulates migration into protective niches and promotes survival of CLL cells. Although none of the sialylated markers examined correlated with migration mediated by VCAM1 and FN and stimulated by SDF1α, removal of sialic acids from the cell surface inhibited VCAM1- and FN-dependent migration. Moreover, since the neuraminidase used in this study primarily cleaves α2-3 Sia, this form of sialic acid is clearly implicated in modulating CLL migration, although involvement of α2-8 Sia, not examined in this study, cannot be completely ruled out. By analyzing the mobility of CD49d on SDS PAGE, we observed a reduction in its apparent molecular weight, indicating that

CD49d is modified by sialylation. This result is similar to that found in MM, where global desialylation interferes with CD49d maturation, and impairs adhesion and rolling on VCAM1 and MADCAM1.[25] The mechanisms by which sialylation modulates the adhesive properties of CD49d still have to be explored. CD49d presents multiple conformations characterized by different binding affinities that allow leukocytes to tether, roll or firmly adhere to the endothelium.[40] It could be that sialylation modulates one or more of these different conformations regulating the af-

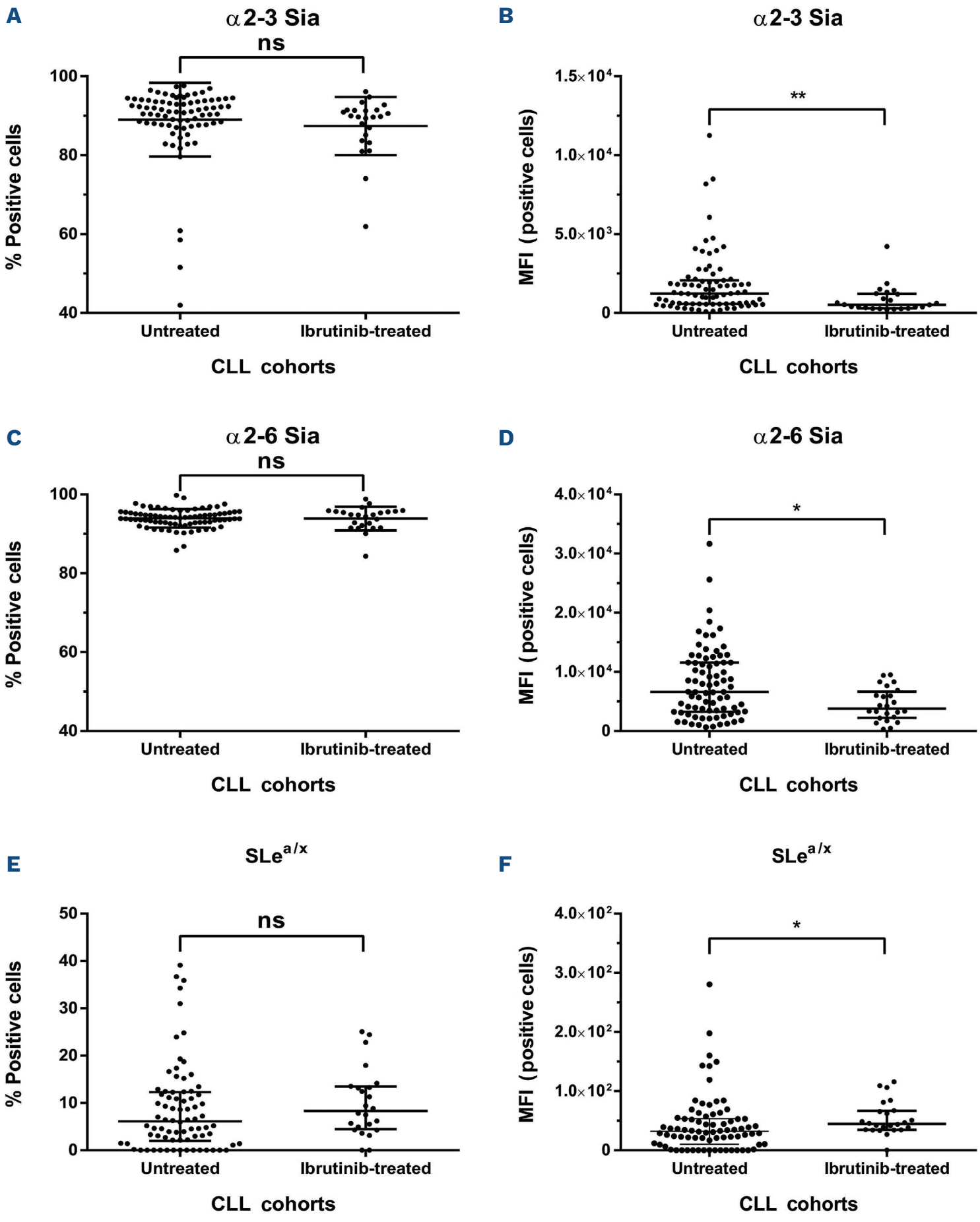

Figure 5. Expression of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ between chronic lymphocytic leukemia cells from untreated and ibrutinib-treated patients. Peripheral blood (PB) collected from 79 untreated and 24 ibrutinib-treated chronic lymphocytic leukemia (CLL) patients were lysed and stained for flow cytometry as described above. Graphs display the percentages and the median fluorescence intensity (MFI) of α2-3 linked sialic acid (α2-3 Sia) (A and B), α2-6 linked sialic acid (α2-6 Sia) (C and D), and Sialyl Lewis$^{a/x}$ (SLe$^{a/x}$) (E and F) expression levels. Dots represent individual measurements. Horizontal lines depict median and interquartile range. Mann-Whitney test: *P<0.05; **P<0.01; ns: not significant.

finity of VLA4 for its substrates. How sialylation of CD49d impacts its prognostic value is still not known. Clearly, the presence of a post-translational modification on CD49d adds another layer of complexity. Sialylation seems to increase the affinity of CD49d for its counter-receptors; thus, the presence of a constitutively sialylated form of CD49d in CLL may represent one of the biological determinants underlying the importance of this marker in the clinic. Screening of the CD49d sialylation status in a large cohort of CLL patients will be instrumental in defining the contribution of sialylation on the prognostic power of CD49d.

In contrast to α2-3 Sia and α2-6 Sia, a large proportion CLL cells were negative or weakly positive for the expression of SLe$^{a/x}$, which is consistent with a previous study.[41] However, we observed that α2-3 Sia, and in particular SLe$^{a/x}$, were expressed at higher levels in CXCR4^{dim} CD5^{bright} CLL cells, which have been shown to be enriched in cells that have recently divided and have left the LN compartment.[9] SLe$^{a/x}$ is a tetrasaccharide composed of an N-acetyl-D-glucosamine, β1-4 galactose, α2-3 Sia and α1-3 or α1-4 fucosylation and represents an essential determinant for selectin binding. It is constitutively expressed on granulocytes and monocytes whereas it can be induced on T and B cells upon activation.[42-46] In hematologic malignancies, SLe$^{a/x}$ is expressed at high levels in diseases characterized by a more immature phenotype such as AML[27] and acute lymphoblastic leukemia.[41] It is also expressed on a subpopulation of MM cells that give rise to an aggressive disease and resistance to bortezomib *in vivo* in nude mice.[47] Given this amount of data, it is tempting to speculate that SLe$^{a/x}$ marks CLL cells that are more prone to proliferate and to home into the LN or BM, which could be facilitated by selectin binding. The higher expression of α2-3 Sia and the SLe$^{a/x}$ in LN-like CLL cells suggests that sialylation may be modulated by microenvironmental signals. It has been shown that CLL cells express surface IgM (sIgM) as a mature glycosylated form similar to normal B cells and as an immature mannosylated form that is characteristic of persistent sIgM engagement.[48] These data indicate that, in CLL, the microenvironment can modulate the glycan composition of the cell surface. It will be of interest to understand whether sialylation can be similarly regulated.

Finally, we examined whether sialylation could be modulated by therapeutic agents, such as ibrutinib, that target essential signaling pathways provided by the microenvironment. Indeed, by comparing the untreated CLL cohort to a cohort of patients treated with ibrutinib, we observed that CLL cells from ibrutinib-treated patients displayed lower levels of α2-3 Sia and α2-6 Sia compared to those from untreated individuals. These results were also confirmed in a comparison between matched samples taken before and after ibrutinib treatment. Whether the decrease in α2-3 Sia and α2-6 Sia is due to a direct effect of ibrutinib on the expression levels of the STs or is a consequence of an ibrutinib-mediated down-regulation of sialylated adhesion molecules[17-19] is still not known. Notably, we found that the levels of SLe$^{a/x}$ were increased in CLL cells from ibrutinib-treated patients. This could be explained by the CLL egress from the LN induced by ibrutinib treatment.[49] Indeed, if SLe$^{a/x}$ marks "activated", dividing CLL cells, it should mainly be expressed in CLL cells resident in the LN. Therefore, by inducing cells to egress from the LN, ibrutinib may force them into the PB. This could apparently create a paradoxical situation whereby ibrutinib releases into the circulation CLL cells more prone to proliferate and disseminate. However, it should be emphasized that not only does ibrutinib promote egress of CLL cells, but it also inhibits re-entry and homing into protective niches by down-modulating pro-adhesive molecules,[18,49] and possibly by inducing their desialylation. Moreover, by inducing desialylation, ibrutinib and similar agents could sensitize circulating CLL cells to the immune system.[23,31] In this context, it will be of interest to examine whether treatment with ibrutinib down-modulates Siglec 7 ligands on CLL cells and sensitizes them to NK-mediated cytotoxicity, or facilitates RTX-mediated CDC.

In conclusion, we have shown that sialylation is an important feature of CLL cells regulating migration through modification of relevant molecules such as CD49d, and may represent a novel therapeutic target to block CLL dissemination and homing, maximizing the efficacy of standard and novel clinical agents. For instance, neuraminidase-conjugated antibodies have been developed to selectively remove sialic acids from the surface of tumor cells potentiating the anticancer immune response.[50] Such a strategy could be implemented in CLL by conjugating therapeutically relevant antibodies, such as RTX, with neuraminidase, combining the mechanisms of action of the therapeutic antibody with the targeted removal of sialic acids, which may lead to a superior clinical response.

Disclosures

No conflicts of interest to disclose.

Contributions

AN and MC performed the experiments and analyzed the data. MSDP, SI and MLM carried out the diagnostic CLL immune phenotype. RS performed the IGVH mutational status. NP and AG reviewed the manuscript. AN, IDG and RB designed the study and wrote the paper. RF supervised and funded the research.

Acknowledgments

We would like to acknowledge Prof. Michael O'Dwyer from the National University of Ireland, Galway, for scientific in-

puts, Dr. Antonio Agostini and Dr. Carmine Carbone from the Fondazione Policlinico Universitario Agostino Gemelli IRCCS for technical assistance, and Roberta Raponi for administrative support.

Funding

The project leading to these results has received funding from the Associazione Italiana Ricerca sul Cancro (AIRC) Special 5x1000 Program Metastases (21198), Milan, Italy (to RF), and from the iCARE-2 2019 program co-founded by the AIRC and the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement n. 800924 (to AN).

Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic leukaemia. Nat Rev Cancer. 2016;16(3):145-162.
2. Herishanu Y, Perez-Galan P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. Blood. 2011;117(2):563-574.
3. Lampert IA, Wotherspoon A, Van Noorden S, Hasserjian RP. High expression of CD23 in the proliferation centers of chronic lymphocytic leukemia in lymph nodes and spleen. Hum Pathol. 1999;30(6):648-654.
4. Haselager MV, Kielbassa K, Ter Burg J, et al. Changes in Bcl-2 members after ibrutinib or venetoclax uncover functional hierarchy in determining resistance to venetoclax in CLL. Blood. 2020;136(25):2918-2926.
5. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. Blood. 2009;114(16):3367-3375.
6. ten Hacken E, Burger JA. Molecular pathways: targeting the microenvironment in chronic lymphocytic leukemia-focus on the B-cell receptor. Clin Cancer Res. 2014;20(3):548-556.
7. Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. Blood. 1999;94(11):3658-3667.
8. Burger JA, Kipps TJ. Chemokine receptors and stromal cells in the homing and homeostasis of chronic lymphocytic leukemia B cells. Leuk Lymphoma. 2002;43(3):461-466.
9. Calissano C, Damle RN, Marsilio S, et al. Intraclonal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. Mol Med. 2011;17(11-12):1374-1382.
10. Kriston C, Plander M, Mark A, et al. In contrast to high CD49d, low CXCR4 expression indicates the dependency of chronic lymphocytic leukemia (CLL) cells on the microenvironment. Ann Hematol. 2018;97(11):2145-2152.
11. Dal Bo M, Tissino E, Benedetti D, et al. Microenvironmental interactions in chronic lymphocytic leukemia: the master role of CD49d. Semin Hematol. 2014;51(3):168-176.
12. Peragine N, De Propris MS, Intoppa S, et al. Early CD49d downmodulation in chronic lymphocytic leukemia patients treated front-line with ibrutinib plus rituximab predicts long-term response. Leuk Lymphoma. 2022;63(12):2982-2986.
13. Gattei V, Bulian P, Del Principe MI, et al. Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. Blood. 2008;111(2):865-873.
14. Bulian P, Shanafelt TD, Fegan C, et al. CD49d is the strongest flow cytometry-based predictor of overall survival in chronic lymphocytic leukemia. J Clin Oncol. 2014;32(9):897-904.
15. Shanafelt TD, Geyer SM, Bone ND, et al. CD49d expression is an independent predictor of overall survival in patients with chronic lymphocytic leukaemia: a prognostic parameter with therapeutic potential. Br J Haematol. 2008;140(5):537-546.
16. Dal Bo M, Bulian P, Bomben R, et al. CD49d prevails over the novel recurrent mutations as independent prognosticator of overall survival in chronic lymphocytic leukemia. Leukemia. 2016;30(10):2011-2018.
17. de Rooij MF, Kuil A, Geest CR, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. Blood. 2012;119(11):2590-2594.
18. Peragine N, De Propris MS, Intoppa S, et al. Modulated expression of adhesion, migration and activation molecules may predict the degree of response in chronic lymphocytic leukemia patients treated with ibrutinib plus rituximab. Haematologica. 2020;106(5):1500-1503.
19. Herman SE, Mustafa RZ, Jones J, Wong DH, Farooqui M, Wiestner A. Treatment with ibrutinib inhibits BTK- and VLA-4-dependent adhesion of chronic lymphocytic leukemia cells in vivo. Clin Cancer Res. 2015;21(20):4642-4651.
20. Harduin-Lepers A, Vallejo-Ruiz V, Krzewinski-Recchi MA, Samyn-Petit B, Julien S, Delannoy P. The human sialyltransferase family. Biochimie. 2001;83(8):727-737.
21. Rodrigues E, Macauley MS. Hypersialylation in cancer: modulation of inflammation and therapeutic opportunities. Cancers (Basel). 2018;10(6):207.
22. Daly J, Sarkar S, Natoni A, et al. Targeting hypersialylation in multiple myeloma represents a novel approach to enhance NK cell-mediated tumor responses. Blood Adv. 2022;6(11):3352-3366.
23. Chang LY, Liang SY, Lu SC, et al. Molecular basis and role of Siglec-7 ligand expression on chronic lymphocytic leukemia B cells. Front Immunol. 2022;13:840388.
24. Schultz MJ, Swindall AF, Bellis SL. Regulation of the metastatic cell phenotype by sialylated glycans. Cancer Metastasis Rev. 2012;31(3-4):501-518.
25. Natoni A, Farrell ML, Harris S, et al. Sialyltransferase inhibition leads to inhibition of tumor cell interactions with E-selectin, VCAM1, and MADCAM1, and improves survival in a human multiple myeloma mouse model. Haematologica. 2020;105(2):457-467.
26. Natoni A, Macauley MS, O'Dwyer ME. Targeting selectins and their ligands in cancer. Front Oncol. 2016;6:93.
27. Barbier V, Erbani J, Fiveash C, et al. Endothelial E-selectin inhibition improves acute myeloid leukaemia therapy by disrupting vascular niche-mediated chemoresistance. Nat

Commun. 2020;11(1):2042.
28. Dobie C, Skropeta D. Insights into the role of sialylation in cancer progression and metastasis. Br J Cancer. 2021;124(1):76-90.
29. Brachtl G, Sahakyan K, Denk U, et al. Differential bone marrow homing capacity of VLA-4 and CD38 high expressing chronic lymphocytic leukemia cells. PLoS One. 2011;6(8):e23758.
30. Pasikowska M, Walsby E, Apollonio B, et al. Phenotype and immune function of lymph node and peripheral blood CLL cells are linked to transendothelial migration. Blood. 2016;128(4):563-573.
31. Bordron A, Bagacean C, Mohr A, et al. Resistance to complement activation, cell membrane hypersialylation and relapses in chronic lymphocytic leukemia patients treated with rituximab and chemotherapy. Oncotarget. 2018;9(60):31590-31605.
32. Daly J, Carlsten M, O'Dwyer M. Sugar free: novel immunotherapeutic approaches targeting Siglecs and sialic acids to enhance natural killer cell cytotoxicity against cancer. Front Immunol. 2019;10:1047.
33. Foa R, Fierro MT, Lusso P, et al. Reduced natural killer T-cells in B-cell chronic lymphocytic leukaemia identified by three monoclonal antibodies: Leu-11, A10, AB8.28. Br J Haematol. 1986;62(1):151-154.
34. Foa R, Fierro MT, Raspadori D, et al. Lymphokine-activated killer (LAK) cell activity in B and T chronic lymphoid leukemia: defective LAK generation and reduced susceptibility of the leukemic cells to allogeneic and autologous LAK effectors. Blood. 1990;76(7):1349-1354.
35. Sportoletti P, De Falco F, Del Papa B, et al. NK cells in chronic lymphocytic leukemia and their therapeutic implications. In J Mol Sci. 2021;22(13):6665.
36. Foa R, Lauria F, Lusso P, et al. Discrepancy between phenotypic and functional features of natural killer T-lymphocytes in B-cell chronic lymphocytic leukaemia. Br J Haematol. 1984;58(3):509-516.
37. Veuillen C, Aurran-Schleinitz T, Castellano R, et al. Primary B-CLL resistance to NK cell cytotoxicity can be overcome in vitro and in vivo by priming NK cells and monoclonal antibody therapy. J Clin Immunol. 2012;32(3):632-646.
38. Parry HM, Stevens T, Oldreive C, et al. NK cell function is markedly impaired in patients with chronic lymphocytic leukaemia but is preserved in patients with small lymphocytic lymphoma. Oncotarget. 2016;7(42):68513-68526.
39. Hofland T, Endstra S, Gomes CKP, et al. Natural killer cell hyporesponsiveness in chronic lymphocytic leukemia can be circumvented in vitro by adequate activating signaling. Hemasphere. 2019;3(6):e308.
40. Chigaev A, Waller A, Zwartz GJ, Buranda T, Sklar LA. Regulation of cell adhesion by affinity and conformational unbending of alpha4beta1 integrin. J Immunol. 2007;178(11):6828-6839.
41. Ohmori K, Yoneda T, Ishihara G, et al. Sialyl SSEA-1 antigen as a carbohydrate marker of human natural killer cells and immature lymphoid cells. Blood. 1989;74(1):255-261.
42. Munro JM, Lo SK, Corless C, et al. Expression of sialyl-Lewis X, an E-selectin ligand, in inflammation, immune processes, and lymphoid tissues. Am J Pathol. 1992;141(6):1397-1408.
43. Silva M, Fung RKF, Donnelly CB, Videira PA, Sackstein R. Cell-specific variation in E-selectin ligand expression among human peripheral blood mononuclear cells: implications for immunosurveillance and pathobiology. J Immunol. 2017;198(9):3576-3587.
44. Postigo AA, Marazuela M, Sanchez-Madrid F, de Landazuri MO. B lymphocyte binding to E- and P-selectins is mediated through the de novo expression of carbohydrates on in vitro and in vivo activated human B cells. J Clin Invest. 1994;94(4):1585-1596.
45. Montoya MC, Holtmann K, Snapp KR, et al. Memory B lymphocytes from secondary lymphoid organs interact with E-selectin through a novel glycoprotein ligand. J Clin Invest. 1999;103(9):1317-1327.
46. Ohta S, Hanai N, Habu S, Nishimura T. Expression of sialyl Lewis(x) antigen on human T cells. Cell Immunol. 1993;151(2):491-497.
47. Natoni A, Smith TAG, Keane N, et al. E-selectin ligands recognised by HECA452 induce drug resistance in myeloma, which is overcome by the E-selectin antagonist, GMI-1271. Leukemia. 2017;31(12):2642-2651.
48. Krysov S, Potter KN, Mockridge CI, et al. Surface IgM of CLL cells displays unusual glycans indicative of engagement of antigen in vivo. Blood. 2010;115(21):4198-4205.
49. Palma M, Mulder TA, Osterborg A. BTK inhibitors in chronic lymphocytic leukemia: biological activity and immune effects. Front Immunol. 2021;12:686768.
50. Gray MA, Stanczak MA, Mantuano NR, et al. Targeted glycan degradation potentiates the anticancer immune response in vivo. Nat Chem Biol. 2020;16(12):1376-1384.