Modulated expression of adhesion, migration and activation molecules may predict the degree of response in chronic lymphocytic leukemia patients treated with ibrutinib plus rituximab

B-cell receptor (BCR) signaling has emerged as a pivotal pathway in the pathogenesis and progression of chronic lymphocytic leukemia (CLL) and the introduction of small molecules targeting the BCR signalosome has dramatically changed the treatment landscape of CLL. Among such molecules, ibrutinib, a potent and selective inhibitor of the Bruton tyrosine kinase (BTK) protein, a central mediator of BCR signaling, has been associated with high efficacy and an acceptable toxicity profile in patients with CLL, including those with high-risk genetic features who respond poorly to chemoimmunotherapy. 1

Ibrutinib, as well as other BCR inhibitors, produces an early redistribution of tissue-resident CLL cells into the blood, resulting in a transient, treatment-induced lymphocytosis. 2 Previous in vitro studies have shown that this phenomenon is correlated to the direct effect of the drug on B-cell adhesion and migration which leads to attenuated microenvironment retention and homing of CLL cells. 3 A significant downmodulation in the gene expression and plasma concentration of tissue-homing chemokines has also been observed in vivo following combination therapies with ibrutinib and anti-CD20 monoclonal antibodies, currently being explored to improve patients’ clinical outcome. 4 No data are, however, available on the modulation of CLL cell surface markers in this context. In the present study (ClinicalTrials.gov identifier NCT02232386), we analyzed the in vivo effects of ibrutinib on the expression of adhesion, migration and activation molecules in peripheral blood samples collected from 119 CLL patients enrolled in the GIMEMA LLC 1114 phase II front-line ibrutinib plus rituximab (IR) trial. In particular, the mean fluorescent intensity (MFI) of CD11a, CD18, CD38, CD40, CD43, CD44, CD49d, CD62L, CD69, CD80, CD81, CD86, CD154, CD184 and CD185 on leukemic B cells was evaluated on day 0 (D0) and day 14 (D14) of IR therapy. The CLL patients’ characteristics and experiments are detailed in Online Supplementary Table S1 and in the Online Supplementary Methods, respectively.

We found that 14 days of IR therapy were sufficient to induce a significant change in the levels of expression of the majority of the markers analyzed (10/15, 66.7%). As shown in Figure 1, among the antigens that were modulated, nine were downmodulated while only one was upmodulated after in vivo treatment. We observed a significant downmodulation of CD62L (467±437 vs. 162±134, \( P < 0.00001 \)), a key molecule in CLL cell migration, adhesion and transendothelial migration. 5 The expression of the CXCL13 chemokine receptor CD185, another mediator of CLL tissue homing, also decreased significantly after in vivo treatment with IR (1396±1340 vs. 810±679, \( P < 0.00001 \)), while we unexpectedly observed upmodulation of the CXCL12 chemokine receptor CD184 (2309±1951 vs. 3127±1799, \( P < 0.00001 \)). These data are consistent with recent reports of both a greater dependency of CD184low CLL cells on microenvironmental stimuli for survival and a correlation between

Figure 1. Effects of ibrutinib plus rituximab in vivo treatment on the expression of chronic lymphocytic leukemia cell adhesion molecules, chemokine receptors and activation markers. Box plots show the comparison between the expression levels for each of the antigens analyzed on primary leukemic B cells before and after 14 days of in vivo treatment with ibrutinib plus rituximab. Data are presented as mean fluorescent intensity values obtained with specific monoclonal antibodies compared with values given by isotype controls. Significant differences are indicated as ****\( P < 0.00001 \), ***\( P < 0.001 \), **\( P < 0.01 \), *\( P < 0.05 \); NS: not significant (paired Student t-test). D0: before treatment, D14: after 14 days of treatment; MFI: mean fluorescent intensity.
BCR-mediated CD184 downregulation and lymphadenopathy in CLL patients. In addition, ibrutinib, by inhibiting CD184-mediated signaling through blockade of CXCL12-mediated phosphorylation at CD184 Ser339, could reduce stromal tethering and further enhance CLL cell egress from lymph nodes in vivo.

CLL cells also showed significantly reduced expression of CD38 and CD49d (n=48/119 CD38+ cases: 874±941 vs. 353±289, P=0.0002 and n=62/119 CD49d+ cases: 1449±1087 vs. 811±790, P<0.00001; respectively), which are associated with a worse prognosis as mediators of leukemic B-cell localization in tissue protective niches, promoting rapid growth and longer cell survival. Furthermore, among cell activation and co-stimulatory molecules, IR treatment induced significant downmodulation of CD44 (10999±6845 vs. 8844±5858, P=0.0011), a surface glycoprotein receptor for hyaluronic acid, CD40 (820±493 vs. 445±309, P<0.00001) and CD69 (825±1069 vs. 464±699, P<0.0001). Since the expression of these markers is characteristic of tissue-resident CLL cells, these results, in line with previous in vitro and in vivo observations, suggest that ibrutinib may reduce leukemic cell activation, interfering with B-cell survival and proliferation. We also recorded a significant downmodulation in the expression of CD43 (3508±2535 vs. 2714±1813, P=0.00005); contrariwise, CD81 expression resulted unchanged after 14 days of treatment (527±661 vs. 423±553, P=0.085). As both antigens are employed in CLL for the flow cytometric detection of minimal residual disease, our data suggest that CD43 is not a reliable marker under ibrutinib therapy and that the identification of different fluorimetric panels excluding CD43 should be considered in this context.

In agreement with the changes in MFI levels, ibrutinib treatment also induced significant decreases in the percentages of CLL cells expressing CD38 (P<0.00001), CD40 (P<0.00001), CD44 (P<0.0001), CD49d (P=0.004), CD62L (P<0.0001), CD69 (P<0.00001) and CD185 (P<0.00001). In contrast, no differences in either MFI or percentage values were observed for CD11a, CD18a,
CD80, CD86 and CD154, as these antigens were barely expressed on leukemia B cells (data not shown).

With regard to the main biological prognostic factors in CLL, no significant differences in antigen modulation were observed according to the IGHV status. This finding is in agreement with the evidence that CLL patients with unmutated or mutated IGHV genes respond equally well to treatment with ibrutinib10 and supports the hypothesis that peripheral lymphocytosis is more related to the role of BTK in cell adhesion and migration, and less to its function in BCR signaling.11 On the contrary, the presence of TP53 mutations was associated with a lower down-modulation of CD62L MFI (21/119 TP53 mutated vs. 98/119 TP53 wild-type cases, ΔMFI: 20±172 vs. 479±424, P=0.039).

Taking into account a lesser dependency of TP53-mutated CLL cells on the BCR pathway for survival and proliferation,12 these data may help to elucidate the biological basis for the suboptimal response of TP53-disrupted patients to ibrutinib alone or in combination with anti-CD20 monoclonal antibodies.13,14

A differential response was also observed with regard to cytogenetic aberrations. On D14, CLL samples harboring an isolated trisomy 12 (tris12+ CLL, n=21/119) showed a significantly greater down-modulation of CD49d and CD62L when compared to CLL cases with other fluorescence in situ hybridization patterns, with the exception of CD49d in del17p+ cases (Figure 2A). These results reflected the increased expression of integrins on tris12+ cells. A higher baseline MFI was indeed observed for CD49d (tris12+ vs. normal karyotype, P=0.016; tris12+ vs. del13q14+, P=0.002; tris12+ vs. del11q14+, P=0.0003; tris12+ vs. del17p+, P=0.029) and CD62L (tris12+ vs. del11q14+, P=0.003; tris12+ vs. del17p+, P=0.026) compared to tris12- samples, which may clarify the specific tropism of tris12+ cells towards lymph nodes and the shortened lymphocytosis observed during ibrutinib monotherapy.15

Lymphocytosis was observed in 36 of the 119 CLL patients (30.3%), with D0 vs. D14 absolute lymphocyte counts being 42.6±26.4x10^9/L vs. 92.8±57.3x10^9/L, respectively (P<0.00001). The lymphocytosis appeared associated with the downmodulation of at least four antigens (P=0.050), independently of the type of antigens involved. No significant differences in antigen modulation were recorded according to the presence (36/119 cases) or absence (83/119 cases) of lymphocytosis, suggesting that ibrutinib-mediated inhibition of microenvironmental interactions could not always turn into an increase in absolute lymphocyte count, probably because of the cytotoxic effect on both tissue-resident and mobilized CLL cells. Accordingly, when CLL patients were subdivided on the basis of level of CD20 expression into those with higher (CD20^hi MFI ≥1000, n=62/119) or lower (CD20^lo MFI <1000, n=57/119) expression, CD20^hi cases showed both a greater clearance of CLL cells (CLL median absolute cell counts on D14 in CD20^hi vs. CD20^lo cases: 38.9±46.7x10^9/L vs. 58.3±48.6x10^9/L, P=0.030) (Figure 2B) and a lower incidence of lymphocytosis (15/62 [24.6%] vs. 21/57 [36.8%], P=0.083) on D14. In addition, CD20^lo CLL samples were characterized by a significantly greater downmodulation of CD38, CD44, CD49d and CD62L antigens (Figure 2C), suggesting that these cells may be more prone to mobilize into the periphery and be exposed to monoclonal antibody-mediated killing.

Finally, in order to assess a possible relationship between antigen expression changes and response to treatment, the MFI modulation on D14 was correlated to the amount of residual CLL cells detected in the peripheral blood 8 months after the start of IR treatment.
Among 91 of 119 cases with available data, three of the 91 (3.3%) were negative for minimal residual disease while 88 (96.7%) were positive with a median minimal residual disease 8 months after starting IR treatment of 18.7%±23.7 (range, 0.01%–94%; mean 0.64%±0.80). These differences became statistically significant for CD44 and CD49d downmodulation (CLL ≤0.1x10^3/μL vs. CLL ≥0.1x10^3/μL: ΔMFI 583±3800 vs. 3655±3226, P=0.025 and 1415±1146 vs. 648±626, P=0.009; respectively), as well as for CD184 upmodulation (CLL <0.1x10^3/μL vs. CLL ≥0.1x10^3/μL: ΔMFI 2111±1635 vs. 1291±1070, P=0.016) (Figure 3), suggesting a potential role for ibrutinib-mediated early modulation of CLL surface antigen expression in predicting the degree of response to IR therapy.

Taken together, the present data demonstrate the profound immunophenotypic changes induced in vivo on primary CLL cells by 14 days of IR therapy. Since most of the molecules involved mediate the interaction of leukemic B cells with the microenvironment, our results may help to elucidate the mobilization process of CLL cells observed during ibrutinib treatment and the relationship between antigen modulation and peripheral lymphocytosis in the context of treatment approaches combining ibrutinib with anti-CD20 antibodies. The ongoing extended follow-up will further clarify the influence of the early antigen modulation on long-term therapeutic efficacy in vivo, in order to better understand the biological basis of the effect of ibrutinib on time and eventually to provide further specific antigen targets for CLL treatment.

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