

1 **Expression of functional sphingosine-1-phosphate receptor 1 is reduced by B-cell receptor**
2 **signalling and increased by inhibition of PI3 kinase δ but not SYK or BTK in chronic**
3 **lymphocytic leukaemia cells**

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13 **Running Title**

14 BCR signalling and S1PR1 expression in CLL

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23 **Abstract**

24

25 B-cell receptor (BCR) signalling pathway inhibitors such as ibrutinib, idelalisib and
26 fostamatinib (respective inhibitors of BTK, phosphatidylinositol-3 kinase δ and SYK)
27 represent a significant therapeutic advance in B-cell malignancies including chronic
28 lymphocytic leukaemia (CLL). These drugs are distinctive in increasing blood lymphocytes
29 whilst simultaneously shrinkage of enlarged lymph nodes, suggesting anatomical
30 redistribution of CLL cells from lymph nodes into the blood. However, the mechanisms
31 underlying this phenomenon are incompletely understood. Here, we showed that the egress
32 receptor, sphingosine-1-phosphate (S1P) receptor 1 (S1PR1), was expressed at low levels in
33 normal germinal centres and CLL lymph nodes *in vivo* but became up-regulated on normal B
34 cells and, to a variable and lesser extent, CLL cells following *in-vitro* incubation in S1P-free
35 medium. Spontaneous recovery of S1PR1 expression on normal B and CLL cells was
36 prevented by BCR cross-linking, whereas treatment of CLL cells with idelalisib increased
37 S1PR1 expression and migration towards S1P, the greatest increase occurring in cases with
38 un-mutated IGHV genes. Intriguingly, ibrutinib and fostamatinib had no effect on S1PR1
39 expression or function. Conversely, chemokine-induced migration, which requires integrin
40 activation and is essential for the entry of lymphocytes into lymph nodes as well as their
41 retention, was blocked by ibrutinib and fostamatinib but not idelalisib. In summary, our
42 results suggest that different BCR signalling inhibitors redistribute CLL cells from lymph
43 nodes into the blood through distinct mechanisms: idelalisib actively promotes egress by
44 up-regulating S1PR1 whereas fostamatinib and ibrutinib may reduce CLL-cell entry and
45 retention by suppressing chemokine-induced integrin activation.

46

47 Introduction

48 Chronic lymphocytic leukaemia (CLL) is a malignancy of mature B cells that can follow
49 either a progressive or an indolent clinical course. Studies of mutational status and gene usage
50 of the immunoglobulin heavy chain variable region (IGHV) of the B cell receptor (BCR) on CLL cells
51 have not only revealed a relationship between IGHV mutation and clinical course, but have also led
52 to wide acceptance of a key role for BCR engagement in disease pathogenesis and clinical behaviour
53 (1). One manifestation of progressive disease in CLL is the development of
54 lymphadenopathy, which results from entry of malignant cells into lymph nodes where they
55 receive signals for survival and proliferation. In a normal lymph node, transendothelial
56 migration (TEM) of B cells from high endothelial venules (HEV) into the interfollicular area is
57 stimulated by the chemokine CCL21 and is dependent on cell adhesion mediated by the
58 integrin $\alpha\text{L}\beta\text{2}$ (Supplementary Figure 1A) (2-5). Once inside the lymph node, B cells then
59 migrate to the follicles in a CXCL13-dependent manner in search of antigen (6). Exit, or
60 egress, of B cells from lymph nodes depends on migration towards sphingosine-1 phosphate
61 (S1P)-rich tissues such as the blood and occurs when the S1P receptor-1 (S1PR1; S1P₁) is up-
62 regulated (7-11). S1PR1 is not expressed by peripheral blood cells as high levels of its ligand
63 S1P cause receptor internalisation. However, when lymphocytes enter the S1P-depleted
64 lymph node environment, the receptor is up regulated and mediates lymphocyte egress
65 (11). In T cells, this process is prevented by activation of the T-cell receptor which results in
66 down-regulation of S1PR1 (11). Importantly, the transit time of normal lymphocytes through
67 lymph nodes is determined by levels of S1PR1 on the cell surface. Thus, lymphocytes that
68 enter the lymph nodes but do not encounter antigen rapidly up-regulate S1PR1 and transit
69 through the node without delay. In contrast, T cells that encounter antigen down-regulate
70 S1PR1 due to repression by TCR signalling and can remain within the lymph node for much

71 longer periods of time (8, 10). The regulation of S1PR1 expression on normal B cells is
72 unclear. However, normal B cells which have been chronically stimulated through their B-
73 cell receptor (BCR) do not migrate towards S1P (12), suggesting that S1PR1 expression may
74 be repressed by BCR signalling.

75 The development of lymphadenopathy in CLL implies either enhanced entry of the
76 malignant cells into lymph nodes and/or their retention within the node (13). As is the case
77 with normal B cells, entry of CLL cells into lymph nodes is also driven by CCL21
78 (Supplementary Figure 1B) (14-16). However, unlike normal B cells, CLL cells additionally
79 require expression and activation of the integrin $\alpha 4\beta 1$ in order to undergo TEM (15, 16).
80 Once inside lymph nodes, CLL cells may respond to CXCL13 because they express high levels
81 of CXCR5 (17). However, the relevance of CXCL13/CXCR5-dependent migration is uncertain
82 since the nodal architecture of CLL lymph nodes is effaced. Retention of CLL cells in lymph
83 nodes may result from enhanced adhesion to extracellular matrices (18) or from reduced
84 expression of S1PR1 (19).

85 Recently, new therapeutic agents have been developed that target kinases involved in
86 the BCR signalling pathway. These include idelalisib (CAL-101; GS-1101), ibrutinib (PCI-
87 32765) and fostamatinib (R406) which inhibit, respectively, phosphoinositol 3-kinase δ
88 (PI3K δ) (20), Bruton's tyrosine kinase (BTK) (21) and spleen tyrosine kinase (SYK), although
89 fostamatinib has additional activity against some other kinases (22, 23). All of these kinase
90 inhibitors induce a rapid lymphocytosis associated with a reduction in lymphadenopathy
91 when given to patients with CLL (24, 25). This strongly implies that these kinase inhibitors
92 produce a mobilising effect by redistributing CLL from the lymph nodes into the blood. In
93 the case of ibrutinib, this effect has been attributed to blockade of BCR- and chemokine-
94 induced integrin activation resulting in reduced adhesion of CLL cells to lymph node stroma

95 (26). However, given that CLL cells are chronically stimulated *in-vivo* through their BCR (27-
96 29) and that antigen receptor stimulation prevents up-regulation of S1PR1 expression on
97 normal T cells in lymph nodes, another possible explanation for the mobilising effect of BCR
98 pathway inhibitors presents itself. Namely, we hypothesized that S1PR1 expression is down-
99 regulated on CLL cells as a result of chronic BCR signalling and that this contributes to their
100 retention within lymph nodes. We further speculated that BCR inhibitors relieve this BCR-
101 mediated repression of S1PR1 expression resulting in the egress of CLL cells from affected
102 lymph nodes. We reasoned that these effects should be most evident in those cases with
103 unmutated IGHV where BCR signalling is particularly active (30).

104 We tested this hypothesis by examining the effects of BCR stimulation and BCR
105 signalling pathway inhibitors on CLL cells of defined IGHV mutational status cultured in the
106 absence of S1P. In keeping with our predictions, idelalisib increased the expression of S1PR1
107 and induced migration towards S1P, the greatest effect being observed in IGHV-unmutated
108 CLL cells. In contrast, fostamatinib and ibrutinib had no such effect but, unlike idelalisib,
109 inhibited CCL21-induced migration. Together, our findings suggest that idelalisib induces
110 CLL-cell mobilisation by actively promoting S1P-directed egress from lymph nodes, whereas
111 fostamatinib and ibrutinib mobilise CLL cells by releasing adhesive interactions and blocking
112 chemokine-directed entry into lymph nodes.

113

114 **Materials and Methods**

115

116 **Patient samples**

117 This study was performed using peripheral blood samples from 20 patients with CLL;
118 clinical data is shown in Supplementary Table 1. Normal B cells were obtained from healthy
119 donors (n=6). Lymph node tissue was obtained from 5 patients with CLL and 4 healthy
120 donors. The IGHV gene usage and extent of somatic hypermutation were determined by
121 comparison to the nearest germline counterpart sequence in the international
122 ImMunoGeneTics (IMGT) information system with IMGT/V-QUEST. A cut-off of 2% IGHV
123 mutation was used to separate cases into mutated (M-CLL) and unmutated (UM-CLL) groups
124 (31, 32). HUVEC were purified from umbilical veins. All samples were obtained with
125 informed consent and with the approval of the Liverpool Research and Ethics Committee,
126 Royal Liverpool and Broadgreen University Hospitals NHS Trust and the Research and
127 Development Committee, Liverpool Women's Hospital NHS Trust.

128

129 **Cell preparation and culture**

130 Primary B lymphocytes (normal and CLL) were used in all the experiments described. Cells
131 were isolated from peripheral blood and buffy coats by centrifugation over Lymphoprep
132 (Axis-Shield, Oslo, Norway). Normal B cells were purified by negative selection using a B-cell
133 isolation kit (Miltenyi Biotech, Biscy, UK) (>98% CD19⁺). All cultures involving primary
134 lymphocytes employed RPMI medium containing 1% fatty acid-free bovine serum albumin
135 (BSA; Sigma, Poole, UK); HUVEC were cultured in IMDM containing 20% foetal calf serum
136 (FCS), whereas HS-5 and CD40L-transfected fibroblasts were cultured in DMEM containing
137 10% FCS. All culture media were supplemented with 2 mM L-glutamine, 100 U/ml penicillin

138 and 100 µg/ml streptomycin (Invitrogen, Paisley, Scotland). Ibrutinib, fostamatinib and
139 idelalisib were all used at 1 µM (Selleckchem, Texas). These drug concentrations were
140 sufficient to maximally inhibit the respective target kinases following BCR ligation (data not
141 shown); IC50 values and peak plasma concentrations are shown in Supplementary Table 2.
142 Goat anti-human IgM [F(ab)2 fragments (Jackson ImmunoResearch, Pennsylvania)] was used
143 at 20 µg/ml.

144

145 **Immunohistochemistry**

146 Tissue staining for S1PR1 was performed using formalin-fixed, paraffin-embedded tissue
147 sections mounted on glass slides. S1PR1 (Abcam, Cambridge, UK) and IgG1 isotypic control
148 (R&D systems Abbingdon, UK) antibodies were used at 4µg/ml, whereas CD20 and CD23
149 were supplied ready to use (Dako, Cambridge, UK). As previously described (33), de-waxing
150 of the sections and antigen retrieval were performed with EnVision™ FLEX target retrieval
151 solution with the Dako PT-link module. Slides were stained with an autostainer using the
152 EnVision™ FLEX convenience kit (Dako) and counterstained in Meyers' haematoxylin
153 (Sigma). Isotypic control staining is shown in Supplementary Figure 2A.

154

155 **Flow cytometry**

156 Cells were simultaneously stained with directly conjugated MAbs to S1PR1 (Clone
157 218713; R&D Systems), CCR7, CD49d ($\alpha 4$ integrin) and CD19 (all from Becton Dickinson,
158 Oxford, UK) together with appropriate isotypic control antibodies and analysed by
159 multicolour flow cytometry. The percentage and mean fluorescence intensity (MFI) for
160 S1PR1, CCR7 and CD49d were determined on CD19⁺ cells. In addition, viability of the cells

161 after culture was assessed using propidium iodide. None of the drugs used decreased the
162 viability of CLL cells following culture. Since cells were cultured in conditions to minimise cell
163 death viability was >75% in the majority of cases.

164

165 **Migration assays**

166 HUVECs were grown to confluence on the inserts of Transwell plates (5 µm pore size;
167 Corning, High Wycombe, UK). S1P (Sigma) or CCL21 (R & D) were added to the bottom wells
168 at concentrations (100 ng/ml and 1 µg/ml, respectively) shown to induce maximum
169 migration (data not shown). CLL cells were added to the inserts, and the number of B cells
170 that had migrated to the bottom wells was counted after 6 h incubation. The migration
171 index (no. of CD19⁺ cells transmigrating with chemokine divided by no. of cells
172 transmigrating in the absence of chemokine) was then calculated.

173

174 **Statistical analysis**

175 The Mann-Witney U test was used to analyse differences in continuous measurement
176 data between treated and untreated samples. Chi squared analysis was used to relate
177 categorical *in-vitro* responses to patient characteristics.

178 **Results**

179

180 **S1PR1 is under-expressed in normal germinal centres and CLL lymph nodes**

181 Expression of S1PR1 within human lymphoid tissues has hitherto not been described.

182 We therefore began our study by staining normal lymph nodes for S1PR1. As is shown in

183 Figures 1A and B, S1PR1 was expressed by all cells within the outer follicle, but not by CD23⁺

184 cells in the germinal centre (GC). In addition, the endothelial cells lining the sinus also

185 stained for S1PR1, as did cells (presumably lymphocytes) within the sinus itself. We next

186 examined lymph nodes from 5 different patients with CLL. In contrast to lymph nodes from

187 healthy individuals, the majority of cells in the CD20⁺ CLL cells within the lymph nodes,

188 including those in the proliferation centres, did not express S1PR1, although the sinus-lining

189 endothelial cells were clearly positive (Figures 1C-F). Thus, the pattern of expression of

190 S1PR1 is as expected in normal lymph node tissue, but in CLL lymph nodes expression of this

191 receptor appears to be down-regulated.

192

193 **S1PR1 expression on normal B and CLL cells is down-regulated by BCR signalling**

194 Most of the research describing S1PR1 expression and function in lymphocytes has

195 been performed on mouse T cells. We therefore continued our characterisation of this

196 receptor on human cells by examining normal B cells cultured in the presence and absence

197 of S1P, the presence of which is known to cause S1PR1 internalisation (11). We found that

198 S1PR1 expression spontaneously increased on normal B cells cultured in the absence of S1P

199 (Figure 2A; Supplementary Figure 2B), an effect that is inhibited when S1P is present

200 (Supplementary Figure 2C). Consistent with the results of others studying the effects of

201 antigen receptor engagement on S1PR1 expression in mouse T cells (11), we found that

202 spontaneous up-regulation of S1PR1 on human B cells cultured in the absence of S1P is
203 similarly repressed by BCR crosslinking (Figure 2B). Importantly, repression of S1PR1
204 expression by BCR crosslinking is reversed by pre-treatment of normal B cells with idelalisib
205 (Figure 2B), suggesting a key role of PI3K δ within the mechanism of this repression. Taken
206 together, our culture and BCR stimulation experiments involving normal B cells are entirely
207 in keeping with the observed distribution of S1PR1 expression in normal lymph node tissues
208 (Figure 1A), and support the notion that S1PR1 is internalised by its ligand in blood but re-
209 expressed in the lymph node where the levels of S1P are low unless it is down-regulated by
210 BCR signalling in the GC.

211 We next examined the effect of S1P withdrawal and BCR crosslinking on CLL cells.
212 Similar to our findings in normal B cells, S1PR1 expression increased spontaneously on CLL
213 cells when they were cultured in the absence of S1P (Figure 2C). However, although the
214 changes in S1PR1 expression reached statistical significance within the group of 20 cases
215 tested ($P<0.002$), the observed increase was delayed (no increase was seen at 4 h and
216 maximal levels were observed after 16 h) and variable, being of significantly smaller
217 magnitude than the increase observed in normal B cells ($P<0.001$; Figure 2D). This variability
218 could not be explained by IGVH mutational status since spontaneous up-regulation of S1PR1
219 was similar in mutated and un-mutated cases ($P=0.387$; Supplementary Figure 2D). Since the
220 greatest increase in S1PR1 expression on CLL cells was seen at 16 h, we used this time point
221 for all subsequent experiments.

222 We next sought to establish whether spontaneous S1PR1 up-regulation expression
223 was repressed by BCR signalling in CLL cells. To do this, the 7 samples displaying the greatest
224 spontaneous up-regulation of S1PR1 at 16 h were cultured in the presence or absence of
225 anti-IgM. In keeping with our observations in normal B cells, the spontaneous increase in

226 S1PR1 was prevented in all 7 cases of CLL by BCR cross-linking ($P=0.018$; Figures 2E and F).
227 Collectively, these findings strongly support a role for BCR signalling in repressing the
228 spontaneous up-regulation of S1PR1 that is observed in the absence of S1P in both normal
229 and CLL B cells.

230

231 **S1PR1 expression on CLL cells is increased by idelalisib but not fostamatinib or ibrutinib**

232 We next addressed the question of whether BCR signalling could modulate S1PR1
233 expression in cases of CLL that displayed little or no spontaneous up-regulation of S1PR1
234 during cell culture. We reasoned that failure of S1PR1 to up-regulate in these cases likely
235 resulted from repression by constitutive BCR signalling. To test this idea, cells from the 20
236 CLL cases shown in Figure 2C were cultured in S1P-free medium in the absence or presence
237 of idelalisib at a concentration (1 μM) known to specifically inhibit PI3K δ (34). In keeping
238 with our hypothesis, we found that treatment of CLL cells with idelalisib resulted in a
239 significant enhancement of spontaneous S1PR1 expression (Figure 3A; Supplementary
240 Figure 2E). This result was in keeping with our observations in BCR-stimulated normal B cells
241 where idelalisib restored spontaneous expression of S1PR1 (Figure 2B). The magnitude of
242 S1PR1 up-regulation induced by idelalisib varied between CLL cases, as did the time required
243 for the maximum increase in S1PR1 expression to be observed. Nevertheless, the increase
244 reached overall statistical significance at both 8 h ($P=0.002$) and 16 h ($P<0.0001$). In contrast
245 to spontaneous up-regulation of S1PR1, up-regulation induced by idelalisib correlated with
246 IGVH status, being higher in cases with un-mutated IGHV genes (Figure 3B; $P=0.0397$).
247 Importantly, inhibitors of other isoforms of PI3K [PI3K α (A66) and PI3K β (TGX-221)] used at
248 the same concentration (1 μM), did not promote spontaneous S1PR1 expression in any of

249 the cases examined (Supplementary Figure 2F; $P>0.14$), indicating that the effect was
250 isoform specific. Similarly, when we compared the effects of idelalisib with those of either
251 fostamatinib or ibrutinib (all at $1\mu\text{M}$), we found that treatment of CLL cells with either of the
252 latter two compounds had no effect on spontaneous S1PR1 expression (Supplementary
253 Figures 2G and H, respectively). Finally, co-culture of CLL cells on endothelial cells (HUVEC),
254 stromal cells (HS-5) or CD40L-expressing fibroblasts to mimic cell interactions in the lymph
255 node microenvironment showed that none of these accessory cells had any effect on the
256 expression of S1PR1 or its up-regulation by idelalisib, nor did they render CLL cells
257 responsive to S1PR1 modulation by fostamatinib or ibrutinib (Figure 3C and Supplementary
258 Figures 2G-2I). Taken together, these results indicate a specific role for PI3K δ in the *in-vivo*
259 regulation of S1PR1 in both normal and CLL B cells that have been stimulated via the BCR.

260

261 **Migration of CLL cells towards S1P is enhanced by idelalisib but not fostamatinib or** 262 **ibrutinib**

263 Having shown that idelalisib increases S1PR1 expression, we next sought to examine
264 its effect on S1PR1 function. To do this, we examined the effect of the 3 BCR signalling
265 inhibitors on CLL-cell transendothelial migration (TEM) towards S1P using a transwell
266 system. As is shown in Figure 4A, untreated CLL cells did not migrate towards S1P. When
267 CLL-cells were treated with idelalisib for 16 h before the assay they displayed marked TEM
268 towards S1P ($P=0.043$); the amount of TEM observed correlating with the level of S1PR1
269 expression at the end of the pre-incubation period. In keeping with the inability of ibrutinib
270 or fostamatinib to up-regulate S1PR1, pre-incubation of CLL cells with either of these drugs
271 did not enhance migration towards S1P (Supplementary Figure 3A). These results

272 demonstrate that the S1PR1 induced by idelalisib is functional and suggest that the CLL-cell
273 mobilising effect of idelalisib observed *in vivo* results at least in part from enhanced S1PR1-
274 dependent egress of CLL cells from lymph nodes.

275

276 **Migration of CLL cells towards CCL21 is inhibited by ibrutinib and fostamatinib but not**
277 **idelalisib**

278 Having elucidated the differential effects of idelalisib and fostamatinib/ibrutinib on
279 S1PR1 expression and function, we next investigated the effects of these BCR signalling
280 inhibitors on the processes responsible for entry of CLL cells into lymph nodes. Our previous
281 work has shown that the latter process is dependent on $\alpha 4\beta 1$ and CCL21 (15). We therefore
282 examined the effect of the 3 inhibitors on CLL-cell TEM towards CCL21. In keeping with the
283 findings of other groups (35, 36), migration was blocked by pre-incubation of CLL cells with
284 either fostamatinib or ibrutinib (Figure 4B). This inhibitory effect did not result from altered
285 expression of either $\alpha 4\beta 1$ or CCR7 since no such change was observed (Supplementary
286 Figure 3B and C). In contrast to fostamatinib and ibrutinib, idelalisib had no effect on
287 migration towards CCL21. Taken together with the experiments examining the effects of the
288 3 BCR signalling inhibitors on S1P migration, it can be deduced that the CLL-cell mobilising
289 effects of these drugs are mediated by different mechanisms; fostamatinib and ibrutinib
290 inhibit signals required for entry and retention of CLL cells into lymph nodes, whereas
291 idelalisib promotes egress by facilitating the up-regulation of functional S1PR1.

292 Discussion

293 The aim of this study was to elucidate the possible role of impaired egress as a
294 determinant of lymphadenopathy in CLL, and relief of impaired egress as an explanation for
295 the mobilising effect of BCR signalling pathway inhibitors. We hypothesized that retention of
296 CLL cells in the lymph nodes results at least in part from an inability to exit due to repression
297 of S1PR1 expression by chronic BCR signalling, and that the CLL-cell mobilising effects of BCR
298 signalling inhibitors results from the reversal of such repression. To test this hypothesis, we
299 first sought to confirm previous reports that that S1PR1 expression is reduced in CLL as
300 compared with normal B cells (19, 37, 38) and in addition demonstrate that S1PR1
301 expression is regulated by BCR signalling. To do this, we cultured the normal B and CLL cells
302 in the absence of S1P to prevent receptor internalisation and showed that spontaneous up-
303 regulation of S1PR1 could be prevented by BCR stimulation. These results are in keeping
304 with a recent report which demonstrated that after long-term culture S1PR1 expression by
305 CLL cells was down-regulated by factors present in the microenvironment, including BCR
306 signalling (37). For those cases of CLL that displayed little or no spontaneous recovery of
307 S1PR1 expression during cell culture, we adopted a complementary approach involving
308 treatment with BCR signalling inhibitors to block presumed endogenous BCR signalling. We
309 tested the effects of 3 different BCR signalling inhibitors, all of which are in clinical
310 development and have a potent CLL-cell mobilising activity. We provide evidence that the
311 mechanisms through which these inhibitors mediate their mobilising effects is profoundly
312 different: idelalisib (targets PI3K δ) increased expression of S1PR1 and stimulated S1P-
313 mediated migration, a process required for exit from lymph nodes; in contrast, fostamatinib
314 (targets SYK) and ibrutinib (targets BTK) blocked chemotaxis towards chemokine, a process
315 required for entry into lymph nodes and subsequent retention. This study therefore

316 provides novel insight into the mobilising effects of BCR signalling inhibitors, and, in
317 particular, allows us to propose that the CLL-cell mobilising effect of idelalisib results, at
318 least in part, from reversal of BCR-mediated repression of S1PR1 expression on the
319 malignant cells leading to enhanced egress from affected lymph nodes, whereas the
320 mobilising effect of ibrutinib is mediated by blockade of integrin-mediated signals required
321 for tissue entry and retention. Our proposal is supported by clinical trial data demonstrating
322 that the blood lymphocytosis observed in patients treated with idelalisib (39) reaches peak
323 levels more rapidly than that induced by ibrutinib (40).

324 The BCR signalling pathway is central to the pathogenesis of CLL not only by providing
325 pro-survival and proliferation signals (27, 28, 41) but also in maintaining malignant-cell
326 residency within lymph nodes (26, 42, 43). For example, BTK lies within the pathway
327 controlling $\alpha4\beta1$ -mediated adhesion in BCR-stimulated cells (44) and can also mediate
328 chemokine-induced migration and homing of normal B, mantle-cell lymphoma and CLL cells
329 (26, 42, 45). Similarly, SYK is necessary for chemokine-induced migration and BCR-mediated
330 adhesion of CLL cells (43, 46). Our demonstration that inhibition of SYK or BTK blocks the
331 TEM of CLL cells towards chemokine is in agreement with these previous studies and
332 supports the notion that the mobilising effects of fostamatinib and ibrutinib result partly
333 from enhanced exit from lymph nodes due to release of $\alpha4\beta1$ -dependent adhesive
334 interactions and partly from reduced lymph node entry due to blockade of CCL21-directed
335 TEM across the HEV.

336 In contrast to the effects seen with fostamatinib and ibrutinib, treatment of CLL cells
337 with idelalisib had no effect on chemokine-induced migration in our experiments. Instead,
338 we found that this compound strongly up-regulated S1PR1 expression on CLL cells and
339 significantly enhanced their migration towards S1P. Importantly, the increase in S1PR1

340 expression and function induced by idelalisib was still observed in the presence of accessory
341 cells similar to those found in the micro-environmental niches where CLL cells reside *in vivo*.
342 The *in-vivo* relevance of our findings is further supported by evidence from normal B and T
343 lymphocytes suggesting that S1PR1-dependent egress overrides the pro-adhesive effects of
344 antigen receptor engagement and chemokines. Thus, lymphocytes are retained in lymphoid
345 and thymic tissues in the absence of functional S1PR1 expression (8, 47). Conversely, B-cell
346 responsiveness to chemokines in lymph nodes is reduced by enforced expression of S1PR1
347 (48). In summary, our results strongly suggest that idelalisib actively promotes S1P-directed
348 egress by up-regulating S1PR1.

349 Our study also provides insight into the mechanisms through which BCR signalling
350 suppresses S1PR1 expression on CLL cells, and by extension, also on normal B cells. In
351 particular, the differential effects of idelalisib and fostamatinib/ibrutinib on S1PR1
352 expression on CLL cells suggests that expression of the latter receptor is regulated by
353 proximal BCR signals mediated by PI3K δ but not distal BCR signals mediated by SYK and BTK.
354 Our data also indicate that the regulation of S1PR1 by PI3K is isoform specific and mediated
355 by the δ isoform specifically. These observations are in alignment with the specific role of
356 PI3K δ in transducing BCR-mediated signals that has been described elsewhere (49, 50) but
357 do not illuminate the process any further. The mechanism through which active PI3K δ
358 regulates S1PR1 expression on lymphocytes is unclear. However, it could potentially involve
359 guanine nucleotide-binding protein-coupled receptor kinase-2 (GRK2) which has been
360 reported to desensitize S1PR1 (51).

361 Our results with idelalisib differ from those obtained in previous *in-vitro* studies which
362 have suggested that the compound is cytotoxic to CLL cells and blocks chemotaxis (20, 52).
363 This discrepancy can be explained by the higher concentrations of idelalisib (5 μ M and 10

364 μM respectively) that were used in these other studies, with the potential for off-target
365 effects. In contrast, the concentration of idelalisib selected for our study ($1 \mu\text{M}$) was the
366 lowest required to block BCR-induced activation of Akt. It is also close to the peak plasma
367 concentration of $2 \mu\text{M}$ predicted from a simplistic one-compartment model of drug
368 disposition involving the administration of a standard dose of idelalisib (150 mg) to a patient
369 of average size (75 kg)(34). Consequently, we believe that our observations with $1 \mu\text{M}$
370 idelalisib result exclusively from inhibition of PI3K δ and are therefore more physiologically
371 relevant than those resulting from higher drug concentrations.

372 Our findings with fostamatinib were only partially in keeping with those of Borge et al
373 (37). Thus, although the latter study showed that $1 \mu\text{M}$ fostamatinib had no effect on S1PR1
374 expression in CLL cells after 24 h culture, incubation with $5 \mu\text{M}$ fostamatinib resulted in
375 increased S1PR1 expression. These findings need to be interpreted in the context of two
376 important facts: first, our own findings (data not shown) and those of others (53) indicate
377 that $1 \mu\text{M}$ fostamatinib can induce total blockade of BCR-induced signalling downstream of
378 SYK (BTK and BLNK) in intact cells; second, the standard therapeutic dose of fostamatinib
379 (500mg) achieves a peak plasma concentration of $1.6 \mu\text{M}$ (21). Consequently, we believe
380 that observations obtained with $1 \mu\text{M}$ fostamatinib are more physiologically relevant than
381 those obtained with the $5 \mu\text{M}$ concentration.

382 By showing that the spontaneous up-regulation of S1PR1 that was observed in a
383 proportion of CLL cases could be reversed by BCR cross-linking and that idelalisib had the
384 greatest effect on CLL cells that did not spontaneously up-regulate S1PR1 in culture, the
385 present study adds further weight to the growing idea that the BCR of CLL cells is chronically
386 stimulated *in vivo*. The notion that CLL cells are subjected to on-going *in-vivo* antigenic
387 stimulation is supported by studies of BCR glycosylation. Thus, the BCR expressed in CLL

388 samples experiencing *in-vivo* BCR engagement contains mannosyl residues consistent with
389 receptor recycling (30). Our demonstration that the up-regulation of S1PR1 by idelalisib is
390 greater in UM- CLL cells compared with M-CLL cells is in keeping the notion that UM CLL
391 cells have greater *in-vivo* BCR signalling activity (30). It also provides an explanation for the
392 particular clinical benefit of idelalisib that is observed in UM CLL (54).

393 In summary, our study is the first to show that BCR signalling represses S1PR1
394 expression and function on CLL cells, potentially leading to delayed egress from lymphoid
395 tissues. It is also the first study to suggest that different inhibitors of BCR signalling induce
396 CLL-cell mobilisation through different mechanisms. In particular, by specifically blocking
397 BCR-induced activation of PI3K δ , we propose that idelalisib activity promotes S1P-mediated
398 egress of CLL cells by relieving BCR-mediated repression of S1PR1 expression. Further work
399 is now required to characterise the underlying mechanisms in the expectation that this may
400 lead to the elucidation of new therapeutic targets.

401 **Conflict of Interest**

402 ARP has received research funding from Gilead. KJT and JS declare no competing financial
403 interests.

404

405 **Authorship**

406 KJT, designed the experimental approaches and assays and performed the experiments. KJT,
407 ARP and JRS designed the study and wrote the manuscript.

408

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596 **Figure legends**

597 **Figure 1. Expression of S1PR1 in normal and CLL node.** A and B. Normal lymph node. The
598 cells of the germinal centre (GC) are identified by staining for CD23 which is mainly
599 expressed on activated B cells. Staining of the adjacent section for S1PR1 shows that CD23⁺
600 cells do not express S1PR1, whereas the cells within the outer follicle (OF) stain positively. C-
601 F. Sequential section of CLL lymph nodes stained for S1PR1 and CD20. In C and D, it can be
602 clearly seen that the endothelial cells lining the sinus (S) and other endothelial cells express
603 S1PR1, whereas CD20⁺ CLL cells lack the receptor. E and F show a representative
604 proliferation centre in a CLL lymph node. CD20⁺ CLL cells do not express S1PR1. Parallel
605 staining with the relevant isotypic control antibody is shown in Supplementary Figure 2A.
606 Bar 50µM; original magnification x20.

607 **Figure 2. S1PR1 expression on normal and CLL B cells cultured in the absence of S1P. A.**

608 Normal B cells from 6 healthy individuals were cultured for 16 h in medium lacking S1P and
609 examined for S1PR1 expression by flow cytometry. An increase in S1PR1 was observed
610 between 2 and 4 h and reached a peak at 8-16 h. **B.** Normal B cells from 6 donors were
611 cultured in S1P-free medium for 16 h in the presence or absence of anti-IgM and/or
612 idelalisib (1 µM). S1PR1 expression was measured by flow cytometry. IgM cross-linking
613 prevented the spontaneous increase in S1PR1 expression ($P=0.0039$). Idelalisib had little
614 effect on S1PR1 expression on normal B cells in the absence of IgM ($P=0.177$), however
615 treatment reversed the anti-IgM-mediated suppression of S1PR1 expression ($P=0.014$) with
616 levels returning to those of untreated cells at 16 h ($P=0.177$). **C.** CLL cells from 20 patients
617 were cultured for 16 h in S1P-free medium and examined for S1PR1 levels by flow
618 cytometry. There was an overall increase in S1PR1 expression ($P < 0.002$) but the increase

619 was variable, delayed and generally of lower magnitude compared to that observed in
620 normal B cells. **D.** Comparison of S1PR1 up-regulation in normal and CLL B cells at 8 and 16
621 h. The increase in expression was significantly greater in normal B cells at both time points.
622 In the box-and-whisker plot, the bar indicates the median of the MFI values for S1PR1
623 expression, whereas an asterisk (*) identifies outlying data points which do not fall within
624 the interquartile range. **E.** 7 of the CLL samples showing most spontaneous increase in
625 S1PR1 expression (highlighted in grey in C) were cultured in S1P-free medium in the
626 presence or absence of anti-IgM and examined for S1PR1 expression by flow cytometry. Up-
627 regulation of S1PR1 was prevented by BCR cross-linking in all cases. **F.** Pooled analysis of the
628 7 cases of CLL described in E showing near-complete abrogation of spontaneous up-
629 regulation of S1PR1 expression ($P=0.73$).

630 **Figure 3. Effect of idelalisib on S1PR1 expression in CLL cells.** **A.** CLL cells from 20 cases
631 were cultured in the presence or absence of idelalisib (1 μ M) and examined by flow
632 cytometry for S1PR1 levels. The chart shows the fold increase in MFI compared with cells
633 cultured for the same amount of time in the absence of idelalisib. The increase in S1PR1
634 expression was statistically significant at both time points ($P<0.002$). **B.** Box and whiskers
635 plot of idelalisib-induced up-regulation of S1PR1 at 16hrs. Idelalisib induced an increase in
636 S1PR1 expression in both IGHV mutated ($n=11$) and un-mutated ($n=8$) CLL samples but the
637 effect was greater in the latter cases ($P=0.0397$). The bar represents the grand median of all
638 samples. **C.** CLL cells displaying little or no spontaneous recovery of S1PR1 expression ($n=4$)
639 were cultured in the presence or absence of idelalisib on different cell monolayers and
640 examined by flow cytometry for S1PR1 levels. A representative example (patient 2141) is
641 shown (data from individual cases are shown in Supplementary Figure 2H). Idelalisib (1 μ M)
642 produced a marked increase in S1PR1 expression at 8 h irrespective of whether the CLL cells

643 were cultured on endothelial cells (HUVEC), stromal cells (HS-5) or CD154-expressing
644 fibroblasts.

645 **Figure 4. Effect of BCR inhibitors on TEM towards S1P and CCL21. A.** CLL cells from 5
646 patients were incubated for 16 h in the presence or absence of idelalisib (1 μ M) and then
647 examined for migration towards S1P using HUVEC-coated transwells. The numbers above
648 bars indicate MFI values for S1PR1 staining at the end of the incubation period. Idelalisib
649 increased TEM towards S1P in all cases, the amount of migration correlating with levels of
650 S1PR1. Untreated CLL cells underwent little or no migration. **B.** CLL cells from 3 patients
651 were cultured in the absence or presence of idelalisib, fostamatinib or ibrutinib (all at 1 μ M)
652 and examined for migration towards CCL21 using HUVEC-coated transwells. Untreated CLL
653 cells underwent TEM towards CCL21. Migration was reduced by fostamatinib and ibrutinib
654 but not idelalisib.

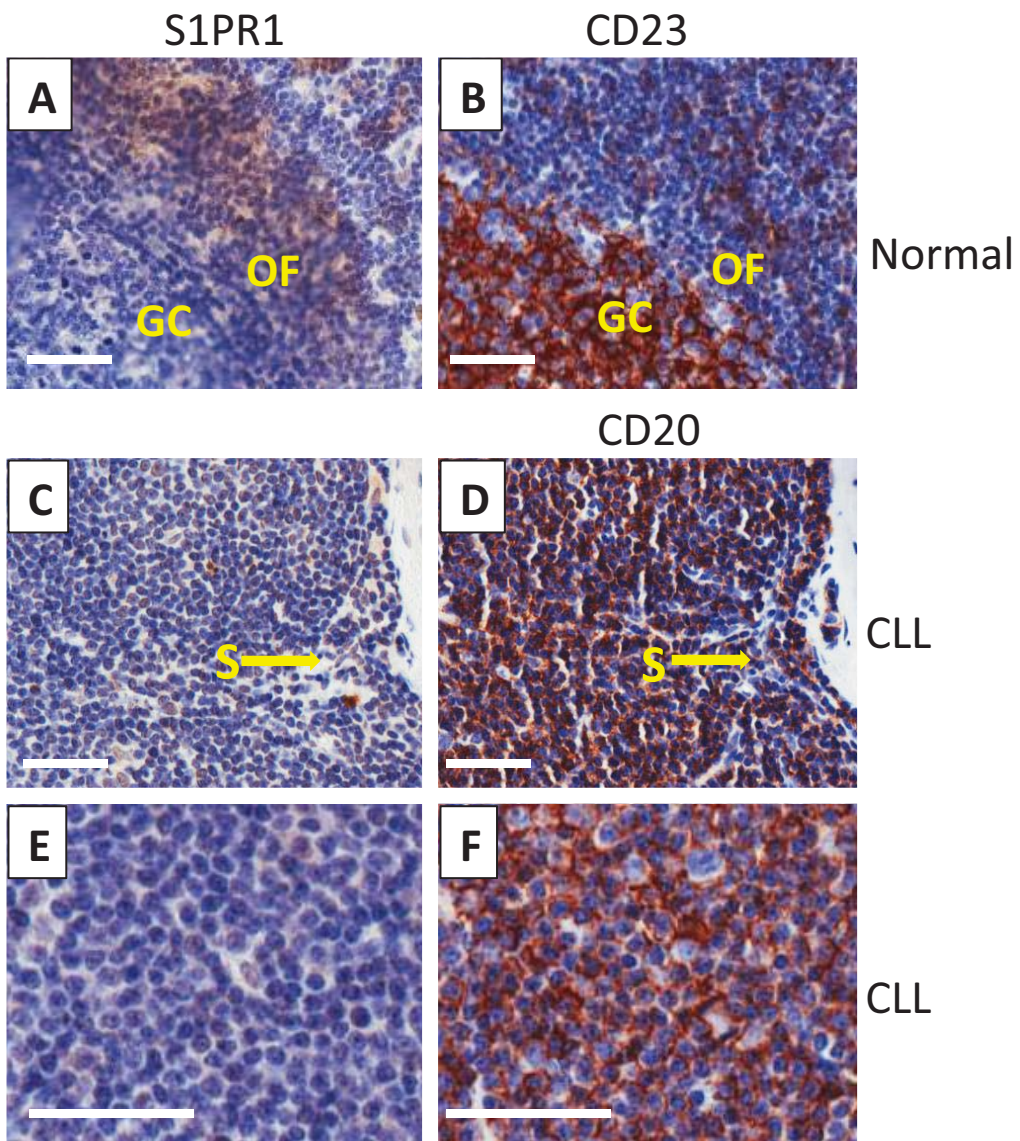


Figure 1. Expression of S1PR1 in CLL and normal lymph nodes.

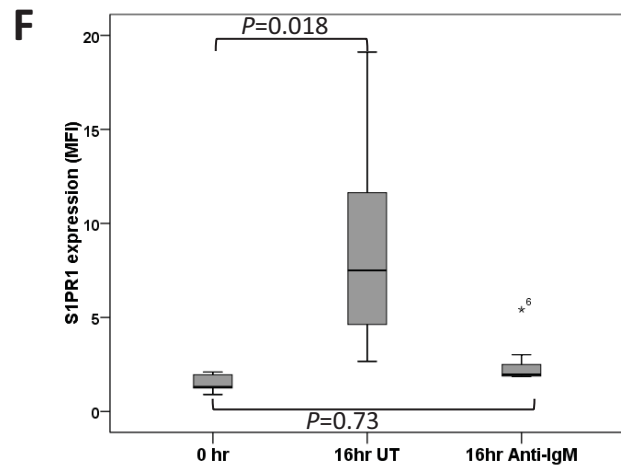
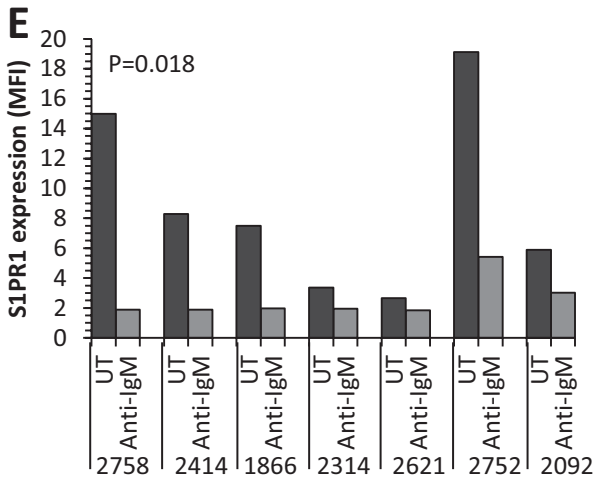
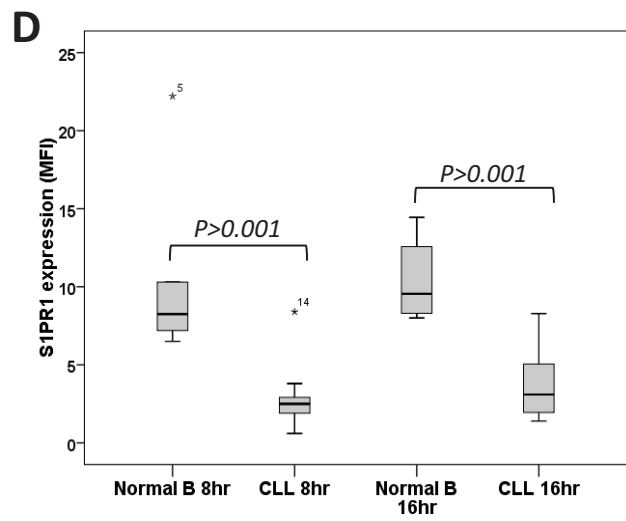
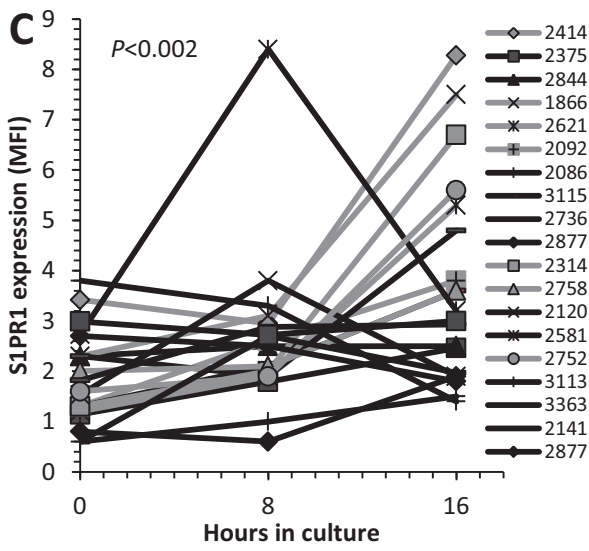
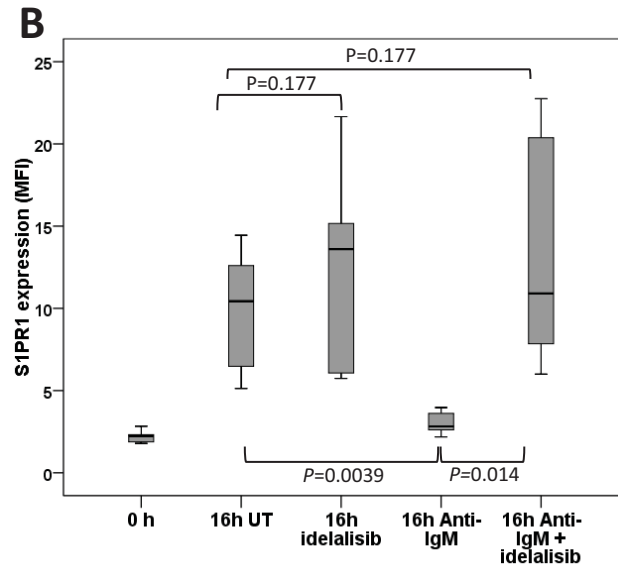
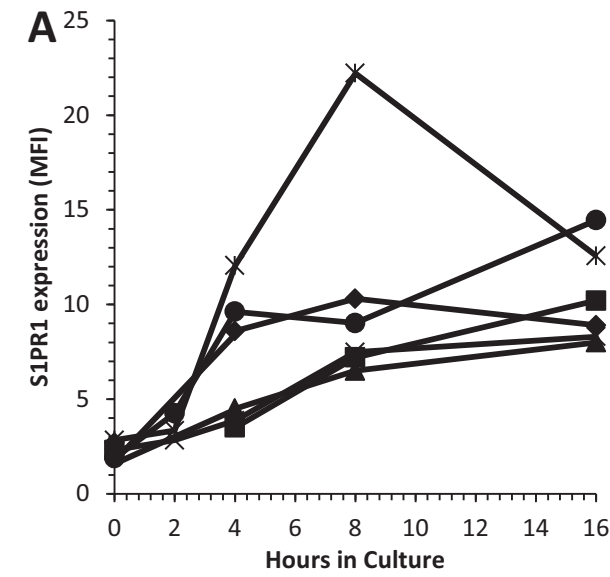


Figure 2. S1PR1 expression on normal and CLL B cells cultured in the absence of S1P.

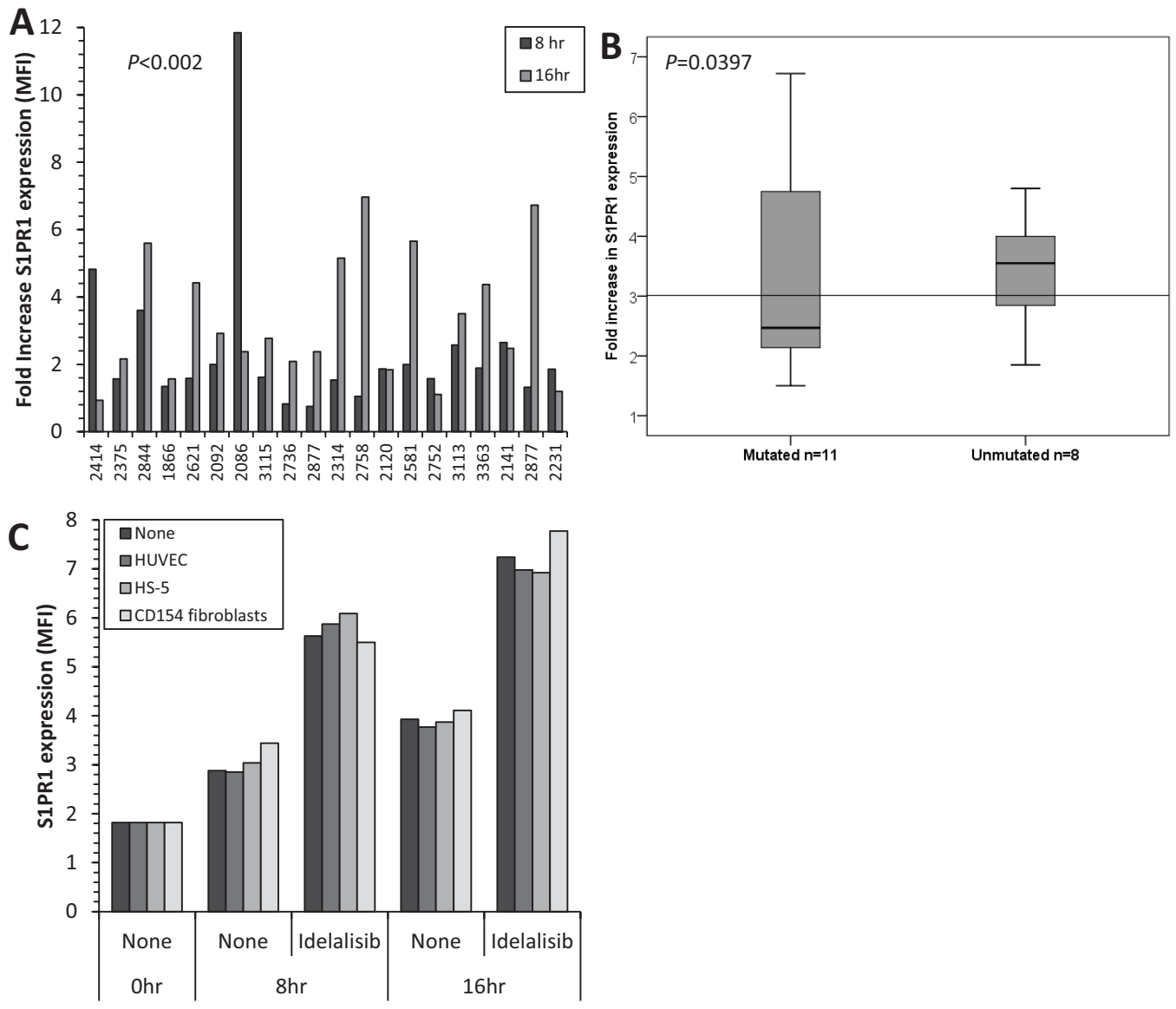


Figure 3. Effect of idelalisib on S1PR1 expression in CLL cells.

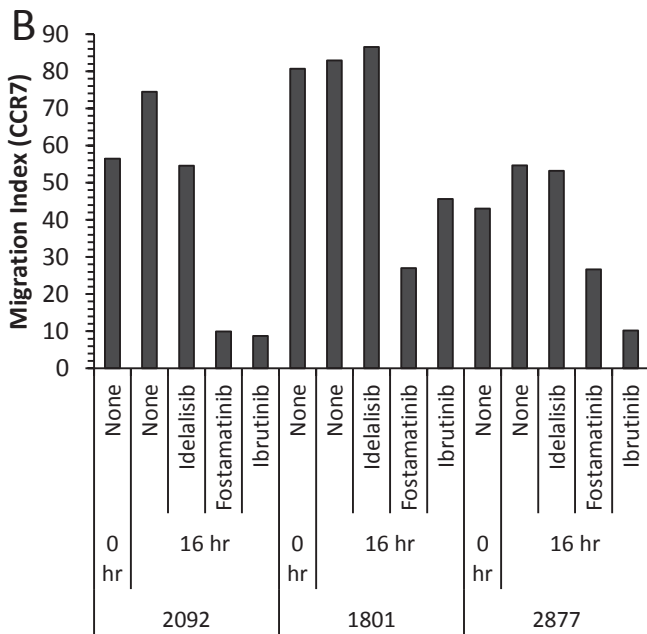
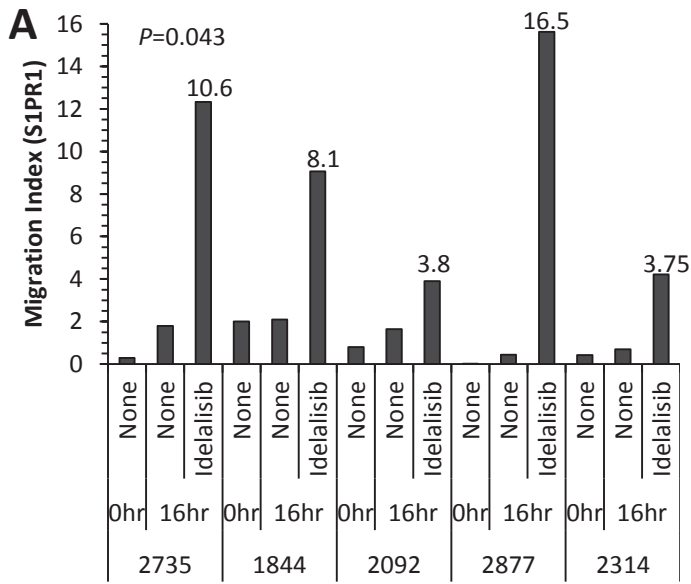


Figure 4. Effect of BCR inhibitors on TEM towards S1P and CCL21.