Ibrutinib and idelalisib target B cell receptor- but not CXCL12/CXCR4-controlled integrin-mediated adhesion in Waldenström macroglobulinemia

The Bruton tyrosine kinase (BTK) inhibitor ibrutinib and the phosphatidylinositol 3-kinase δ (PI3Kδ) inhibitor idelalisib show promising clinical efficacy in the treatment of Waldenström macroglobulinemia (WM), a lymphoplasmacytic lymphoma.[1-3] Very recently, ibrutinib became the first FDA- and EMA-approved treatment for WM patients. Herein, we investigated the molecular and cellular mechanisms underlying the clinical efficacy of ibrutinib and idelalisib in WM patients. We show that, at clinically relevant concentrations, idelalisib, but not ibrutinib, reduced proliferation of WM cells, whereas cytotoxicity was not observed. Furthermore, we demonstrate that WM cells express a signaling-competent B-cell antigen receptor (BCR) which controls integrin-mediated adhesion, and that ibrutinib and idelalisib both inhibit BCR-controlled signaling and integrin-mediated adhesion, whereas chemokine (CXCL12/CXCR4)-controlled signaling, adhesion and migration are not affected. Our data indicate that ibrutinib and idelalisib target BCR-controlled retention of WM cells in the lymphoid organs, resulting in the clinically observed mobilization of malignant cells from these protective niches into the circulation; this may deprive the WM cells from essential microenvironmental growth and survival factors, resulting in WM regression. In addition, our results provide a molecular explanation for the relative ibrutinib resistance of WM patients with gain-of-function CXCR4 mutations.

WM, a lymphoplasmacytic lymphoma, is characterized by the accumulation of post-germinal center B cells in bone marrow, spleen, liver and lymph nodes, which produce a monoclonal IgM M-protein. Apart from genetic lesions in the malignant cells, such as *MYD88* and *CXCR4* mutations, the bone marrow and lymphoid microenvironments play a critical role in the survival and proliferation of these cells. The CXCL12/CXCR4-axis plays a major role in the homing of WM cells to these protective niches. Furthermore, WM cells express a biased IgHV repertoire,[4] suggesting that (antigen-dependent) BCR signaling plays a role in the pathogenesis of WM.

In CLL and MCL, BCR signaling plays a prominent role in the regulation of integrin-mediated retention of malignant cells in lymphoid organs.[5-8] In these patients, the BCR signalosome inhibitors ibrutinib and idelalisib induce a rapid decrease in lymphadenopathy, accompanied by transient lymphocytosis.[6,9-11] In CLL and MCL, we have previously demonstrated that ibrutinib and idelalisib target BCR-controlled - and ibrutinib also chemokine-controlled - integrin-mediated adhesion, resulting in mobilization of the malignant cells from their protective niches in the lymphoid organs into the circulation, followed by lymphoma regression.[5,6,8] Recently, ibrutinib received FDA and EMA approval for the treatment of CLL and MCL, and idelalisib for small lymphocytic lymphoma and follicular lymphoma. Clinical trials for WM were also very promising, with an overall response rate of 90.5% (n=63) for ibrutinib,[3] and 55-80% (n=9 and n=10) for idelalisib,[1,2] and recently, ibrutinib became the first ever FDA-approved treatment for WM patients. Interestingly, in WM patients ibrutinib also induces lymphocytosis, but patients with *CXCR4* mutations are relatively resistant against ibrutinib.[3] Herein, we investigated the molecular and cellular mechanisms underlying the

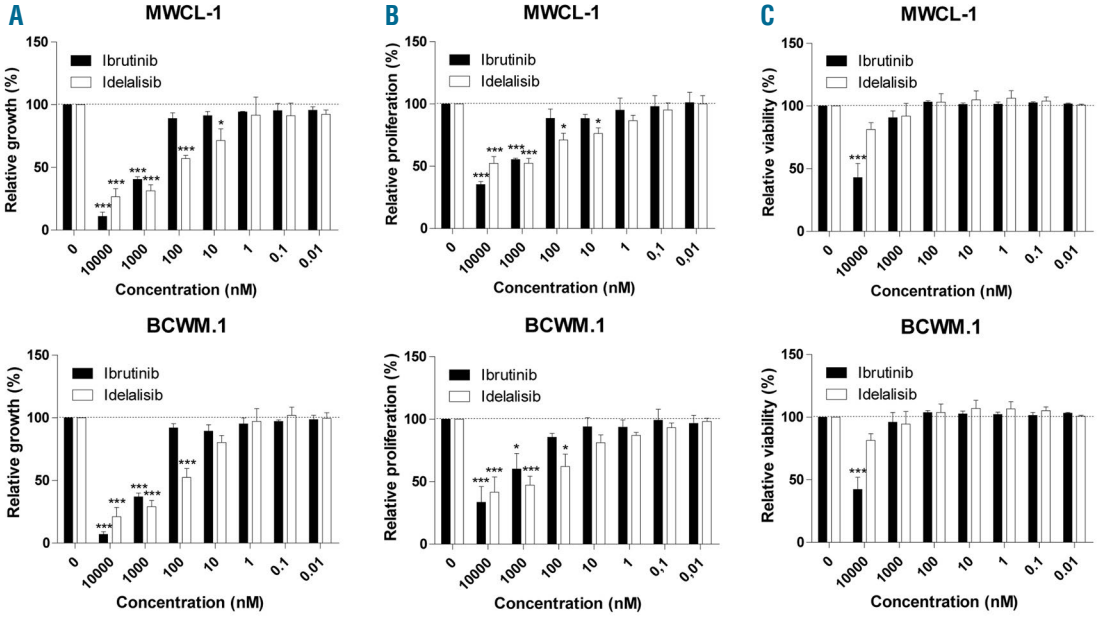

Figure 1. Idelalisib, but not Ibrutinib, strongly inhibits WM proliferation. MWCL-1 and BCWM.1 cells were labelled with CFSE and cultured in the presence of different concentrations of ibrutinib or idelalisib. After 5 days, the numbers of viable cells were counted (A), proliferation was measured by analyzing the CFSE dilution (B), and the viability was determined (C), (n=3 independent experiments). Graphs are presented as normalized mean + SEM (100% = cells treated with only DMSO). **P*<0.05; ***P*<0.01; ****P*<0.001, significantly different from DMSO controls (one-way ANOVA followed by Dunnett's *t*-test).

clinical efficacy of ibrutinib and idelalisib in WM patients.

First we assessed the possible effect of ibrutinib and idelalisib on cell growth in the WM cell lines MWCL-1 and BCWM.1, which both carry the WM characteristic MYD88^{L265P} mutation (*Online Supplementary Figure S1A*).[12] Cell growth was already reduced at 10-100nM idelalisib, but only at 1μM ibrutinib (Figure 1A and *Online Supplementary Figure S2A, S2B*). The observed dose-dependency of ibrutinib was in agreement with Yang *et al.*[13] Distinguishing between proliferation and viability revealed that at clinically relevant/achievable concentrations (i.e., C_{max} ibrutinib 170nM (dose 420 mg/day)[14] and idelalisib 6μM (dose 350 mg/day)[10]) only idelalisib inhibited proliferation, whereas neither drug affected cell viability (Figure 1B,C and *Online Supplementary Figure S2A, S2B*). The differential effect of idelalisib and ibrutinib may reflect the capacity of PI3K to regulate not only BTK- but also AKT-mediated signaling (Figure 2A),

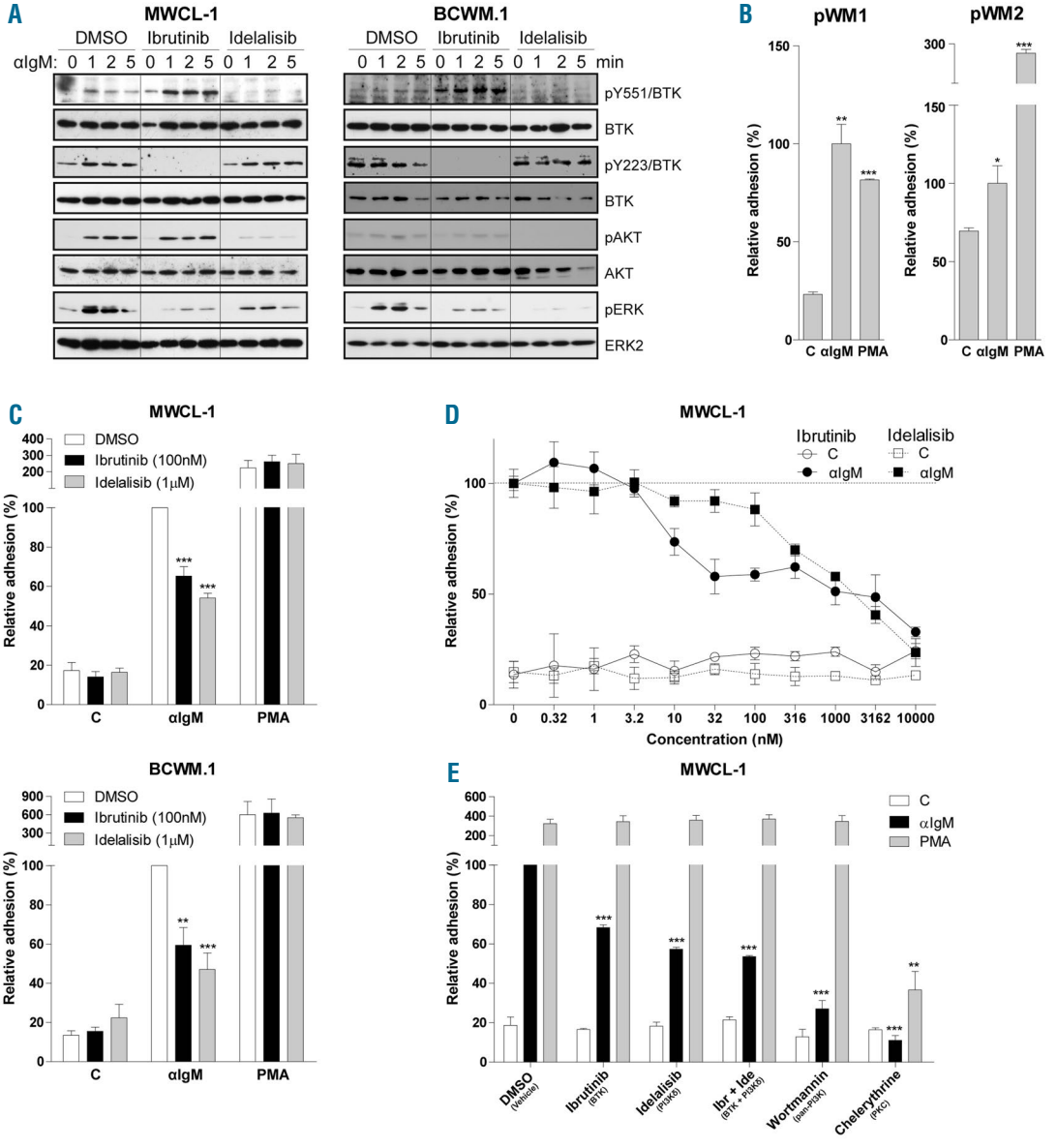

Figure 2. Ibrutinib and idelalisib target BCR-controlled signaling and integrin-mediated adhesion of WM cells. (A) MWCL-1 and BCWM.1 cells pretreated with 100 nM ibrutinib or 1 μM idelalisib were stimulated with 500 ng/ml αIgM, and immunoblotted for p-BTK (pY551 and pY223), p-AKT, and p-ERK. Total BTK, AKT, and ERK2 were used as loading controls. (B) Bone marrow mononuclear cells from 2 WM patients were stimulated with αIgM or PMA, and allowed to adhere to fibronectin-coated surfaces for 30 minutes. Adherence of CD19$^+$ WM cells was quantified by flow cytometry. (C) MWCL-1 and BCWM.1 cells pretreated with 100 nM ibrutinib or 1 μM idelalisib were stimulated with αIgM or PMA, and allowed to adhere to fibronectin-coated surfaces for 30 minutes. The means are from 6 (BCWM.1) or 10 (MWCL-1) independent experiments. (D) MWCL-1 cells pretreated with different concentrations of ibrutinib or idelalisib were stimulated with αIgM and allowed to adhere to fibronectin-coated surfaces for 30 minutes. (E) MWCL-1 cells pretreated with different BCR signalosome inhibitors were stimulated with αIgM or PMA, and allowed to adhere to fibronectin-coated surfaces for 30 minutes (n=3 independent experiments). Graphs are presented as normalized mean + SEM (100% = stimulated cells without inhibitors). *P<0.05; **P<0.01; ***P<0.001, significantly different from DMSO controls (one-way ANOVA followed by Dunnett's t-test).

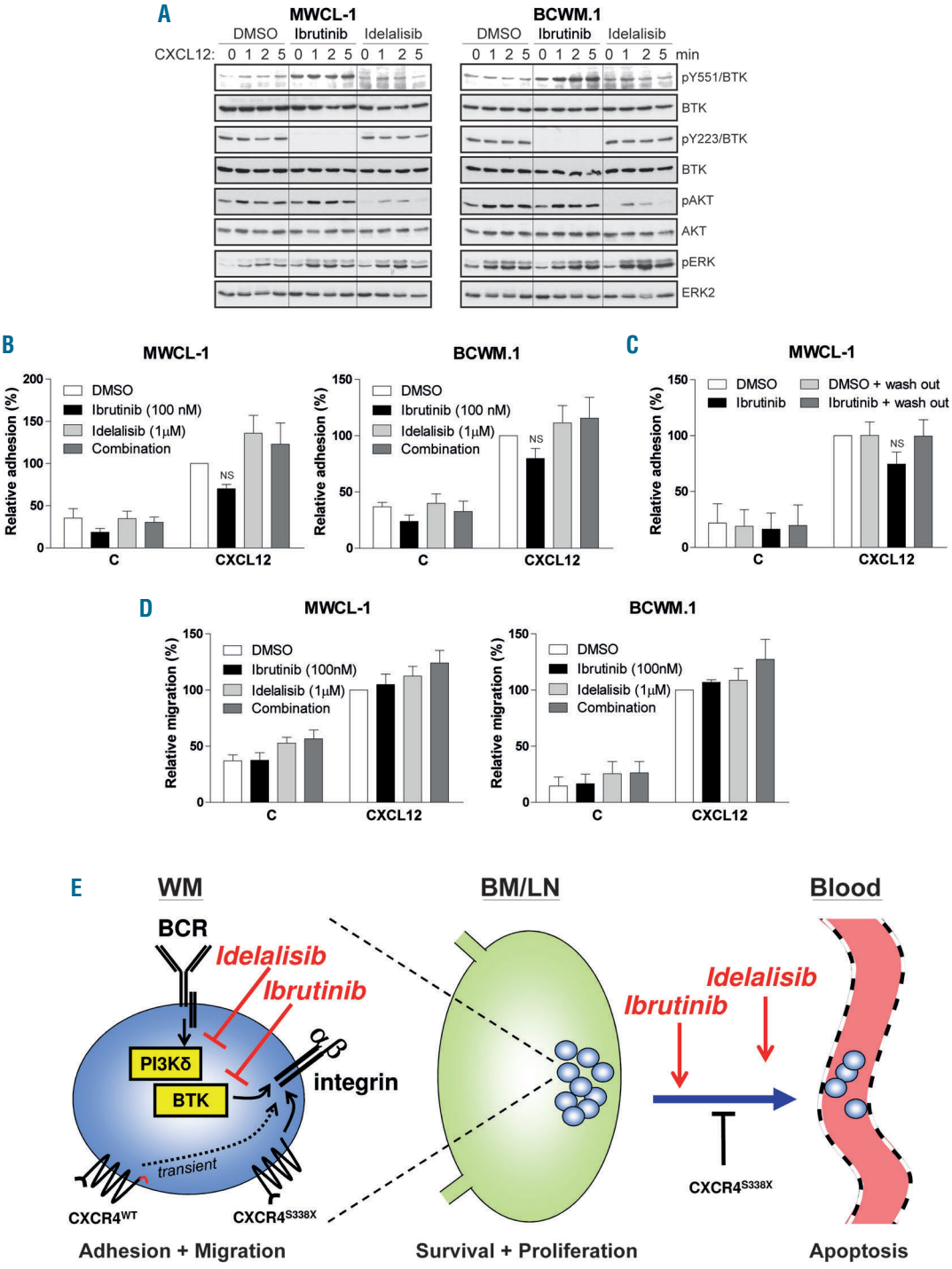

Figure 3. Ibrutinib and idelalisib do not target CXCL12-controlled integrin-mediated adhesion and migration of WM cells. (A) MWCL-1 and BCWM.1 cells pretreated with 100 nM ibrutinib or 1 μM idelalisib were stimulated with 100 ng/ml CXCL12, and immunoblotted for p-BTK (pY551 and pY223), p-AKT, and p-ERK. Total BTK, AKT, and ERK2 were used as loading controls. (B) MWCL-1 and BCWM.1 cells pretreated with 100 nM ibrutinib and/or 1 μM idelalisib were allowed to adhere to VCAM-1- and CXCL12-cocoated surfaces for 5 minutes (n=3 independent experiments). (C) MWCL-1 cells pretreated with 100 nM ibrutinib were subsequently washed (to remove any non-covalently bound ibrutinib) and allowed to adhere to VCAM-1- and CXCL12-cocoated surfaces for 5 minutes (n=3 independent experiments). (D) MWCL-1 and BCWM.1 cells pretreated with 100 nM ibrutinib and/or 1 μM idelalisib were allowed to migrate towards CXCL12 in VCAM-1-coated transwells for 5 hours (n=3 independent experiments). (E) Model of the mechanism of the action of ibrutinib and idelalisib in WM. Inhibition of BTK by ibrutinib or PI3Kδ by idelalisib impairs BCR-controlled integrin-mediated adhesion of WM cells in bone marrow (BM) and lymph nodes (LN), which results in their egress from these protective niches into the circulation, resulting in WM regression. The homing receptor CXCR4 is normally desensitized upon binding of CXCL12, which is highly expressed within the lymphoid organ microenvironment; however, this is prevented by the WHIM-like mutation (S338X), lacking the regulatory domain. Consequently, $CXCR4^{S338X}$ might aberrantly support retention of WM cells in the lymphoid organs. Since CXCR4-controlled integrin-mediated adhesion is insensitive to ibrutinib and idelalisib, this would counteract their ability to inhibit BCR-controlled integrin activation, thus explaining the clinically observed ibrutinib resistance of WM patients with the $CXCR4^{S338X}$ mutation. Graphs are presented as normalized mean + SEM (100% = stimulated cells without inhibitors). NS: not significantly different from DMSO controls (one-way ANOVA).

including mTOR, GSK3 and FOXO pathways. Furthermore, it is tempting to suggest that aberrant NFκB activation by mutant MYD88 may compensate for ibrutinib treatment, since combining IRAK inhibitors with ibrutinib enhances NFκB inhibition and WM cytotoxicity.[13]

BCR signaling controls survival, proliferation, and adhesion of B cells. After having established that the BCR is expressed (*Online Supplementary Figure S1B, S1C*) and functional in MWCL-1 and BCWM.1 cells (Figure 2A,C) and in primary WM cells (Figure 2B), we investigated how ibrutinib and idelalisib affect BCR-controlled signaling. Ibrutinib completely abrogated BTK autophosphorylation (Y223), whereas phosphorylation of the activating LYN/SYK substrate site Y551 of BTK was actually augmented (Figure 2A). A similar potentiation of BTK-Y551 phosphorylation upon ibrutinib treatment, indicative for the inhibition of BTK-mediated negative feedback of proximal BCR signaling, has been observed in other B cell lines [(e.g. Namalwa Burkitt's cells,[5] and MCL cell lines (de Rooij, M *et al.*, manuscript in preparation)]. Interestingly, this occurred in the absence of additional BCR cross-linking of the WM cells (Figure 2A), suggesting substantial basal BCR signaling (tonic/chronic BCR signaling). In addition, ERK activation was inhibited by ibrutinib, but AKT activation was not affected. Previously, we and others have reported that ibrutinib abrogated AKT signaling in CLL and MCL cells,[5,6] but we have recently demonstrated that this was not related to specific BTK inhibition (de Rooij, M *et al.*, manuscript in preparation). Idelalisib completely abrogated AKT activation. The activation of BTK by LYN/SYK, which requires PIP3-mediated membrane association, and the activation of ERK were also inhibited by ibrutinib, but BTK autophosphorylation was not affected (Figure 2A).

An important function of the BCR, which is of major relevance for the clinical efficacy of ibrutinib and idelalisib in CLL and MCL, is the control of integrin-mediated adhesion/retention. Indeed, BCR stimulation induced adhesion of the WM cell lines as well as primary WM cells to the extracellular matrix component fibronectin and the cell adhesion molecule VCAM-1 (Figure 2B,C and *Online Supplementary Figure S3A*), which is mediated by integrin α4β1 (being expressed on MWCL-1 and BCWM.1 cells (*Online Supplementary Figure S1C*)). Moreover, BCR-controlled adhesion was inhibited by 40-50% upon ibrutinib and idelalisib treatment (Figure 2C and *Online Supplementary Figure S3A*). Adhesion in response to the PKC activator PMA was not attenuated (Figure 2C and *Online Supplementary Figure S3A*), demonstrating that the observed effects of ibrutinib and idelalisib on BCR-controlled integrin activation were selective, and not caused by cytotoxicity. The inhibition of adhesion by ibrutinib and idelalisib was already observed at 3.2nM and 100nM, respectively (Figure 2D), well within their clinically achievable ranges. Furthermore, the ibrutinib effect persisted upon wash out (*Online Supplementary Figure S3B*), indicating it is BTK-specific, involving covalent irreversible binding of ibrutinib to BTK. Unlike in CLL and MCL,[8] combining the drugs did not enhance the inhibitory effect (Figure 2E and *Online Supplementary Figure S4A, S4B*). Nevertheless, combination (or sequential) therapy could still be beneficial in WM as it may possibly prevent or overcome single drug resistance, e.g. due to mutations in BTK or PLCγ2. The partial effects of ibrutinib and idelalisib on adhesion can be explained by the involvement of parallel pathways, implicating other kinases. In support of this, the pan-PI3K inhibitor wortmannin and the more distally acting PKC inhibitor chelerythrine completely abolished BCR-controlled adhesion (Figure 2E). Given the previously reported interaction of BTK with $MYD88^{L265P}$,[13] it is tempting to speculate that BTK engaged in either the TLR or BCR signalosome may control different cellular functions.

Many components of the BCR signalosome are also involved in CXCL12/CXCR4-signaling. CXCR4 is expressed on MWCL-1 and BCWM.1 cells, although at low levels (*Online Supplementary Figure S1C-S1E*). Membrane expression and CXCL12-induced internalization of CXCR4 were not affected by ibrutinib and idelalisib (*Online Supplementary Figure S5*). As mentioned, treatment with ibrutinib completely abrogated BTK autophosphorylation (Y223), whereas phosphorylation of Y551 was augmented; however, both occurred already in the absence of CXCL12, indicating that it may rather reflect the inhibition of (tonic) BCR signaling (Figure 3A). Furthermore, ibrutinib did not affect CXCL12-induced activation of ERK and AKT. Idelalisib reduced AKT phosphorylation, but again already in the absence of CXCL12, most likely reflecting inhibition of (tonic) BCR signaling (Figure 3A). The activation of BTK and ERK were not affected. Moreover, CXCL12-induced adhesion and migration were not (specifically) inhibited by ibrutinib and idelalisib (Figure 3B-D); the effect of ibrutinib on CXCL12-induced adhesion was reversible upon wash out, which demonstrates it does not reflect a BTK-specific action of ibrutinib (Figure 3C). Together, these data indicate that the BCR signalosome components are not critical for CXCL12-induced responses in WM.

Although CXCL12 is important for homing, most likely it is not involved in retention: in lymphoid tissues CXCL12 is abundantly expressed, causing CXCR4 desensitization and internalization. Interestingly however, approximately 30% of WM patients carry WHIM-like mutations in CXCR4 (e.g. S338X); this mutation prevents CXCR4 desensitization,[15] which may result in aberrant CXCL12-controlled adhesion and sustained retention of WM cells in lymphoid organs (Figure 3E). Combined with our observation that ibrutinib (or idelalisib) does not target CXCL12-controlled adhesion of WM cells, this may explain why WM patients with these gain-of-function CXCR4 mutations show a strongly reduced lymphocytosis upon, and are less responsive to, ibrutinib treatment as compared to patients with wild-type CXCR4.[3]

Taken together, our data show that ibrutinib and idelalisib target tonic and antigen-controlled BCR signaling in WM cells, thereby inhibiting BCR-controlled integrin-mediated adhesion (Figure 3E). *In vivo* this would result in impaired retention of WM cells in lymphoid tissues, explaining the lymphocytosis observed upon ibrutinib treatment.[3] Thus, our data indicate that ibrutinib and idelalisib do not directly kill WM cells but rather target their BCR-controlled adhesion, thereby causing mobilization of the malignant cells from the protective niches in the lymphoid organs into the circulation, resulting in the deprivation of microenvironmental growth and survival factors, and clinically evident WM regression.

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