

CXCR4 WHIM-like frameshift and nonsense mutations promote ibrutinib resistance but do not supplant *MYD88*^{L265P}-directed survival signalling in Waldenström macroglobulinaemia cells

Yang Cao,^{1,2} Zachary R. Hunter,^{1,3}
Xia Liu,^{1,2} Lian Xu,¹ Guang Yang,^{1,2}
Jie Chen,¹ Nickolas Tsakmaklis,¹
Sandra Kanan,¹ Jorge J. Castillo^{1,2} and
Steven P. Treon^{1,2}

¹Bing Center for Waldenström's Macroglobulinemia, Dana Farber Cancer Institute, ²Department of Medicine, Harvard Medical School, and ³Department of Pathology, Boston University Medical School, Boston, MA, USA

Received 6 August 2014; accepted for publication 12 September 2014

Correspondence: Steven P. Treon, Bing Center for Waldenström's Macroglobulinemia, Dana Farber Cancer Institute, M548, 450 Brookline Avenue, Boston, MA 02115, USA.

E-mail: steven_treon@dfci.harvard.edu

Summary

CXCR4^{WHIM} frameshift and nonsense mutations follow *MYD88*^{L265P} as the most common somatic variants in Waldenström Macroglobulinaemia (WM), and impact clinical presentation and ibrutinib response. While the nonsense (*CXCR4*^{S338X}) mutation has been investigated, little is known about *CXCR4* frameshift (*CXCR4*^{FS}) mutations. We engineered WM cells to express *CXCR4*^{FS} mutations present in patients, and compared their CXCL12 (SDF-1a) induced signalling and ibrutinib sensitivity to *CXCR4*^{wild-type (WT)} and *CXCR4*^{S338X} cells. Following CXCL12 stimulation, *CXCR4*^{FS} and *CXCR4*^{S338X} WM cells showed impaired *CXCR4* receptor internalization, and enhanced AKT1 (also termed AKT) and MAPK1 (also termed ERK) activation versus *CXCR4*^{WT} cells ($P < 0.05$), though MAPK1 activation was more prolonged in *CXCR4*^{S338X} cells ($P < 0.05$). *CXCR4*^{FS} and *CXCR4*^{S338X} cells, but not *CXCR4*^{WT} cells, were rescued from ibrutinib-triggered apoptosis by CXCL12 that was reversed by AKT1, MAPK1 or *CXCR4* antagonists. Treatment with an inhibitor that blocks *MYD88*^{L265P} signalling triggered similar levels of apoptosis that was not abrogated by CXCL12 treatment in *CXCR4*^{WT} and *CXCR4*^{WHIM} cells. These studies show a functional role for *CXCR4*^{FS} mutations in WM, and provide a framework for the investigation of *CXCR4* antagonists with ibrutinib in *CXCR4*^{WHIM}-mutated WM patients. Direct inhibition of *MYD88*^{L265P} signalling overcomes CXCL12 triggered survival effects in *CXCR4*^{WHIM}-mutated cells supporting a primary role for this survival pathway in WM.

Keywords: Waldenström macroglobulinaemia, WHIM, *CXCR4*, *MYD88*, ibrutinib.

CXCR4 WHIM-like mutations constitute the second most common somatic mutations after *MYD88*^{L265P} in Waldenström Macroglobulinaemia (WM; Treon *et al*, 2012; Hunter *et al*, 2014). Approximately 30–35% of WM patients harbour somatic mutations in the regulatory C-terminal domain of *CXCR4* that extends from amino acids 308–352. Both nonsense and frameshift somatic mutations in this region have been described in WM patients (Hunter *et al*, 2014; Treon *et al*, 2014). The location of these somatic mutations in WM patients is similar to germline mutations found in patients with WHIM (Warts, Hypogammaglobulinaemia, Infections and Myelokathexis) syndrome, a congenital immunodeficiency disorder characterized by chronic noncyclic neutropenia (Dotta *et al*, 2011).

In WM cells, expression of the *CXCR4*^{S338X} nonsense mutation blocks CXCL12 (also termed SDF-1a) induced *CXCR4* receptor internalization, leading to a persistent activation state (Cao *et al*, 2014; Roccaro *et al*, 2014). Both AKT1 (also termed AKT) and MAPK1 (also termed ERK) are activated following SDF-1a engagement of *CXCR4*, and the presence of the *CXCR4*^{S338X} mutation results in enhanced and prolonged activation of AKT1 and MAPK1 in comparison to *CXCR4*^{WT} (Cao *et al*, 2014). CXCL12-triggered activation of AKT1 and MAPK1 contributes to enhanced WM cell survival and resistance to ibrutinib and other WM-used therapeutics in *CXCR4*^{S338X} engineered cells (Cao *et al*, 2014; Roccaro *et al*, 2014). Clinical resistance to ibrutinib in WM patients with *CXCR4* nonsense

or frameshift (FS) mutations has recently been reported, with persistent AKT1 activation observed in patients with *CXCR4*^{S338X} mutations (Treon *et al*, 2013; Cao *et al*, 2014). While the impact of the nonsense *CXCR4*^{S338X} mutation on CXCL12-induced signalling and drug resistance has been examined in WM cells, no informative studies on the impact of *CXCR4*^{FS} mutations have been reported.

A pro-survival role of the *MYD88*^{L265P} mutation has been demonstrated in WM, a somatic mutation found in 90–95% of WM patients (Treon *et al*, 2012; Jimenez *et al*, 2013; Poulain *et al*, 2013; Varettoni *et al*, 2013; Yang *et al*, 2013; Ansell *et al*, 2014). Nearly all WM patients with *CXCR4*^{WHIM} mutations, regardless of nonsense or frameshift mutation status, express the *MYD88*^{L265P} mutation (Hunter *et al*, 2014; Treon *et al*, 2014). It remains unclear if CXCL12-triggered survival in WM cells supplants the pro-survival signalling of the *MYD88*^{L265P} mutation in WM. We therefore examined the impact of *CXCR4*^{FS} mutations by engineering WM cells to express *CXCR4*^{FS} mutations identified by whole genome sequencing (WGS) in WM patients, and studied their impact in WM cells on CXCL12-triggered CXCR4 signalling and ibrutinib-mediated apoptosis. We also examined the impact of blocking MYD88 signalling on the pro-survival effects of *CXCR4*^{WHIM} mutations in order to clarify their relative role in supporting WM cell survival.

Materials and methods

CXCR4^{WT}, *CXCR4*^{FS} and *CXCR4*^{S338X} cDNAs were subcloned into plenti-IRES-GFP vector, and transduced using a lentiviral based strategy into BCWM.1 WM cells as before (Yang *et al*, 2013; Cao *et al*, 2014). Frameshift mutations proximal and distal to *CXCR4*^{S338X} that were identified in WM patients by WGS were studied (Fig 1). One frameshift variant resulted from insertion of T at position 136872570 resulting in T311fs; the other frameshift variant resulted from GAAGACTCAG>AC at position 136872467 resulting in S344fs. The nonsense mutation *CXCR4*^{S338X} resulted from a C>G change at 136872485 (Hunter *et al*, 2014). Five days after transduction, green-fluorescent protein (GFP) positive cells were sorted and used for functional studies. Surface CXCR4 expression was determined at baseline and following stimulation for 30 min with CXCL12 (10–100 nmol/l), as before (Cao *et al*, 2014). Phosphoflow experiments were performed using conjugated antibodies to phospho-MAPK11/2 (T²⁰²/Y²⁰⁴) and phospho-AKT1(S⁴⁷³) (BD Biosciences, Can Jose, CA, USA) as previously described (Cao *et al*, 2014). Ibrutinib was obtained from MedChem Express (Monmouth Junction, NJ, USA). Cell signalling and survival studies related to CXCR4 signalling were performed in the presence or absence of CXCL12 (20–50 nmol/l) and ibrutinib (5.0 μmol/l). For survival studies, WM cells were incubated

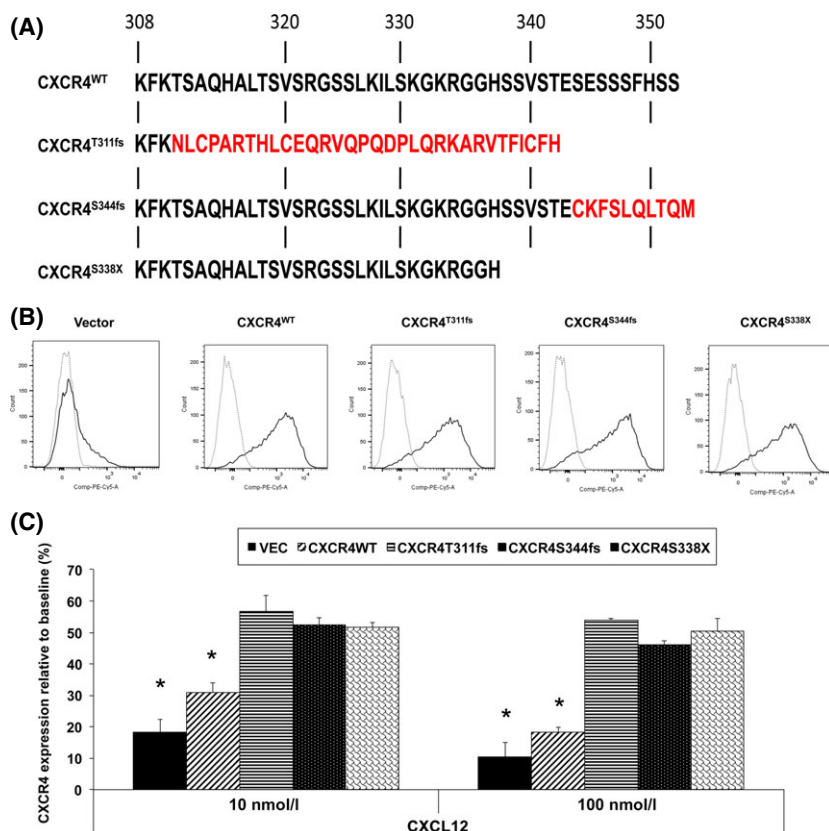


Fig 1. CXCR4 cell surface expression following CXCL12 stimulation of *CXCR4*^{WT}, *CXCR4*^{FS} and *CXCR4*^{S338X} receptor expressing Waldenström Macroglobulinaemia (WM) cells. (A) Depiction of C-terminal domain of CXCR4 showing *CXCR4*^{WT}, *CXCR4*^{T311fs}, *CXCR4*^{S344fs} and *CXCR4*^{S338X} amino acid sequences. Amino acids shown in red denote novel predicted sequences resulting from frameshift mutations. (B) Cell surface CXCR4 receptor expression on vector only, *CXCR4*^{WT}, *CXCR4*^{T311fs}, *CXCR4*^{S344fs} and *CXCR4*^{S338X} transduced BCWM.1 WM cells by flow cytometric analysis using anti-CXCR4 monoclonal antibody (12G5) (dark line) or isotype control (grey line). (C) Changes in cell surface CXCR4 receptor expression following stimulation of vector only, *CXCR4*^{WT}, *CXCR4*^{T311fs}, *CXCR4*^{S344fs} and *CXCR4*^{S338X} transduced BCWM.1 cells for 30 min at 37°C with CXCL12 (10 nmol/l, 100 nmol/l). Surface CXCR4 expression was assessed by flow cytometry and expression relative to baseline levels are shown. Data represent the median of at least three independent experiments; *P < 0.05 for comparisons against *CXCR4*^{T311fs}, *CXCR4*^{S344fs} or *CXCR4*^{S338X} receptor expressing WM cells.

for 18 h with BCL2 inhibitor (GDC-0199; Selleck Chemicals Inc., Houston, TX, USA) to optimize ibrutinib-related apoptotic effects in CXCL12 rescue experiments, as previously described (Cao *et al*, 2014). Survival studies related to AKT1 and MAPK1 were performed in the presence or absence of AKT1- (MK-2206, 0.5 $\mu\text{mol/l}$) or MAP2K1 (MEK) (AS-703026, 0.25 $\mu\text{mol/l}$) specific inhibitors (Selleck Chemicals Inc.), as previously reported (Cao *et al*, 2014). For MYD88 blocking studies, cells were incubated with a peptide inhibitor for MYD88 homodimerization or control peptide (100 $\mu\text{mol/l}$) for 18 h, as before, in the presence or absence of 20 nmol/l of CXCL12 (Treon *et al*, 2012; Yang *et al*, 2013). Survival studies were performed using Annexin V staining (R&D Systems, Minneapolis, MN, USA).

Results

Receptor internalization is impaired in WM cells engineered to express frameshift and nonsense CXCR4^{WHIM} mutations

Non-transfected BCWM.1 cells express very low levels of the CXCR4 receptor. We therefore transfected BCWM.1 cells with vector alone, CXCR4^{WT}, as well as CXCR4^{S338X}, CXCR4^{T311fs}, and CXCR4^{S344fs} expressing vectors. The predicted C-terminal amino acid sequences and truncation sites for the CXCR4 mutations evaluated in this study are shown in Fig 1. Flow cytometry confirmed similar levels of CXCR4 receptor expression for transfected cells (Fig 1). Stimulation of transfected WM cells with CXCL12 for 30 min resulted in significantly greater internalization of CXCR4 receptor expression on CXCR4^{WT} vs. CXCR4^{S338X}, CXCR4^{T311fs} and CXCR4^{S344fs} expressing WM cells ($P < 0.05$; Fig 1). Similar reduced levels of CXCR4 receptor internalization were observed for CXCR4^{S338X}, CXCR4^{T311fs} and CXCR4^{S344fs} expressing cells following CXCL12 stimulation. A representative flow histogram for CXCL12 internalization studies is provided in Figure S1.

CXCL12 triggers enhanced AKT1 and MAPK1 activation in WM cells engineered to express frameshift and nonsense CXCR4^{WHIM} receptors

Because AKT1 and MAPK1 are important survival factors in WM, as well as downstream mediators for CXCR4 receptor signalling, we next interrogated their signalling (Busillo & Benovic, 2007; Leleu *et al*, 2007, 2008). WM cells were stimulated with CXCL12 for 2, 15 and 30 min and evaluated by phospho-flow analysis. Stimulation with CXCL12 showed enhanced AKT1 activation at 2, 10 and 30 min for CXCR4^{T311fs}, CXCR4^{S344fs}, and CXCR4^{S338X} receptor expressing cells *versus* vector only ($P < 0.04$ for all time point comparisons) and *versus* CXCR4^{WT} receptor expressing WM cells ($P \leq 0.05$ for all time point comparisons; Fig 2). Levels of AKT1 activation showed no significant differences between CXCR4^{WT} receptor and vector only expressing cells, as well as for CXCR4^{FS} and

CXCR4^{S338X} receptor expressing cells for all time point comparisons. Significant increases in CXCL12 triggered MAPK1 activation for CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptor expressing cells *versus* vector and CXCR4^{WT} receptor expressing cells was observed for all time point comparisons ($P \leq 0.04$). No significant differences in CXCL12 triggered MAPK1 activation between CXCR4^{WT} receptor and vector only expressing cells were observed at any time points. While no significant differences in CXCL12-triggered MAPK1 activation were observed for CXCR4^{T311fs} or CXCR4^{S344fs} vs. CXCR4^{S338X} receptor expressing cells at 2 and 10 min, higher levels of MAPK1 activation were observed for CXCR4^{S338X} cells vs. CXCR4^{T311fs} or CXCR4^{S344fs} receptor expressing cells at 30 min ($P < 0.04$; Fig 2). As with our previous studies in CXCR4^{S338X} receptor expressing cells (Cao *et al*, 2014), the relevance of CXCL12 induced AKT1 and MAPK1 activation for CXCR4^{T311fs} and CXCR4^{S344fs} cell survival was investigated by co-incubation of cells with ibrutinib and either AKT1 (MK-2206)- or MAP2K1/MAPK1 (AS-703026)-specific inhibitors. Similar to our findings with CXCR4^{S338X} receptor expressing cells, CXCL12 rescue of ibrutinib-treated CXCR4^{T311fs} and CXCR4^{S344fs} cells was attenuated in the presence of either AKT1 (MK-2206) or MAP2K1/MAPK1 (AS-703026) specific inhibitors (Fig 2).

CXCL12 rescues WM cells engineered to express frameshift and nonsense CXCR4^{WHIM} receptors from ibrutinib-triggered apoptosis

We next examined the pro-apoptotic effects of ibrutinib on CXCR4^{WT}, CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptor-expressing WM cells in the presence or absence of CXCL12 and the CXCR4 antagonist plerixafor. CXCR4^{WT}, CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptor-expressing WM cells showed similar levels of ibrutinib-triggered apoptosis ($P < 0.01$ *versus* DMSO control; Fig 3). In contrast to CXCR4^{WT} cells, CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptor-expressing cells were rescued by CXCL12 from ibrutinib-induced apoptosis ($P < 0.02$ for all CXCR4^{WHIM} cells and $P = 0.169$ for CXCR4^{WT} *versus* non-CXCL12 stimulated control cells). Concurrent treatment of CXCL12 exposed CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptor expressing cells with the CXCR4 antagonist plerixafor restored the pro-apoptotic effects of ibrutinib ($P \leq 0.05$). In contrast, no differences in levels of ibrutinib triggered apoptosis for CXCR4^{WT} cells treated with CXCL12 and plerixafor *versus* CXCL12 alone were observed ($P = 0.16$; Fig 3).

Inhibition of MYD88^{L265P} signalling triggers apoptosis of WM cells regardless of CXCR4^{WT} or CXCR4^{WHIM} receptor expression

Lastly, we investigated if MYD88-related survival signalling could be supplanted by CXCL12 in WM cells engineered to express CXCR4^{WT}, CXCR4^{T311fs}, CXCR4^{S344fs} and

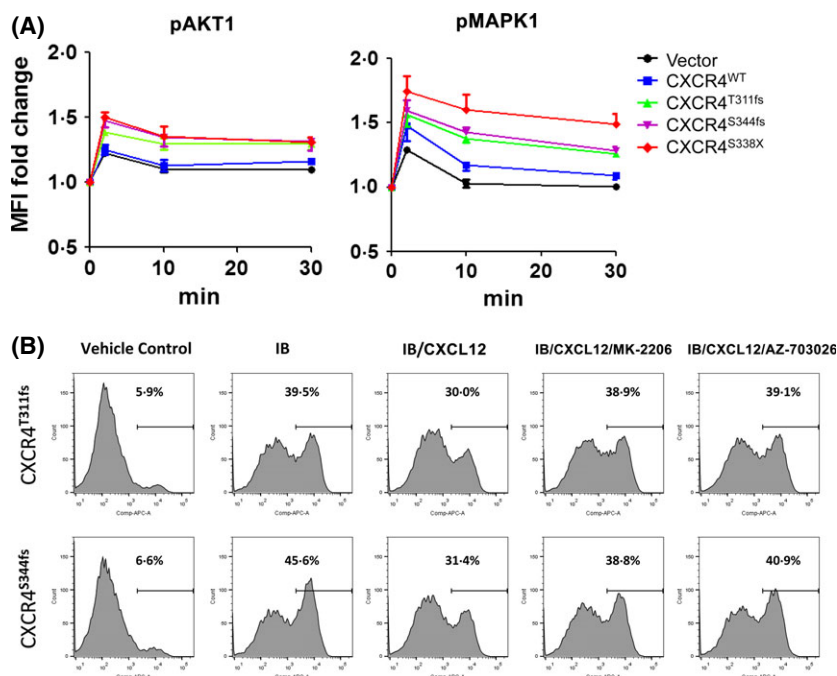


Fig 2. Impact of CXCL12 on AKT1 and MAPK1 signalling and survival in CXCR4^{WT} and CXCR4^{WHIM} receptor expressing Waldenström Macroglobulinaemia (WM) cells. (A) Vector only, CXCR4^{WT}, CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} expressing WM cells were treated with CXCL12 (20 nmol/l) for 2, 15, and 30 min and phosphoflow analyses performed using phospho-AKT1 (S⁴⁷³) or phospho-MAPK1 (T²⁰²/Y²⁰⁴) directed antibodies. Data represent the mean of at least three experiments \pm standard error of the mean. $P \leq 0.05$ and $P < 0.04$ for vector and CXCR4^{WT} comparisons against CXCR4^{WHIM} receptor expressing cells for phospho-AKT1 and phospho-MAPK1 respectively for all time point comparisons. $P < 0.04$ for CXCR4^{S338X} vs. CXCR4^{T311fs} or CXCR4^{S344fs} cells at 30 min. MFI, mean fluorescence intensity. (B) CXCR4^{T311fs} and CXCR4^{S344fs} expressing WM cells were treated for 18 h with vehicle control (dimethyl sulfoxide); ibuprofen (IB; 0.5 μ mol/l) in the presence or absence of CXCL12 (20 nmol/l) and/or the AKT1 (MK-2206; 0.5 μ mol/l) and MAP2K1/MAPK1 (AZ-703026; 0.25 μ mol/l) inhibitors. Annexin V staining was performed to assess apoptosis. Study was performed in triplicate, and results from a representative study set are shown. $P \leq 0.05$ for CXCR4^{FS} cells treated with CXCL12 and/or AKT1 or MER/MAPK1 inhibitor *versus* CXCL12 alone.

CXCR4^{S338X} receptors. Use of an inhibitor that targets MYD88 homodimerization and blocks MYD88 L265P signalling (Treon *et al*, 2012; Poulain *et al*, 2013; Yang *et al*, 2013) triggered significantly higher levels of apoptosis *versus* a control peptide ($P < 0.01$; Fig 4). Co-treatment of cells expressing the CXCR4^{WT}, CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptors with CXCL12 showed no rescue effects, and no intergroup differences in the level of apoptosis triggered by the MYD88 signalling inhibitor were observed (Fig 4).

Discussion

We sought to address the functional significance of CXCR4^{FS} mutations that constitute half of the WHIM-like somatic mutations in WM (Hunter *et al*, 2014; Treon *et al*, 2014). We show that CXCR4^{FS} receptor expressing cells exhibited diminished CXCR4 receptor internalization in response to CXCL12 akin to that observed for CXCR4^{S338X} receptor expressing cells. These findings are consistent with earlier work in WHIM patients establishing the terminal 10 amino acids as critical determinants of CXCL12 triggered CXCR4 receptor internalization (Futahashi *et al*, 2007). Particularly critical in this region are Ser^{346/347}, which are phosphorylated by ADRBK1/2

(GRK2/3), and required for subsequent phosphorylation of more proximal sites (Ser^{324/325} and Ser^{338/339} that regulate CXCR4 receptor internalization and desensitization (Mueller *et al*, 2013). As shown in Fig 1, all three mutants examined in this study impact the Ser^{346/347} site by introduction of a stop codon leading to protein truncation (CXCR4^{S338X}), by frameshift mutation leading to a stop codon and protein truncation (CXCR4^{T311fs}) and by frameshift mutation resulting in replacement of amino acids (CXCR4^{S344fs}).

AKT1 and MAPK1 are important survival factors in WM (Leleu *et al*, 2007, 2008). Both AKT1 and MAPK1 showed higher levels of activation in all three of the CXCR4^{WHIM} receptor-expressing cells relative to CXCR4^{WT} receptor-expressing cells. However, differences in MAPK1 activation were observed between CXCR4^{FS} and CXCR4^{S338X} expressing cells, with more prolonged MAPK1 activation observed for CXCR4^{S338X} vs. CXCR4^{T311fs} and CXCR4^{S344fs} receptor expressing cells. Differences in MAPK1 activation may explain why WM patients with nonsense mutations inclusive of CXCR4^{S338X} present with higher burdens of disease *versus* those with CXCR4^{FS} mutations (Treon *et al*, 2014). MAPK1 activation is dependent on β -arrestin binding to the CXCR4 C-terminal domain, which is prompted by recruitment of G

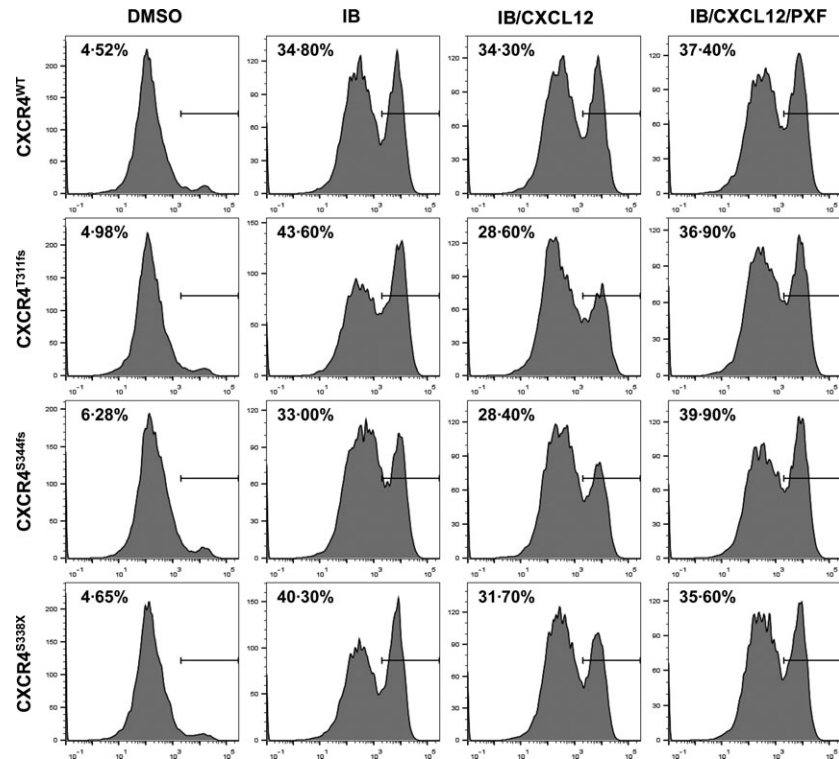


Fig 3. Impact of CXCL12 and plerixafor on CXCR4^{WT} and CXCR4^{WHIM} receptor expressing WM cells treated with ibrutinib. CXCR4^{WT}, CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptor expressing WM cells were treated for 18 h with vehicle control (dimethyl sulfoxide, DMSO); ibrutinib (IB; 0.5 $\mu\text{mol/l}$) and/or the CXCR4 receptor antagonist plerixafor (PXF; 30 $\mu\text{mol/l}$). Annexin V staining was performed to assess apoptosis. The study was performed in triplicate, and results from a representative study set are shown. $P < 0.01$ for CXCR4^{WT}, CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptor expressing WM cells treated with ibrutinib *versus* DMSO control; $P < 0.02$ for all CXCR4^{WHIM} cells and $P = 0.169$ for CXCR4^{WT} treated with CXCL12 vs. non-CXCL12 stimulated control cells; $P \leq 0.05$ for all CXCR4^{WHIM} cells treated with plerixafor and CXCL12 vs. CXCL12 alone.

protein-coupled receptor kinases (GRKs) in response to CXCL12 (Busillo *et al*, 2010). Loss of the distal CXCR4 C-terminal domain in WHIM patients sustains β -arrestin recruitment and MAPK1 signalling (Busillo *et al*, 2010). Variations in MAPK1 activation could therefore reflect differences in GRK phosphorylation and β -arrestin binding sites within the C-terminus, and more detailed studies to clarify their contribution to MAPK1 signalling in CXCR4^{WHIM}-mutated WM cells will be required.

Differences in heterodimer formation for CXCR4^{FS} or CXCR4^{S338X} with CXCR4^{WT} proteins may also contribute to variations in downstream signalling following CXCL12 ligation. HEK cells transfected with both CXCR4^{WT} and CXCR4^{S338X} expressing vectors show preferential existence of heterodimers (Lagane *et al*, 2008). Novel amino acid sequences introduced by frameshift mutations into the C-terminal domain could be more disruptive than truncations introduced by nonsense mutations, thereby differentially impacting CXCR4 dimer formation and downstream signalling. More work is clearly needed to clarify such possibilities.

CXCL12-triggered AKT1 and MAPK1 activation was recently shown by us to impact ibrutinib-triggered apoptosis in WM cells engineered to express the CXCR4^{S338X} receptor as well as other agents used in WM therapy (Cao *et al*, 2014). We show in these studies that CXCR4^{FS} mutations conferred CXCL12-mediated resistance to ibrutinib similar to that observed in CXCR4^{S338X} receptor-expressing WM cells. Taken together, these findings provide a molecular basis for the diminished clinical activity of ibrutinib observed in WM patients bearing

both nonsense and frameshift mutations (Trean *et al*, 2013). Moreover, these findings also suggest that CXCR4 receptor inhibitors could potentially restore sensitivity to ibrutinib, as well as other therapeutics impacted by CXCR4^{FS} mutations. Plerixafor, a US Food and Drug Administration (FDA) approved agent for stem cell mobilization in lymphoma patients, reversed myelokathexis-related leucopenia in WHIM patients treated daily for 6 months, thereby demonstrating both clinical efficacy and long-term safety of CXCR4 inhibition (McDermott *et al*, 2014). Several other antagonists to CXCR4 including BMS-936564, AMD-070, TG-0054 are also in clinical trials.

A notable finding in this study was the primary survival function of MYD88^{L265P} signalling relative to CXCL12-triggered CXCR4^{WT} or CXCR4^{WHIM} signalling. Treatment with an inhibitor that blocks MYD88^{L265P} signalling triggered similar levels of apoptosis, and was not abrogated by CXCL12 in CXCR4^{WT} or CXCR4^{WHIM} receptor expressing cells. These findings support a primary role for MYD88^{L265P} signalling for WM cell survival. Additional studies will invariably be needed to confirm these results. Nonetheless, these findings have important implications for WM pathogenesis and treatment. Virtually all patients with CXCR4^{WHIM} mutations harbour the MYD88^{L265P} mutation (Hunter *et al*, 2014; Trean *et al*, 2014), and CXCR4^{WHIM} mutations are subclonal to MYD88^{L265P} in most WM patients bearing both mutations (L. Xu, unpublished observations). These findings, taken with the results of the MYD88 inhibitor studies presented herein, support a primary survival role for the MYD88^{L265P} mutation in WM, and suggest that therapeutic strategies targeting

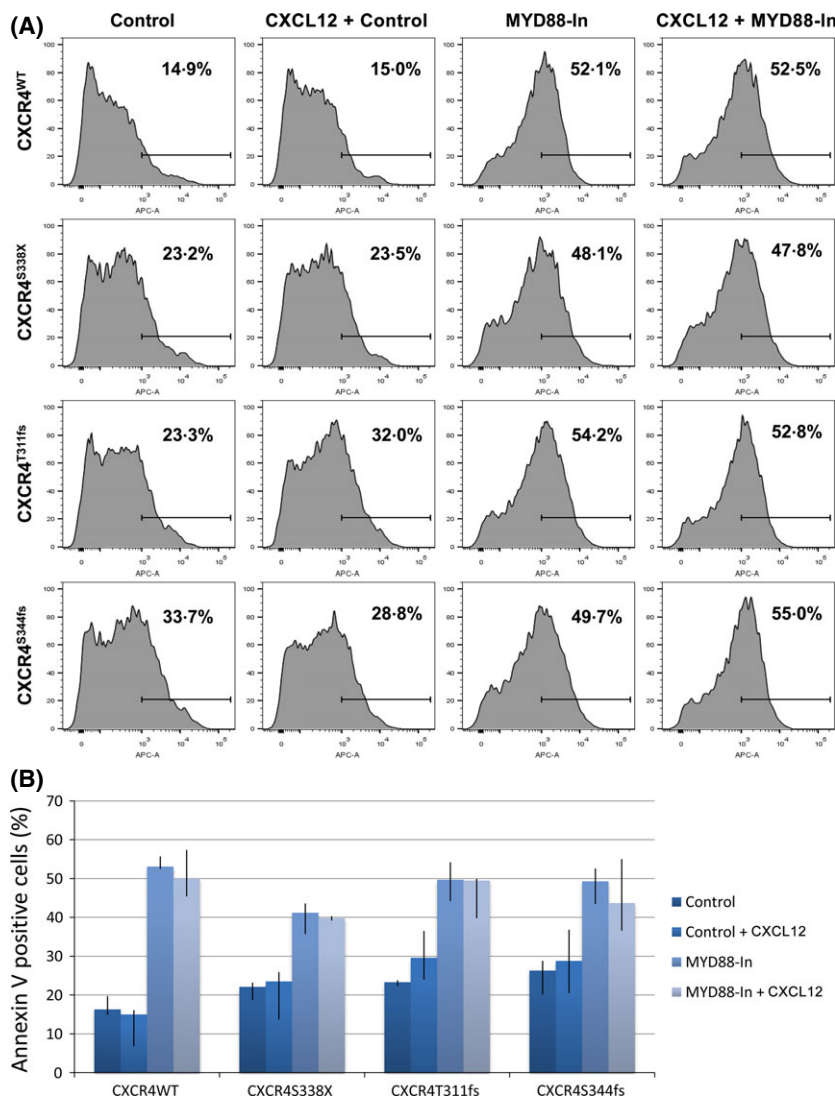


Fig 4. Inhibition of MYD88 signalling on survival of CXCL12-treated CXCR4^{WT} and CXCR4^{WHIM} receptor expressing WM cells. CXCR4^{WT}, CXCR4^{T311fs}, CXCR4^{S344fs} and CXCR4^{S338X} receptor expressing WM cells were treated for 18 h with either control or MYD88 inhibitor peptides (100 μmol/l) in the presence or absence of CXCL12 (20 nmol/l). Annexin V staining was performed to assess apoptosis. Representative histograms are shown (A), and results from three experiments are shown with standard error of the mean (B). *P* < 0.01 for all CXCR4 expressing cells treated with MYD88 inhibitor *versus* control peptide, and not significant for all cells treated with MYD88 inhibitor alone or in combination with CXCL12, and for all intergroup comparisons.

MYD88 signalling may overcome any survival benefits conferred by CXCR4^{WHIM} mutations.

In conclusion, our findings show that frameshift and nonsense CXCR4^{WHIM} mutations confer decreased CXCL12-triggered CXCR4 receptor internalization, enhanced AKT1 and MAPK1 activation, and CXCL12-mediated resistance to ibrutinib-triggered apoptosis in WM cells. Use of inhibitors targeting CXCR4 receptor restored the sensitivity of CXCR4^{WHIM} receptor-expressing WM cells to ibrutinib, thereby providing a framework for the investigation of CXCR4 receptor antagonists with ibrutinib in this WM patient population. Direct inhibition of MYD88^{L265P} signalling overcomes CXCL12-triggered survival effects in CXCR4^{WHIM}-mutated cells, supporting a primary role for MYD88 signalling in WM.

Acknowledgements

The authors acknowledge the generous support of the Leukemia and Lymphoma Society for a Translational Research

Grant, Peter S. Bing M.D., the Edward and Linda Nelson Fund for WM Research, the Kerry Robertson Fund for WM Studies for their generous support of this research work.

Author contributions

YC, ZRH and SPT conceived and designed the experiments, and performed the data analysis. XL, LX, YC, GY, JC, and NT performed the laboratory experiments. SK, JJC, and SPT participated in patient care, data collection and oversight of these studies. SPT wrote the manuscript.

Disclosure of conflicts of interest

SPT received research funding, speaking honoraria and/or consulting fees from Pharmacylics Inc., Janssen Pharmaceuticals Inc., and Gilead Pharmaceuticals, Inc.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. CXCR4 cell surface expression following CXCL12 stimulation of CXCR4 Wild-Type, Nonsense, and Frameshift mutant receptor expressing WM cells.

References

- Ansell, S.M., Hodge, L.S., Secreto, F.J., Manske, M., Braggio, E., Price-Troska, T., Ziesmer, S., Li, Y., Johnson, S.H., Hart, S.N., Kocher, J.P., Vasmatazis, G., Chanan-Kahn, A., Gertz, M., Fonseca, R., Dogan, A., Cerhan, J.R. & Novak, A.J. (2014) Activation of TAK1 by MYD88 L265P drives malignant B-cell growth in non-Hodgkin lymphoma. *Blood Cancer Journal*, **14**, e183.
- Busillo, J.M. & Benovic, J.L. (2007) Regulation of CXCR4 signaling. *Biochimica Biophysica Acta*, **1768**, 952–963.
- Busillo, J.M., Armando, S., Sengupta, R., Meucci, O., Bouvier, M. & Benovic, J.L. (2010) Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. *Journal of Biological Chemistry*, **285**, 7805–7817.
- Cao, Y., Hunter, Z.R., Liu, X., Xu, L., Yang, G., Chen, J., Patterson, C.J., Tsakmaklis, N., Kanan Rodig, S., Castillo, J.J. & Treon, S.P. (2014) The WHIM-like CXCR4^{S338X} somatic mutation activates AKT and ERK, and promotes resistance to ibrutinib and other agents used in the treatment of Waldenström's Macroglobulinemia. *Leukemia*, doi: 10.1038/leu.2014.187.
- Dotta, L., Tassone, L. & Badolato, R. (2011) Clinical and genetic features of Warts, Hypogammaglobulinemia, Infections and Myelokathexis (WHIM) syndrome. *Current Molecular Medicine*, **11**, 317–325.
- Futahashi, Y., Koman, J., Urano, E., Aoki, T., Hamatake, M., Miyachi, K., Yoshida, T., Matsuda, Z. & Yamamoto, N. (2007) Separate elements are required for ligand-dependent and independent internalization of metastatic potentiator CXCR4. *Cancer Science*, **98**, 373–379.
- Hunter, Z.R., Xu, L., Yang, G., Zhou, Y., Liu, X., Cao, Y., Manning, R.J., Tripsas, C., Patterson, C.J., Sheehy, P. & Treon, S.P. (2014) The genomic landscape of Waldenström's Macroglobulinemia is characterized by highly recurring MYD88 and WHIM-like CXCR4 mutations, and small somatic deletions associated with B-cell lymphomagenesis. *Blood*, **123**, 1637–1646.
- Jimenez, C., Sebastian, E., Chillon, M.C., Giraldo, P., Mariano Hernandez, J., Escalante, F., Gonzalez-Lopez, T.J., Aguilera, C., de Coca, A.G., Murillo, I., Alcoceba, M., Balanztegui, A., Sarasquete, M.E., Corral, R., Marin, L.A., Paiva, B., Ocio, E.M., Gutierrez, N.C., Gonzalez, M., San Miguel, J.F. & Garcia-Sanz, R. (2013) MYD88 L265P is a marker highly characteristic of, but not restricted to, Waldenström's macroglobulinemia. *Leukemia*, **27**, 1722–1728.
- Lagane, B., Chow, K.Y.C., Balabanian, K., Levoe, A., Harriague, J., Planchenault, T., Baleux, F., Gunera-Saad, N., Arenzana-Seisdedos, F. & Bachelier, F. (2008) CXCR4 dimerization and β -arrestin mediated signaling account for the enhanced chemotaxis to CXCL12 in WHIM syndrome. *Blood*, **112**, 34–44.
- Leleu, X., Jia, X., Runnels, J., Ngo, H.T., Moreau, A.S., Farag, M., Spencer, J.A., Pitsillides, C.M., Hatjiharissi, E., Roccaro, A., O'Sullivan, G., McMillin, D.W., Moreno, D., Kiziltepe, T., Carrasco, R., Treon, S.P., Hideshima, T., Anderson, K.C., Lin, C.P. & Ghobrial, I.M. (2007) The Akt pathway regulates survival and homing in Waldenström macroglobulinemia. *Blood*, **110**, 4417–4426.
- Leleu, X., Eeckhoutte, J., Jia, X., Roccaro, A.M., Moreau, A.S., Farag, M., Sacco, A., Ngo, H.T., Runnels, J., Melhem, M.R., Burwick, N., Azab, A., Azab, F., Hunter, Z., Hatjiharissi, E., Carrasco, D.R., Treon, S.P., Witzig, T.E., Hideshima, T., Brown, M., Anderson, K.C. & Ghobrial, I.M. (2008) Targeting NF- κ B in Waldenström macroglobulinemia. *Blood*, **111**, 5068–5077.
- McDermott, D.H., Liu, Q., Velez, D., Lopez, L., Anaya-O'Brien, S., Ulrick, J., Kwatema, N., Starling, J., Fleisher, T.A., Priel, D.A., Merideth, M.A., Giuntoli, R.L., Evbuomwan, M.O., Littell, P., Marquesen, M.M., Hilligoss, D., DeCastro, R., Grimes, G.J., Hwang, S.T., Pittaluga, S., Calvo, K.R., Stratton, P., Cowen, E.W., Kuhns, D.B., Malech, H.L. & Murphy, P.M. (2014) A phase 1 clinical trial of long-term, low-dose treatment of WHIM syndrome with the CXCR4 antagonist plerixafor. *Blood*, **123**, 2308–2316.
- Mueller, W., Schütz, D., Nagel, F., Schulz, S. & Stumm, R. (2013) Hierarchical organization of multisite phosphorylation at the CXCR4 C terminus. *PLoS One*, **8**, e64975.
- Poulain, S., Roumier, C., Decambon, A., Renneville, A., Herbaux, C., Bertrand, E., Tricot, S., Daudignon, A., Galiegue-Zouitina, S., Soenen, V., Theisen, O., Grardel, N., Nibourel, O., Roche-Lestienne, C., Quesnel, B., Duthilleul, P., Preudhomme, C. & Leleu, X. (2013) MYD88 L265P mutation in Waldenström macroglobulinemia. *Blood*, **121**, 4504–4511.
- Roccaro, A.M., Sacco, A., Jimenez, C., Maiso, P., Moschetta, M., Mishima, Y., Aljawai, Y., Sahin, I., Kuhne, M., Cardarelli, P., Cohen, L., San Miguel, J.F., Garcia-Sanz, R. & Ghobrial, I.M. (2014) C1013G/CXCR4 acts as a driver mutation of tumor progression and modulator of drug resistance in lymphoplasmacytic lymphoma. *Blood*, **123**, 4120–4131.
- Treon, S.P., Xu, L., Yang, G., Zhou, Y., Liu, X., Cao, Y., Sheehy, P., Manning, R.J., Patterson, C.J., Tripsas, C., Arcaini, L., Pinkus, G.S., Rodig, S.J., Sohani, A.R., Harris, N.L., Laramie, J.M., Skifter, D.A., Lincoln, S.E. & Hunter, Z.R.M. (2012) YD88 L265P somatic mutation in Waldenström's macroglobulinemia. *New England Journal of Medicine*, **367**, 826–833.
- Treon, S.P., Tripsas, C., Yang, G., Cao, Y., Xu, L., Hunter, Z.R., Cropper, S.J., Mostyn, P., Meid, K., Warren, D., Patterson, C.J., Varma, G., Laubach, J.P., Paba-Prada, C.E., Kunsman, J., Ghobrial, I.M., Kanan, S., Advani, R.H. & Palomba, M.L. (2013) A prospective multicenter study of the Bruton's tyrosine kinase inhibitor ibrutinib in patients with relapsed or refractory Waldenström's macroglobulinemia. *Blood*, **122**, Abstract 251.
- Treon, S.P., Cao, Y., Xu, L., Yang, G., Liu, X. & Hunter, Z.R. (2014) Somatic mutations in MYD88 and CXCR4 are determinants of clinical presentation and overall survival in Waldenström's macroglobulinemia. *Blood*, **123**, 2791–2796.
- Varettoni, M., Arcaini, L., Zibellini, S., Boveri, E., Rattotti, S., Riboni, R., Corso, A., Orlandi, E., Bonfichi, M., Gotti, M., Pascutto, C., Mangiacavalli, S., Croci, G., Fiaccadori, V., Morello, L., Guerrera, M.L., Paulli, M. & Cazzola, M. (2013) Prevalence and clinical significance of the MYD88 L265P somatic mutation in Waldenström's macroglobulinemia and related lymphoid neoplasms. *Blood*, **121**, 2522–2528.
- Yang, G., Zhou, Y., Liu, X., Xu, L., Cao, Y., Manning, R.J., Patterson, C.J., Buhrlage, S.J., Gray, N., Tai, Y.T., Anderson, K.C., Hunter, Z.R. & Treon, S.P. (2013) A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenström macroglobulinemia. *Blood*, **122**, 1222–1232.