

Dasatinib Inhibits CXCR4 Signaling in Chronic Lymphocytic Leukaemia Cells and Impairs Migration Towards CXCL12

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Abstract

Chemokines and their ligands play a critical role in enabling chronic lymphocytic leukaemia (CLL) cells access to protective microenvironmental niches within tissues, ultimately resulting in chemoresistance and relapse: disruption of these signaling pathways has become a novel therapeutic approach in CLL. The tyrosine kinase inhibitor dasatinib inhibits migration of several cell lines from solid-organ tumours, but effects on CLL cells have not been reported. We studied the effect of clinically achievable concentrations of dasatinib on signaling induced by the chemokine CXCL12 through its' receptor CXCR4, which is highly expressed on CLL cells. Dasatinib pre-treatment inhibited Akt and ERK phosphorylation in CLL cells upon stimulation with CXCL12. Dasatinib also significantly diminished the rapid increase in actin polymerisation observed in CLL cells following CXCL12 stimulation. Moreover, the drug significantly inhibited chemotaxis in a transwell assay, and reduced the percentage of cells able to migrate beneath a CXCL12-expressing murine stromal cell line. Dasatinib also abrogated the anti-apoptotic effect of prolonged CXCL12 stimulation on cultured CLL cells. These data suggest that dasatinib, akin to other small molecule kinase inhibitors targeting the B-cell receptor signaling pathway, may redistribute CLL cells from protective tissue niches to the peripheral blood, and support the investigation of dasatinib in combination strategies.

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Introduction

It has long been appreciated that chronic lymphocytic leukaemia (CLL) cells are dependent on a number of microenvironmental stimuli for survival and proliferation [1]. The chemokine CXCL12, the ligand for the receptor CXCR4, has a key physiological role in controlling mature B lymphocyte trafficking through germinal centres [2]. CLL cells express high levels of functional CXCR4 [3]; signaling through this receptor reduces spontaneous and drug-induced apoptosis [4] and also facilitates CLL cell migration beneath stromal cells [3]. In addition to promoting chemoresistance, the ability of CLL cells to access and be retained within the bone marrow (BM) and lymph node (LN) microenvironment increases their chance of encountering proliferative signals such as antigenic stimulation of the B cell antigen receptor (BCR), or the T cell factors CD154 (CD40 ligand) and interleukin 4 (IL-4) [5], ultimately resulting in disease progression.

Dasatinib is a tyrosine kinase inhibitor first developed as a 'second-generation' ATP-competitive inhibitor of the oncogenic BCR-Abl kinase that characterises chronic myeloid leukaemia, having a potency over three hundred-fold greater than imatinib for the kinase [6]. Dasatinib also inhibits all Src-family tyrosine

kinases with an IC₅₀ less than 1 nM, and other targets include c-kit (IC₅₀ 5 nM), platelet-derived growth factor β (IC₅₀ 28 nM), Bruton's tyrosine kinase (BTK; IC₅₀ 5 nM) and Tec kinases (IC₅₀ 297 nM) [6,7]. Dasatinib results in significant clinical responses in patients with imatinib-resistant chronic myeloid leukaemia [8], and due to its' multi-kinase targets, research interest has turned to studying the drug in other haematological and solid-organ cancers. Both our group and others have demonstrated that dasatinib inhibits BCR signal transduction and blocks BCR-mediated survival of CLL cells [9,10,11]. In solid tumour cell lines and models, including melanoma [12], sarcoma [13], and colon carcinoma [14] dasatinib has been shown to exert significant anti-migratory effects, both *in vitro* and *in vivo*. Therefore, we were interested to assess whether dasatinib disrupted CLL cell migration in response to chemokine stimulation. Here, we show that dasatinib significantly impairs migration of CLL cells toward CXCL12, by inhibiting CXCR4 signaling.

Methods

Ethics Statement

Ethical approval was obtained from the West of Scotland Research Ethics Committee. All patients who donated blood

samples gave written informed consent in accordance with the Declaration of Helsinki, and samples were anonymised during the study.

Clinical Samples and Reagents

CLL cells were isolated from peripheral blood as previously described [9]. Clinical details of patients used in these studies are presented in Table 1; none of the patients had received chemotherapy within the preceding 3 months. Immunohistochemistry for ZAP-70 expression and FISH for 11q and 17p deletions was performed by our local clinical pathology laboratory, and results shown in Table 1. Dasatinib was purchased from LC Laboratories (Woburn, MA, USA). As the peak plasma concentration of dasatinib in patients following standard dosing is in the region of 130 nM [15], dasatinib was used at a maximum concentration of 100 nM in experiments.

Assessment of Actin Polymerisation

CLL cells ($\times 10^6$) were incubated in RPMI-1640 supplemented with 0.5% BSA for 30 min with or without 100 nM dasatinib then stimulated by the addition of 100 ng/ml CXCL12 (PeproTech EC Ltd, London, UK). 100 μ l aliquots were removed pre-stimulation, and at 15, 60, 300, and 600 s after stimulation, and fixed/permeabilised in 250 μ l Fix/Perm solution (BD Biosciences, Oxford, UK) according to manufacturer's instructions. Cells were then washed in Perm/WashTM buffer (BD Biosciences) and stained with AlexaFluor[®] 488-labelled phalloidin (Invitrogen Ltd., Paisley, UK) for 10 min. Analysis was performed by acquiring 10,000 events on a FACSCantoII flow cytometer (BD Biosciences).

Assessment of Chemotaxis

CLL cells (5×10^5) were incubated in 100 μ l RPMI-1640/0.5% BSA media with or without 1, 10, or 100 nM dasatinib for 30 min prior to the assay. Cells were then transferred to the upper chamber of a 6.5-mm diameter Transwell culture insert (Costar[®], Fisher Scientific UK.) and placed into wells containing 600 μ l media supplemented with or without 150 ng/ml CXCL12, and incubated for 4 hr at 37°C. Thereafter, three 150 μ l aliquots were removed from each lower chamber for counting by flow cytometry. For each aliquot the total number of events acquired during 20 s on high flow setting was recorded.

Pseudoemperipolesis Assay

CLL cells (2×10^6 /ml) were treated with or without with 100 nM dasatinib for 30 min then transferred to collagen-coated wells containing a confluent layer of M2-10B4 fibroblasts. Each experimental condition was set-up in triplicate, and cells were then incubated for 5 hr at 37°C in 5% CO₂. Following removal of non-migrated cells by thorough washing, the stromal cell layer was trypsinised and stained with an anti-CD19 APC antibody (BD Biosciences) to facilitate isolation of CLL cells by flow cytometry. Pseudoemperipolesed CLL cells were counted by acquiring CD19⁺ events on a FACSCantoII flow cytometer on high flow setting for 30 s. An aliquot of the starting cell population was similarly counted, to enable calculation of the percentage of CLL cells able to transmigrate.

Assessment of Viability

CLL cell viability was assessed by staining with Annexin V fluorescein isothiocyanate and Viaprobe (BD Biosciences) as previously described [9].

Immunoblotting

CLL cells (3×10^6 /ml) were incubated with or without 100 nM dasatinib in RPMI 1640 containing 0.5% BSA for 30 min, then left unstimulated or further treated with 100 ng/ml CXCL12 at 37°C for 3 or 10 min. Protein lysates were subsequently prepared and analysed by immunoblotting as previously described [9]. All antibodies used in western blotting were sourced from Cell Signaling Technology[®] (Danvers, MA, USA) apart from phospho-Lyn³⁹⁶, which was purchased from Epitomics (Cambridge, MA, USA).

CLL Cell Proliferation Assays

To analyse cell division, CLL cells were stained with 1 μ M CFSE prior to culture. Cells (5×10^3) were then co-cultured for up to 12 days with NT-L murine fibroblasts stably transfected to express CD154 [16] in media supplemented with 10 ng/ml IL-4 (the CD154/IL-4 system) in the presence or absence of 100 nM dasatinib. Control cells, to which 50 ng/ml colcemid (Sigma Aldrich, Dorset, UK) was added, were included to define the undivided cell population; media and dasatinib was replenished every 72 hr. To assess both cell divisions and absolute cell numbers, cells were stained with an anti-CD19 APC antibody then resuspended in 450 μ l buffer to which 50 μ l CountBrightTM beads (Invitrogen Ltd.) was added prior to flow cytometry. To assess cell division, 10,000 CD19⁺ events were recorded for each sample on a FACSCantoII flow cytometer and analysed with FACSDiva software (BD Biosciences). Using the mean fluorescence intensity (MFI) of CFSE in the colcemid control to define the gate encompassing undivided cells, further gates were set for a successive halving of MFI, to include cells that had undergone cell division(s). To determine absolute cell counts, 5000 Count-

Table 1. Clinical sample details.

Sample I.D.	Age	Sex	Binet Stage	Treated	ZAP-70Status	FISH
3	55	F	A	No	Neg	-
7	73	M	C	Yes	Pos	11q-
12	59	F	A	No	Neg	-
18	63	F	B	Yes	Pos	11q-
21	67	F	C	Yes	Pos	-
32	67	F	B	No	Pos	-
34	64	M	B	Yes	Pos	11q-
35	68	M	B	No	Pos	-
36	76	M	A	Yes	Pos	11q-
41	59	M	A	No	Neg	-
44	63	F	A	No	Neg	-
45	78	M	B	Yes	Pos	-
46	52	F	A	No	Pos	-
50	61	M	C	No	Pos	-
52	78	F	B	Yes	Neg	11q-
64	64	M	C	Yes	Neg	-
62	84	F	A	Yes	ND	-
68	57	F	A	Yes	Pos	-
69	43	M	A	No	Pos	-
70	74	F	C	No	Neg	-

ND = not determined

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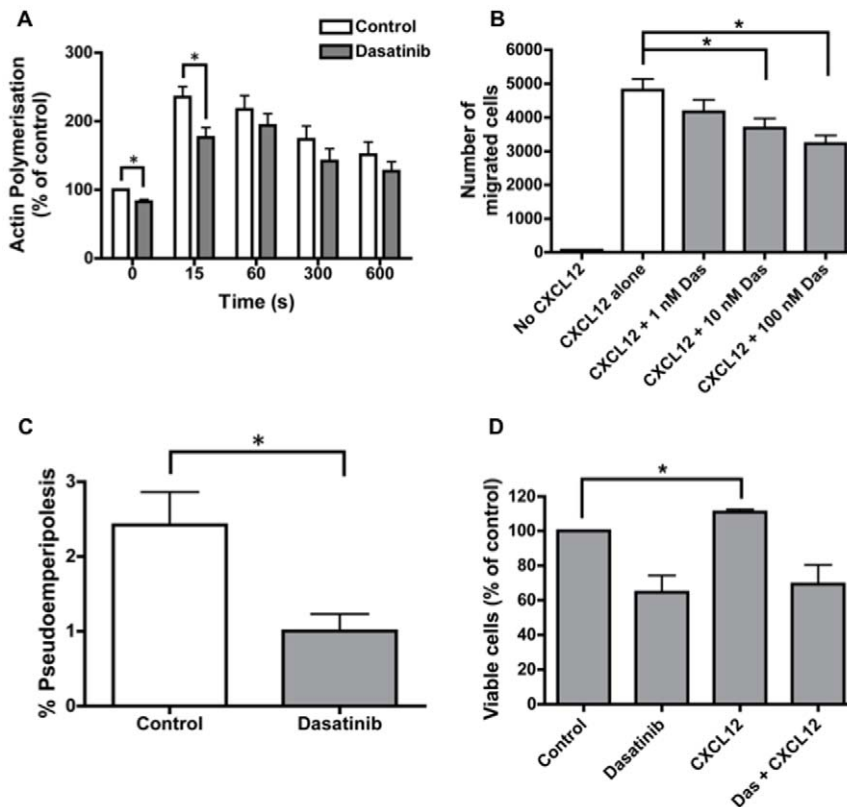


Figure 1. Dasatinib inhibits actin polymerisation and migration of CLL cells in response to CXCL12. **A:** Analysis of the effect of dasatinib on actin polymerisation following CXCL12 treatment of CLL cells. Results are expressed relative to the untreated control prior to CXCL12 stimulation. The graph shows the mean (\pm SEM) values for five independent experiments using different CLL samples (samples 7, 41, 45, 46 and 62). * indicates $p < 0.05$. **B:** The graph shows the effect of increasing concentrations of dasatinib on chemotaxis of CLL cells towards CXCL12. Data are expressed as the average number (\pm SEM) of migrated cells from five independent experiments (samples 21, 35, 45, 46 and 62). **C:** The graph shows the percentage of CLL cells able to migrate beneath a stromal cell layer (pseudoemperipolesis) in the presence or absence of dasatinib. Results are expressed as the mean (\pm SEM) percentage of migrated cells in each condition ($n = 8$; samples 12, 18, 32, 46, 52, 64, 68 and 69). **D:** CLL cells (1×10^6) were incubated in conditions as shown for 48 hr, then apoptosis was assessed by Annexin/Viaprobe staining. The graph shows the mean (\pm SEM) viabilities of cells, relative to the viability of untreated control cells ($n = 6$; samples 12, 36, 41, 44, 45 and 62). doi:10.1371/journal.pone.0048929.g001

Bright™ bead events were acquired. CLL cell counts were then determined using a standard formula based on the ratio of CD19⁺ events to beads, following the manufacturer's instructions.

Statistical Analysis

Data were analysed using the unpaired, 2-tailed, Student *t* test (GraphPad Prism software, La Jolla, CA, USA).

Results and Discussion

Firstly, we assessed the effect of dasatinib pre-treatment on actin polymerisation in response to CXCL12 stimulation. Dasatinib pre-treatment notably reduced the basal level of actin polymerisation within CLL cells, and also significantly blunted the initial increase in polymerisation following CXCL12 stimulation (Figure 1A). CLL cell migration towards CXCL12 was then assessed in a transwell assay. Dasatinib reduced chemotaxis in a concentration-dependent manner, with 100 nM dasatinib resulting in a mean (\pm SEM) number of migrated cells of 3229 (\pm 242) as compared to 4812 (\pm 328) in untreated controls (Figure 1B; $p < 0.001$). Moreover, dasatinib significantly reduced the percentage of CLL cells able to migrate beneath the CXCL12-expressing stromal cell

line M2-10B4 (Figure 1C; $p = 0.02$). No significant differences in actin polymerisation or migration were observed between ZAP-70 positive and negative CLL cells in our study. This confirms a previous report that found ZAP-70 positive CLL cells to be more responsive to the chemokines CCL19 and CCL21, but not CXCL12 [17]. As CXCL12 stimulation increases the viability of CLL cells cultured *in vitro* [18], we were also interested to assess whether dasatinib may inhibit the anti-apoptotic effect of CXCL12. CLL cells were cultured for 48 hr in the presence and absence of dasatinib, CXCL12, or both. CXCL12 significantly increased the viability of cultured CLL cells, confirming previous reports (Figure 1D). Dasatinib completely abrogated the anti-apoptotic effect of CXCL12, with cell viability similar to that of cells treated with dasatinib alone (Figure 1D).

As CXCR4 stimulation results in rapid activation of PI-3K and ERK-MAPK in CLL cells [4,19,20], we next assessed the activation status of these two signaling pathways following CXCL12 stimulation in the presence or absence of dasatinib. Dasatinib completely abrogated Akt phosphorylation, and partially inhibited ERK activation (Figure 2A). There is substantial evidence indicating that PI-3K/Akt signaling is a key regulator of migration toward CXCL12 in CLL cells. Burger *et al.* showed that

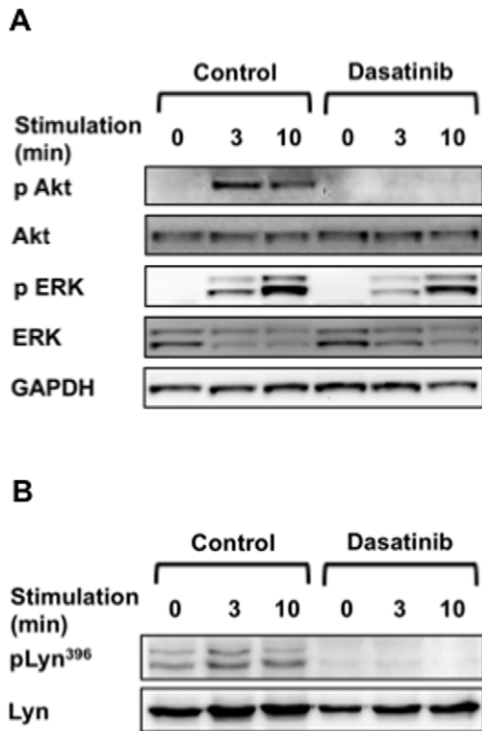


Figure 2. Dasatinib inhibits key signaling events on CXCR4 stimulation. **A:** CLL cells were treated with or without 100 nM dasatinib for 30 min, prior to stimulation with 100 ng/ml CXCL12. Protein lysates were prepared at 3 and 10 min, and immunoblotting performed to determine the level and phosphorylation of Akt and ERK. One representative blot from three independent experiments is shown (samples 35, 45 and 50). **B:** CLL cells were treated as above, and immunoblotting for Lyn³⁹⁶ phosphorylation performed. The immunoblot shown is representative of three independent experiments (samples 44, 45 and 70). doi:10.1371/journal.pone.0048929.g002

the PI-3K inhibitor wortmannin reduced CLL cell migration towards CXCL12, whilst MEK inhibition had no significant effect [3]. More recently, specific PI-3K inhibitors have been shown to inhibit actin polymerisation, chemotaxis, and pseudoemperipolesis in response to CXCL12 [19,21]. We were next interested to investigate the mechanism by which dasatinib may inhibit Akt phosphorylation in CLL cells in response to CXCL12.

Dasatinib exerts its pro-apoptotic effects through inhibition of kinases involved in BCR signaling, including Lyn and Syk [7,9,10,11]. Here, we show that dasatinib inhibits Lyn autophosphorylation in the presence and absence of CXCL12 stimulation (Figure 2B). Interestingly, the Src-family kinase Lyn has been demonstrated to control migration of hematopoietic cells, with chemotaxis of BM mononuclear cells from Lyn^{-/-} mice toward CXCL12 impaired by over 75% [22]. Moreover, siRNA knockdown of Lyn in primary CD34⁺ hematopoietic progenitor cells reduced migration toward CXCL12 three to seven-fold over controls [23]. Therefore Lyn inhibition may contribute to the anti-migratory effect of dasatinib in CLL cells. Of note, the Src kinases Lyn and Fyn interact directly with the p85 subunit of PI-3K through their Src homology 3 (SH3) domains in a B cell lymphoma cell line [24], and Lyn co-localizes with PI-3K in HL-60 cells following CXCL12 stimulation [22]. Buchner et al. recently demonstrated CXCL12 stimulation to induce phosphor-

ylation of Syk and Akt in CLL cells, which was abrogated by the small molecule Syk inhibitor R406 [25]. R406 also significantly reduced CLL cell migration toward CXCL12 [25,26]. Although R406 has a number of off-target effects [27], a recent study demonstrated two novel specific Syk inhibitors, PRT318 and P505-15, to significantly reduce chemotaxis toward CXCL12 or CXCL13 and inhibit pseudoemperipolesis in stromal co-culture experiments [28].

Recently published data suggests that additional dasatinib target kinases may also contribute to the overall anti-migratory effect. BTK activation has been demonstrated following CXCL12 stimulation in a B-cell lymphoma cell line, and the small-molecule BTK inhibitor PCI-32765 blocked CXCL12-induced ERK and Akt phosphorylation in the same cell line [29] and primary CLL cells [30]. Furthermore, PCI-32765 significantly reduced actin polymerization and migration of primary CLL cells toward CXCL12 and CXCL13 [29,30]. It is notable that the IC₅₀ of dasatinib for BTK is 5 nM [7]. In solid tumour cell lines, dasatinib inhibits migration by blocking phosphorylation of Src and the downstream target focal adhesion kinase (FAK) [12,13,14]. Of note, Lopez-Guerra *et al.* recently demonstrated phosphorylation of FAK in response to CXCL12 stimulation in CLL cells, and inhibition of Src and FAK by the multikinase inhibitor sorafenib correlated with reduced chemotaxis [31]. In summary, dasatinib targets several key tyrosine kinases that regulate the migration of CLL cells in response to chemokine stimulation, resulting in a significant impairment of chemotaxis.

Clinical trials of kinase inhibitors targeting BCR signaling in CLL have confirmed that the anti-migratory effects observed *in vitro* also occur *in vivo* and contribute substantially to overall clinical response. Hoellenriegel *et al.* reported that the PI-3K delta inhibitor CAL-101 induced an early reduction in patient LN size accompanied by a significant lymphocytosis that later resolved on therapy [21], suggesting that CLL cells were mobilised from the protective environment of the BM and LN to the peripheral blood where they were then sensitive to the pro-apoptotic effects of the inhibitor. A transient lymphocytosis is also observed in patients responding to the orally-available Syk inhibitor fostamatinib disodium [32], and the BTK inhibitor PCI-32765 [30] in reported phase I/II trials. To date, there is only one published phase II trial of dasatinib in CLL. In this small study, Amrein *et al.* reported significant nodal responses to be achieved more frequently (9/15 patients) than a reduction in peripheral blood leucocytosis (4/15 patients) [33]. The authors postulated that dasatinib may preferentially induce apoptosis of proliferating CLL cells. Of note, we found no inhibitory effect of dasatinib on proliferation or survival of CLL cells cultured for up to 12 days in the CD154/IL-4 system (Figure 3), an *in vitro* co-culture system that approximates the *in vivo* microenvironment of proliferation centres [34]. These data are consistent with previous reports that the pro-apoptotic effect of dasatinib alone is lost on co-culture with stromal cells expressing CD154 [9,35], but more importantly, this is the first published report of the effect of dasatinib on CLL cell proliferation. Our data therefore suggest that an *in vivo* anti-migratory effect contributes to the overall clinical response to dasatinib, as observed with the other targeted kinase inhibitors described above. As dasatinib exhibits synergy with both established and novel chemotherapeutic agents in the absence of a protective microenvironment [9] our present data further support the investigation of dasatinib in future combination clinical trials.

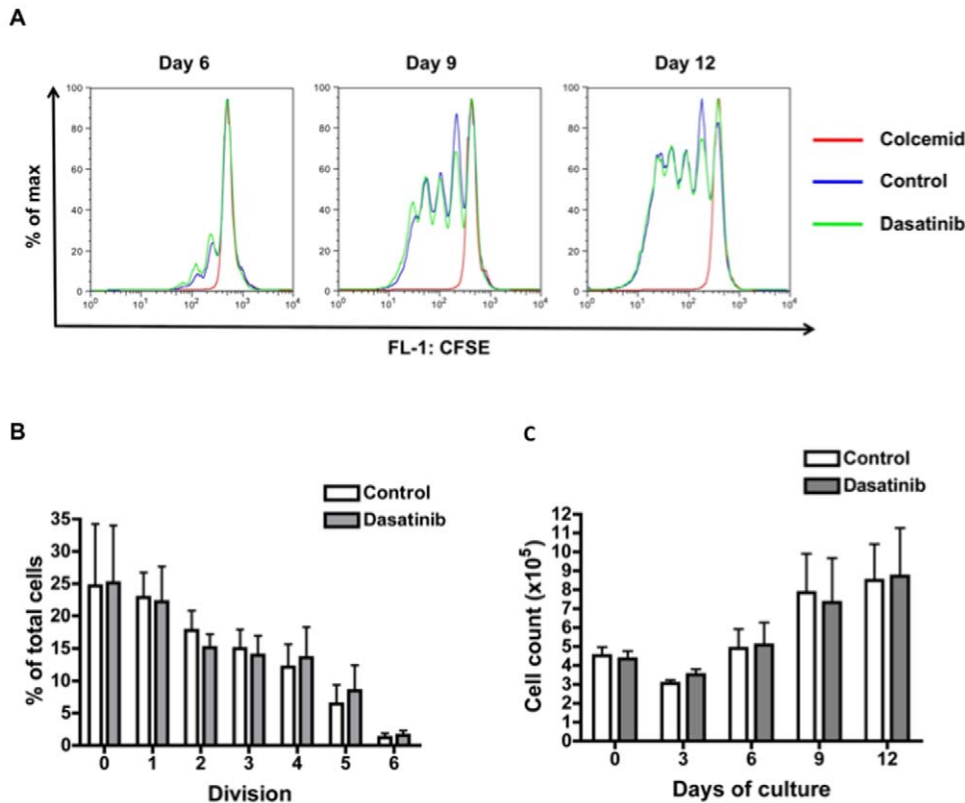


Figure 3. Dasatinib does not inhibit proliferation of CLL cells induced by CD154 and IL-4. **A:** The panels show the assessment of cell division by successive dilution of CFSE at days 6, 9, and 12 of co-culture in the CD154/IL-4 system in one representative experiment. **B:** The graph shows the mean (\pm SEM) percentage of cells in each cell division at day 12 from six independent experiments (samples 34, 41, 46, 68, 69 and 70). **C:** The graph shows the mean (\pm SEM) cell counts determined for each condition and time point in all six experiments. doi:10.1371/journal.pone.0048929.g003

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