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The phosphatidylinositol 3-kinase-δ Inhibitor CAL-101 demonstrates promising pre-clinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals

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Running Title: PI3K-δ inhibitor induces apoptosis in primary CLL cells

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ABSTRACT

Targeted therapy with imatinib in chronic myeloid leukemia (CML) prompted a new treatment paradigm. Unlike CML, chronic lymphocytic leukemia (CLL) lacks an aberrant fusion protein kinase, but instead displays increased phosphatidylinositol 3-kinase (PI3K) activity. To date, development of PI3K inhibitors has been limited due to the requirement of this pathway for many essential cellular functions. Identification of the hematopoietic-selective isoform PI3K-δ unlocks a new therapeutic potential for B-cell malignancies. Herein, we demonstrate that PI3K has increased enzymatic activity and that PI3K-δ is expressed in CLL cells. A PI3K–δ selective inhibitor CAL-101 promoted apoptosis in primary CLL cells *ex vivo* in a dose- and time-dependent fashion that was independent of common prognostic markers. CAL-101 mediated cytotoxicity was caspase dependent and was not diminished by co-culture on stromal cells. Additionally, CAL-101 abrogated protection from spontaneous apoptosis induced by CD40L, BAFF, TNF-α and fibronectin. In contrast to malignant cells, CAL-101 does not promote apoptosis in normal T-cells or NK cells, nor does it diminish antibody-dependent cellular cytotoxicity. However, CAL-101 did decrease activated T-cell production of various inflammatory and anti-apoptotic cytokines. Collectively, these studies provide rationale for the clinical development of CAL-101 as a first-in-class targeted therapy for CLL and related B-cell lymphoproliferative disorders.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of adult leukemia in the United States, with approximately 15,000 new cases and about 4,500 deaths per year [1]. CLL is characterized by a B1 monoclonal lymphocyte immunophenotype with expression of the surface antigens CD19, CD5, CD20, CD23, and dim sIgG. The cell of origin of CLL is uncertain but a gene expression pattern most similar to a mature memory B-cell has been hypothesized [2]. In addition, CLL cells display disrupted apoptosis that is caused both by primary tumor features and co-dependent stromal elements [3]. Although many patients are asymptomatic at diagnosis, CLL is a progressive disease that in most patients eventually will require treatment. Once symptomatic, patients have a relatively short overall survival, ranging from 18 months to six years, with a 22.5% 10-year survival expectation [4]. Common treatments for CLL include alkylating chemotherapeutic drugs (such as chlorambucil and cyclophosphamide), purine analogs (such as fludarabine), and rituximab (used in combined with fludarabine (FR), fludarabine and cyclophosphamide (FCR) or pentostatin and cyclophosphamide (PCR). Newer studies with either single agent bendamustine or alemtuzumab have been demonstrated to have improved response and progression-free survival over alkylator-based therapy. However, no current treatment option results in curative therapy and all patients eventually relapse. This provides strong justification for developing additional types of therapies for CLL. Of particular interest are therapies that target signal transduction pathways essential to CLL cell survival mechanisms that are known to be aberrantly activated.

One such pathway is the phosphoinositide 3-kinase (PI3K) pathway. The PI3K pathway is acknowledged as a key component of cell survival in many cancers, including CLL. It is activated by receptors, or the small GTPase Ras, and is made up of various classes of PI3K isoforms [5]. There are three classes of PI3K isoforms; however, only the class I isoforms phosphorylate inositol lipids to form second messenger phosphoinositides. Specifically, class I PI3K enzymes convert PtdIns(3,4)P_2 into PtdIns(3,4,5)P_3, in the cell membrane that recruit, via

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binding to the amino-terminal pleckstrin homology (PH) domain, downstream signaling proteins such as Tec kinases: PDK, Akt, ILK and Rac GEF. Class I isoforms are made up of two subsets (IA and IB). Class IA encompasses p110α, p110β and p110δ (catalytic domains), bound by p85, p50 or p55 (regulatory domains). Class IB is made up solely of the p110γ (catalytic domain) bound by the regulatory domain p101. The p110α and p110β isoforms are ubiquitously expressed, and knock-out mice for both are embryonic lethal [6]. It is thought that this widespread functionality of PI3K signaling is at least partially responsible for the significant cellular toxicity associated with pan-PI3K inhibitors such as LY294002 [7]. However, in recent years it has been shown that the different Class I isoforms, specifically the four catalytic subunits making up the four isoforms (p110α, p110β, p110δ and p110γ), have non-redundant roles and different expression profiles in different cell types [8-11].

The expression of PI3K-δ is generally restricted to hematopoietic cell types [12]. Mice with deleted or mutated PI3K-δ exhibit a B-cell defect, with a lack of B1 lymphocytes, decreased mature B-cell numbers, and impaired antibody production [6, 8, 13]. Biochemically, B-cells derived from PI3K-δ knock out mice also demonstrate less AKT phosphorylation when activated and have decreased PIP_3 levels and phosphopeptide activity [6]. In contrast, PI3K-γ isoform knock out mice, while not embryonic lethal, have predominately a T-cell defect with no B-cell developmental or functional abnormalities [6]. These mouse studies suggest that isoform-specific targeting of the PI3K-δ isoform may be cytotoxic to B-cells with minimal toxicity to other hematopoietic cell types.

Forced expression of PI3K-δ was shown to be transforming in cell lines [14]. Application of another specific PI3K-δ inhibitor in AML demonstrated both pre-clinical activity and enhancement of the cytotoxic effect with chemotherapy in cells with active PI3K-δ [15, 16]. Previous studies have demonstrated increased general activity of PI3K in the pathogenesis of CLL with convergence of CD40L, BAFF, fibronectin, and BCR signaling through this pathway.

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However, only one study examining the influence of PI3K signaling on the microenvironment has addressed the relevance of specific PI3K isoform signaling. This study demonstrated that both the cytotoxic effects of PI3K inhibitors and also microenvironmental protection were afforded predominately by inhibition of the PI3K-α isoform, but other isoforms also played a distinct role in these processes [17].

Therapeutic targeting of a specific PI3K isoform expressed selectively in hematopoietic cell types represents a potentially promising approach for the treatment of CLL. However until recently, no therapeutic agents that actively target specific PI3K isoforms have been available. CAL-101 is a potent and selective inhibitor of PI3K-δ isoform [18]. To further justify transition of CAL-101 to clinical trials in CLL, we investigated this agent for both its direct therapeutic effect on CLL cells and also its ability to disrupt external survival stimuli provided by the microenvironment. These studies demonstrate that CAL-101 both directly promotes apoptosis in CLL cells and also disrupts multiple external survival pathways that contribute to CLL viability and proliferation *in vivo*. Collectively, these studies provide significant support for development of CAL-101 as a therapeutic agent for CLL.

MATERIALS AND METHODS

Reagents and Antibodies:

CAL-101 and recombinant proteins for p110α, p110β, p110γ and p110δ were provided by Calistoga Pharmaceuticals (Seattle, WA). Phycoerythrin (PE)-labeled isotype control mouse IgG_1, CD19, CD3, CD56, fluorescein isothiocyanate (FITC)-labeled annexin V, anti-CD28 and propidium iodide (PI) were purchased from BD Pharmingen (San Diego, CA) and used according to manufacturer's instructions. LY294002 was purchased from BIOMOL (Plymouth Meeting, PA). Z-VAD-fmk, anti-caspase 3, Human IL-6, IL-4, IL-10, TNF-α and INF-γ Quantikine ELISA Kits, rhIL-4 and rhBAFF were purchased from R&D Systems (Minneapolis, MN). PI3K ELISA assay was purchased from Echelon Biosciences (Salt Lake City, UT). Anti-

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AKT, anti-phospho-AKT (Ser473), anti-phospho-STAT3 (Tyr705), anti-STAT3, anti-phospho-GSK3β (Ser9), anti- GSK3β, and anti-PARP were purchased from Cell Signaling Technologies (Danvers, MA). Anti-actin, anti-mcl-1 and anti-p110δ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH was purchased from Millipore (Billerica, MA). Recombinant human soluble CD40L was purchased from PeproTech (Rocky Hill, NJ). Ac-DEVD-AFC was purchased from Enzyme Systems Products (Solon, OH). Anti-CD3 T-cell activation plates and fibronectin coated multiwall plates were purchased from BD Biosciences (San Jose, CA). TNF-α and 2-fluoroadenine-9-β-D-arabinofuranoside (fludarabine prodrug) were purchased from Sigma Aldrich (St. Louis, MO).

Patient Sample Processing and Cell Culture:

Blood was obtained from patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the Institutional Review Board of The Ohio State University (Columbus, OH). All patients examined in this series had immunophenotypically defined CLL as outlined by the modified 1996 National Cancer Institute criteria [19]. All of the patients had been without prior therapy for a minimum of thirty days at the time of collection. CLL B-cells were isolated from freshly donated blood using ficoll density gradient centrifugation (Ficoll-Plaque Plus, Amersham Biosciences, Piscataway, NJ). Enriched CLL fractions were prepared by using the "Rosette-Sep" kit from Stem Cell Technologies (Vancouver, British Columbia, Canada) according to the manufacturer's instructions. Isolated cells were incubated in RPMI 1640 media supplemented with 10% heat-inactivated human serum (HS, Valley Biomedical, Winchester, VA), 2 mmol/L L-glutamine (Invitrogen, Carlsbad, CA), and 100 U/mL penicillin/100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C in an atmosphere of 5% CO_2. The purity of enriched populations of CLL was routinely checked using CD19-phycoerthrin (PE) staining by flow cytometry. Normal cells were obtained from Red Cross partial leukocyte preparations, and T-cells or NK cells were negatively selected using the

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appropriate “Rosette-Sep” kits. The purity of enriched populations of normal cells was routinely checked using CD19, CD3, and CD56-phycoerthrin (PE) staining by flow cytometry. Normal samples were from anonymous donors as part of a second IRB approved exemption protocol at OSU. The HS-5 cell line was obtained from ATCC (Manassas, VA) and cultured in DMEM media supplemented with 10% fetal bovine serum.

Immunoblot Analysis:

Whole cell lysates were prepared as previously described by our group with the addition of phosphatase inhibitor cocktail 1 and 2, protease inhibitor cocktail P8340 and 1 mM phenylmethylsulfonyl fluoride (all from Sigma-Aldrich, St. Louis, MO) to the lysis buffer [20]. Equivalent amounts of protein (50 μg/lane) were separated on polyacrylamide gels and transferred onto nitrocellulose membranes. Following antibody incubations, proteins were detected with chemiluminescent substrate (SuperSignal, Pierce, Rockford, IL).

PI3K Assay:

PI3K assay was preformed on whole cell lysates from CLL or normal B-cells. A PI3K ELISA assay was performed according to the manufactures instructions (Echelon Biosciences). Briefly, whole cell extracts were added to a mixture of $PI(4,5)P_2$ substrate and reaction buffer containing ATP and allowed to incubate at room temperature. The reaction was stopped by adding $PI(3,4,5)P_3$ detector mixed with EDTA and allowed to incubate at room temperature for 1 hour. After this time, the mixture was transferred from each well to a PI3K ELISA plate and allowed to incubate 1 hour. Plates were washed and then incubated with secondary detector for 30 minutes. Plates were washed again and TMB solution was added for 5 minutes at which time H_2SO_4 was added to stop all reactions. Plates were read at 450 nm on a Labsystems 96-well plate reader (Fisher Scientific, Pittsburgh, PA).

Viability and Flow Cytometry Studies:

MTT (3’[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assays were performed to determine cytotoxicity. Briefly, 1 x 10^5 cells (CLL B-cells or normal volunteer cells

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– T- or NK cells) were incubated for 48 hours with different concentrations of CAL-101, 25 μM LY294002 or vehicle control. MTT reagent (Sigma-Aldrich, St. Louis, MO) was then added and plates were incubated for an additional 20 hours before washing with protamine sulfate in phosphate-buffered saline. DMSO was added and absorbance was measured by spectrophotometry at 540 nm in a Labsystems plate reader (Fisher Scientific). Cell viability was also measured at various time points using annexin/PI flow cytometry (Beckman-Coulter, Miami, FL). Data was analyzed with Expo-ADC32 software package (Beckman-Coulter). At least 10,000 cells were counted for each sample. Results were expressed as percentage of total positive cells over untreated control. Experiments examining caspase-dependent apoptosis included the addition of 100 μM Z-VAD. Experiments examining survival signals included the addition of 1 μg/mL CD40L, 800 U/mL IL-4, 50 ng/mL BAFF, 20 ng/mL TNF-α or co-culturing on fibronectin or stromal (HS-5 cell line) coated plates. Stromal co-culture was done by plating a 75 cm^2 flask (80-100% confluent) per 6 well plate 24 hours prior to the addition of CLL cells.

Analysis of Antibody-dependent Cellular Cytotoxicity (ADCC):

ADCC was determined by standard 4 hour ^{51}Cr-release assay. ^{51}Cr-labeled target cells (1×10^4 isolated CLL B-cells) were incubated with media alone (vehicle only) or in the presence of various antibodies (alemtuzumab, rituximab and herceptin) at 10 μg/mL concentration for 30 minutes at 37°C. Unbound antibody was washed off and the cells plated at 1×10^4 cells/well. Effector cells (negatively isolated NK cells from healthy donors) were pre-treated with 10 μM CAL-101 and then added to the plates at indicated effector to target (E:T) ratios. After 4 hour incubation, supernatants were removed and the radioactivity was counted in a gamma counter. The percentage of specific cell lysis was determined by: % lysis = 100 x (ER – SR)/(MR – SR). ER, SR and MR represent experimental, spontaneous and maximum ^{51}Cr-release. Data was normalized to untreated control.

Detection of Cytokine Production by ELISA Assay:

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Cytokine production was measured using Quantikine ELISA assays (R&D Systems). CD3 stimulation was done using anti-CD3 T-cell activation plates (BD Biosciences, San Jose, CA). CD3 positive T-cells were drugged for 48 hours with media alone (vehicle only) and varying doses of CAL-101. After drugging, 2 x 10^6 cells were added to each well of the anti-CD3 plate in duplicate. After 24 hours the supernatant from each well was collected and an ELISA assay was performed according to the manufacturer's directions.

Quantitative RT-PCR:

RNA was extracted from 2 x 10^7 cells using TRIzol reagent (Invitrogen). cDNAs were prepared using a SuperScript First-Strand Synthesis System (Invitrogen) as previously described [21]. Real-Time PCR was performed using pre-designed TaqMan® Gene Expression Assays and ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA).

Enzymatic Caspase Assay:

The presence of active caspase enzymes was determined by the amino trifluoromethyl coumarin assay (AFC), as previously described [22]. Lysates containing approximately 3 x 10^6 cells were incubated with DEVD-AFC to determine the presence of active caspase-3 in a cyto-buffer (10% glycerol, 50 mM Pipes, pH 7.0, 1 mM EDTA) containing 1 mM DTT and 20 μM tetrapeptide substrate. Caspase-3 activity was measured immediately after addition of substrate. Release of free AFC was determined using a Beckman Coulter DTX 880 multimode detector (Filters: excitation; 405/10 nm, emission; 535/25 nm).

Statistical Analysis:

All reported statistical evaluation was done by the Center for Biostatistics at OSU. As many of the measurements used samples from the same patients, linear mixed effects models were used for analysis to take into consideration the dependency of these observations. To stabilize the variance, the raw Ct value of real time PCR data was normalized to internal control, and the standardized data were analyzed using linear mixed effects models. Holm's procedure was used to correct for multiple comparisons when appropriate [23]. Type I error is strongly

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controlled at α=0.05 for single comparisons and after adjustment for multiple comparisons or multiple endpoints.

RESULTS

PI3K-δ is Expressed Abundantly in CLL Cells

Given the broad importance of the PI3K-δ isoform in B-cell development, we sought to determine its expression profile in primary CLL cells. Expression of PI3K-δ was examined in cells from 20 separate CLL patients. All CLL tumor cells demonstrated expression of PI3K-δ, with relatively little variability among patients (Fig. 1A). In addition to CLL patient B-cells, we also evaluated the expression profile of PI3K-δ in normal hematopoietic cells. We found that all lymphoid cells expressed PI3K-δ with little variation across cell type (Fig 1B). Given the consistent expression of PI3K-δ in CLL cells and normal B-cells, we next sought to determine if the PI3K pathway was more active in tumor cells as compared to normal B-cells. As shown in Figure 1C, CLL cells overall have a statistically higher intrinsic PI3K activity as compared to normal B-cells (p-value=0.006), as previously reported by others [24]. These studies collectively confirm the presence of the PI3K-δ isoform in CLL cells and the activity of PI3K overall, thereby validating further exploration of specific inhibitors of this pathway.

CAL-101 Induces Selective Cytotoxicity in CLL Cells Independent of IgV_H Mutational Status or Interphase Cytogenetics

CAL-101 is a selective PI3K-δ inhibitor whose chemical structure and inhibitory properties have been previously described [18]. To determine the potential *in vitro* activity of CAL-101 against CLL cells, B-cells from 16 patients with CLL were treated with different concentrations of CAL-101 for 48 hours. We found that CAL-101 exhibited a dose dependent induction of cytotoxicity in CLL cells (Fig. 2A) as measured by MTT. Cell death induced by therapeutic agents in CLL can occur though caspase-dependent or -independent apoptosis or by necrosis. Studies using annexin-V/PI staining demonstrate evidence of both early (annexin

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V-positive only) and late (annexin V/PI both positive) apoptosis (data not shown). CAL-101 induced apoptosis of CLL cells was significant compared to vehicle treatment alone (p-value<0.0001) (Figure 2B). These results shown in Figure 2B provide support that the cytotoxicity induced by CAL-101 occurs via the induction of apoptosis, and corroborate the MTT data (Figure 2A). Although cytotoxicity was initially measured at 48 hours, CAL-101 induced cell death increased in a dose- and time-dependent manner, with evidence of apoptosis observed as early as 12 hours (Fig. 2B and 2C).

Chromosomal deletions of del(11q22.3) and del(17p13.1) are one of the strongest laboratory predictors of CLL response to chemotherapy [25]. Similarly, IgV_H gene mutational status influences the duration of remission to standard therapies utilized to treat CLL [26]. Figure 2D demonstrates that there was no significant difference in CAL-101 sensitivity based upon different interphase cytogenetic groups including those with del(17p13.1) (p-value=0.34). Furthermore, we observed no significant differences in CAL-101 cytotoxicity in patients with IgV_H mutated versus IgV_H unmutated CLL cells (p-value=0.51) (Fig. 2E). Similar findings with lower doses of CAL-101 (1μM) and LY294002 (pan-PI3K inhibitor) were observed for both cytogenetic and mutational analysis comparisons (data not shown). In the same patient pools Fludarabine was found to have a significant difference among cytogenetic and mutational status populations (data not shown). These studies suggest that CAL-101 may induce cell death independent of p53 function and other adverse prognostic factors associated with poor response to therapy in patients with CLL; which is in contrast to other available agents.

To examine the selectivity of CAL-101 on CLL cells as compared to normal B-cells, we evaluated the cytotoxicity of CAL-101 on CD19 selected normal B-cells from healthy volunteers. At high concentration (10μM) we found that CAL-101 did produce modest apoptosis (95% CI = -12.1630 to -5.0370) in this cell population that was significantly increased (p-value=0.0005) as compared to media control as shown in Figure 2F. The apoptosis noted in normal B-cells was, however, significantly less than that observed in CLL cells (p-value=0.0003). These data

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provide support that CAL-101 induces cytotoxicity preferentially to CLL cells as compared to normal B-cells.

CAL-101 Cytotoxicity Against CLL Cells is Partially Dependent on Caspase Activity

We next investigated the mechanism by which CAL-101 mediates cell death in CLL cells. Apoptosis with other cytotoxic agents in CLL occurs via caspase-dependent and –independent pathways [27-29]. In an attempt to determine whether the cytotoxicity induced by CAL-101 is due to an increase in caspase-dependent apoptosis, CLL cells were incubated with CAL-101 for 12 hours after which assessment of pro-caspase-3 processing to the active p20 caspase-3 cleavage product was assessed by immunoblot analysis. Treatment of CAL-101 resulted in an increase in active caspase-3 concurrent with a decrease in the pro-form (Fig. 3A). Concurrently, the appearance of the cleaved product of the caspase-3 substrate PARP was detected (Fig. 3A). Enzymatic activity of caspase-3 was also detected after 12 hours of CAL-101 treatment and increased after longer treatments (Fig. 3B and data not shown). In order to determine if apoptosis was occurring through a caspase dependent mechanism we treated CLL cells with CAL-101 in the presence or absence of the pan-caspase inhibitor z-VAD-fmk. We found that the addition of z-VAD-fmk to CAL-101 treated samples significantly decreased the cytotoxicity observed with CAL-101 (Fig. 3C) (p-value=0.004) and diminished the appearance of the cleaved product of PARP (Fig. 3D). Collectively, these studies suggest that CAL-101 mediates cytotoxicity primarily through a caspase dependent mechanism.

CAL-101 Does not Demonstrate Cytotoxicity Toward Other Normal Immune Cells

Treating normal NK cells or T-cells with CAL-101 under conditions described above, we did not observe significant cell death even at a dose of 100 μM (p-values for T-cells and NK cells were 0.16 and 0.48 respectively) (Fig. 4A). This result suggests that CAL-101 lacks significant cytotoxicity toward normal T-cells and NK cells as compared to that observed with CLL cells.

CAL-101 Alters Cytokine Production by T-Cells and NK Cells

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Although no significant cytotoxicity was seen in normal T- or NK cells we sought to determine if CAL-101 affects the function of T- or NK cells. Recent studies have demonstrated that PI3K-δ is essential to cytokine production by immune effector cells [30,31]. We therefore assessed the ability of CAL-101 to disrupt T-cell cytokine production by measuring IL-6, TNF-α, IL-10, and IL-4 production after anti-CD3 stimulation. We found that low doses of CAL-101 could inhibit normal T-cells from producing IL-6, IL-10 and TNF-α (Fig. 4B). Our assays failed to detect production of IL-4 in normal T-cells. Similar results were seen with T-cells isolated from CLL patients for evaluated cytokines (IL-6, IL-4 and IL-10) (data not shown). In addition to the aforementioned cytokines, activated T-cells also produce CD40 ligand (CD40L or CD154). Figure 4B demonstrates that treatment of CD3 ligated T-cells co-stimulated with CD28 results in an increase of CD154 mRNA. The addition of CAL-101 to these activated cells resulted in a dose dependent inhibition of CD154 mRNA induction. In a similar manner, we assessed the ability of CAL-101 to disrupt NK cell cytokine production. NK cells were treated with or without CAL-101 and incubated with plate-immobilized alemtuzumab. As shown in Figure 4C, a modest but significant decrease in IFN-γ production was observed with CAL-101 treatment. Similar findings with lower doses of CAL-101 (1 μM) and LY294002 were observed (data not shown). These studies demonstrate collectively that CAL-101 lacks direct cytotoxic potential to T-cells and NK cells, but can inhibit production of inflammatory cytokines such as IL-6, IL-10, TNF-α and IFN-γ and activation induced cytokines such as CD40L that others have shown promote proliferation or enhance survival of CLL cells [32-36].

CAL-101 Does not Alter Antibody Dependent Cellular Cytotoxicity Against CLL Cells

Therapeutic antibodies such as rituximab and alemtuzumab are widely utilized for the treatment of CLL. The mechanism of cytotoxicity of these antibodies likely involves antibody dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity and direct killing [37, 38]. Because rituximab and alemtuzumab are frequently used in combination therapies, we

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examined NK cell antibody dependent cellular cytotoxicity (ADCC) with these therapeutic agents following treatment with CAL-101. Target CLL cells were incubated with alemtuzumab, rituximab or herceptin. As shown in Figure 4D, pre-treatment of NK cells with CAL-101 did not significantly diminish ADCC of alemtuzumab-labeled CLL target cells. Similar results were observed with rituximab (data not shown).

CAL-101 Induces Apoptosis More Selectively than pan-PI3K Inhibitors

To determine if inhibition of PI3K-δ induced apoptosis comparable to inhibition of all isoforms of PI3K we compared CAL-101 to LY294002, a pan-PI3K inhibitor. We found that CAL-101 and LY294002 were not significantly different in their cytotoxic properties in CLL patient B-cells (Fig. 5A). We next sought to determine if CAL-101 produced more selective cytotoxicity than LY294002 in other hematopoietic cells. We found that CAL-101 and LY294002 did not show significant differences in their cytotoxic properties in normal B- or T-cells; however LY294002 produced significant cytoxicity in normal NK cells (p-value =0.004) which was in contrast to CAL-101 (Fig. 5B). This suggests that inhibition of the PI3K-δ isoform alone, by CAL-101, is sufficient to induce apoptosis in CLL patient cells without producing cytotoxic effects in other hematopoietic cells.

CAL-101 Antagonizes CD40-Ligand Mediated CLL Cell Survival

Cytokines such as CD40 ligand (CD40L, CD154) are produced by $CD4^+$ T-cells and also follicular dendritic cells and have been demonstrated to promote survival of CLL cells in part through the PI3K pathway. While our data with T-cells demonstrate CAL-101 can antagonize production of this cytokine, we hypothesized that it might also disrupt intracellular signaling in CLL cells. Following treatment with CD40L, CLL cells show increased phosphorylation of AKT at the Ser473 site, a finding consistent with PI3K pathway activation (Fig. 6A). In conjunction with CD40L treatment of CLL cells, we found significant protection from spontaneous apoptosis (Fig. 6B) (p-value<0.0001). Treatment of CLL cells with CAL-101 could decrease, although not

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completely prevent, the observed increase in AKT phosphorylation at the Ser473 site seen with CD40L (Fig. 6A). This reversal of induced AKT phosphorylation by CD40L occurred in a dose dependent manner with CAL-101 (Fig. 6C). The CAL-101 antagonism of Ser473 AKT phosphorylation, although not complete, was sufficient to abrogate the protective effect provided by CD40L as shown by annexin/PI flow cytometry (p-value< 0.001) (Fig. 6B). In contrast to CD40L, IL-4 mediated prevention of spontaneous apoptosis of CLL cells was not reversible with CAL-101 treatment (Fig. 6D). Furthermore, IL-4 did not increase basal AKT phosphorylation but rather activated the JAK/STAT pathway via phosphorylation of STAT3 (Fig. 6E), suggesting an alternative mechanism of protection for this cytokine. In addition to changes in the phosphorylation of AKT we also found alteration of downstream proteins. CD40L induced phosphorylation of GSK3β at the Ser9 site. Similar to AKT, CAL-101 reversed the phosphorylation of GSK3β (Fig. 6F). Along the same lines, we also saw an increase in Mcl-1 expression after CD40L that was reversible by CAL-101 treatment (Fig. 6G). These findings support a role for PI3K-δ in the protective effect of CD40L on CLL cells and also the specificity for CAL-101 to this pathway.

CAL-101 Antagonizes Alternative Microenvironment Stimuli

We next sought to further evaluate the role of CAL-101 in regulating alternative microenvironmental stimuli. The TNF family member BAFF has been shown in both mouse and human CLL to effectively prevent spontaneous apoptosis of CLL patient cells [39]. Consistent with previous reports, BAFF ligation protected CLL cells from spontaneous apoptosis (Fig. 7A). However, treatment with CAL-101 abrogated the protection induced by BAFF (Fig. 7A). Similar to BAFF, TNF-α has been shown to protect CLL cells from spontaneous apoptosis and TNF-α levels are frequently elevated in the plasma of CLL patients [40]. As we showed previously that CAL-101 could inhibit the production of TNF-α from T-cells, we assessed whether it could also prevent the direct effect of TNF-α on CLL cells. We found that TNF-α indeed protected CLL

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cells from spontaneous apoptosis, and showed that treatment with CAL-101 abrogated this protection (Fig. 7B). We next evaluated the effect of CAL-101 on fibronectin adhesion. Fibronectin has been shown to be a ligand for CD49d ($\alpha4\beta1$ integrins/VLA4); thus the protective effect elicited by fibronectin adhesion acts through a separate PI3K signaling cascade as compared to ligation of TNF family members. We found that fibronectin adhesion also protected CLL cells from spontaneous apoptosis, and CAL-101 again completely abrogated the protective effect of fibronectin (Fig. 7C). These findings further support the ability of CAL-101 for disrupting microenvironment signals. To confirm that the alterations in survival provided by these compounds were working through a PI3K dependent pathway we evaluated phosphorylation of AKT after stimulation. We found that BAFF, TNF-α and fibronectin all lead to an increase in phosphorylation of AKT at the Ser473 site similarly to what was observed with CD40L treatment (Fig. 7E and data not shown).

CLL viability *in vivo* is not only influenced by soluble factors, but also by co-contact with a variety of cells composing the bone marrow and lymph node microenvironment [41]. This is becoming increasingly recognized as important as many drugs that are brought into the clinic have a lower *in vivo* effect than expected due to the cell survival-promoting mechanisms produced by the microenvironment [42]. Because of this we sought to determine if co-incubation with CLL cells on a stromal cell line (HS-5) would affect the cytotoxic properties of CAL-101. We initially treated HS-5 cells with CAL-101 and determined it had no influence on stromal cell viability or morphology (data not shown). Co-culture of CLL cells on the HS-5 cell line demonstrated diminished spontaneous apoptosis as compared to cells co-cultured in media alone (data not shown). However, CAL-101 treatment of CLL cells co-cultured with HS-5 cells resulted in a similar proportion of cytotoxicity as compared to treatment of CLL cells without co-culture (Fig. 7D) (p-value= 0.499). These data suggest that CAL-101 has the potential to mediate cytotoxicity independent of the protective effect of contact with stromal cells.

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DISCUSSION

Herein, we have described a selective PI3K–δ inhibitor, CAL-101, that lacks off-target effects of other PI3K inhibitors (like LY294002), but still induces apoptosis in primary CLL cells. All CLL patient B-cells tested express PI3K–δ, and PI3K activity was higher in these cells than in B-cells from healthy volunteers. Previous studies have demonstrated that PI3K inhibitors induce apoptosis in a variety of cancer cells; however, these agents have shown global toxicities from off-target effects in non-hematopoietic cells. CAL-101 at low doses targets only the PI3K–δ isoform of PI3K, which is selectively expressed in hematopoietic cells. We have shown that CAL-101 has selective activity against B-cells relative to T-cells and NK cells as measured by cell death, although cytokine production of non B-cells is inhibited by CAL-101. The cytotoxic activity of CAL-101 in CLL occurred independently of the del(17p13.1) and IgV_H mutation prognostic markers. Furthermore, this study suggests that CAL-101 mediates cytotoxicity both by directly inhibiting CLL cell PI3K signaling and also antagonizing extrinsic activation of this pathway by CD40L, BAFF, TNF-α, fibronectin, and stromal cells. Together, these data provide strong justification that CAL-101 will be beneficial in the treatment of CLL.

The importance of the PI3K signaling pathway to mature B-cells has recently been established in a seminal paper showing its role in survival following B-cell stimulation [43]. Similarly, a recent publication demonstrated that PI3K-δ is essential for the production of antibodies by non-transformed B-cells [44]. This paper extends the importance of PI3K-δ signaling to transformed CLL cells where we demonstrate that disruption of this signaling pathway directly promotes apoptosis and also antagonizes stromal cell interactions. The findings put forth by us contrast from a recent paper by Burger and colleagues who suggest that inhibition of PI3K-α is the more essential isoform for effective disruption of microenvironmental

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signals and apoptosis [17]. Whereas we demonstrate the PI3K-δ isoform is expressed in CLL cells, only minimal PI3K-α expression was demonstrated (data not shown). CAL-101 is devoid of PI3K-α inhibitory activity and yet apoptosis and pathway disruption from signaling by multiple cytokines was observed. Additionally, treatment of CLL cells with pan-PI3K inhibitors such as LY294002 did not enhance apoptosis further over that observed with CAL-101. Given the complexities and potential problems of pan-PI3K inhibition from non-tumor specific pathway inhibition, our findings and those reported also in normal B-cells suggest that clinical exploration of PI3K-δ isoform specific inhibitors could have significant benefit to patients with CLL and warrant future investigation.

In addition to demonstrating the effect of CAL-101 on primary CLL cells, herein we were also able to demonstrate that this PI3K-δ specific agent greatly diminishes production of several inflammatory cytokines including TNF-α, CD40L, and IL-6 by T-cells and IFN-γ by NK cells. Recent publications have demonstrated the critical importance of PI3K-δ specific signaling in T-cell [45] and NK cell [30] cytokine production. Blocking production of these different cytokines *in vivo* in CLL patients would potentially have the effect of antagonizing the survival effects of these cytokines on CLL cells. Additionally, these cytokines are also produced following initial administration of several therapeutic antibodies utilized in CLL including rituximab, alemtuzumab and ofatumumab and are associated with life threatening infusion toxicity. Administration of CAL-101 with these therapeutic antibodies may diminish this infusion toxicity by abrogating production of these cytokines. In contrast, we demonstrate that CAL-101 does not interfere with antibody mediated ADCC with rituximab and alemtuzumab. Based upon the unique mechanism of action of CAL-101, exploration of such combination strategies with therapeutic antibodies is indicated.

In CLL primary cells we demonstrate low levels of Ser473 AKT phosphorylation in the majority of patients examined which is increased following CD40 ligation and stromal contact,

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which in turn protects CLL cells from spontaneous apoptosis *in vitro* [46]. We also found that other microenvironmental stimuli (such as BAFF, TNF-α, fibronectin, and IL-6) act in a similar manner to promote phosphorylation of downstream targets of PI3K and thus prevent spontaneous apoptosis *in vitro.* At sub-micromolar concentrations of CAL-101 we observe significant antagonism of these survival signals. This key finding suggests that CAL-101 may inhibit downstream phosphorylation events initiated by multiple survival factors in a fashion similar to what we have shown with CD40L-mediated signaling. Collectively, these findings suggest that CAL-101 may effectively antagonize multiple mechanisms of CLL survival provided by nodal and bone marrow stromal cells. Finally, the ability of CAL-101 to induce apoptosis and antagonize multiple environmental stimuli demonstrates true potential for the treatment of CLL and related lymphoid malignancies. Other compounds targeting proximal or distal signaling involved in PI3K have shown clinical activity in CLL patients including the Syk inhibitor fostamatinib disodium [47] and the mTOR inhibitor RAD001 [48, 49], providing strong rational for targeting this pathway. The data put forward here from our laboratory data clearly justify clinical development of CAL-101 in CLL and related lymphoid malignancies which is now well underway [50].

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AUTHOR DISCLOSURES

BL, KDP, and NG are employees of Calistoga Pharmaceuticals and have financial interests in CAL-101 development; JCB has been a consultant to Calistoga and has financial interests in CAL-101 development.

AUTHOR CONTRIBUTIONS

S.E.M.H. planned the research, performed experiments, analyzed data, drafted the first and subsequent drafts of the paper and approved the final version of the paper.
A.L.G. and A.J.W. were involved in planning components of the research, performing experiments, reviewed drafts and approved the final version of the paper.
N.H. and W.Z. were involved in determining cytogenetics or mutational status of patient samples, reviewed drafts and approved the final version of the paper.
J.M.F., L.A., and J.J. accrued patients to the CLL clinical trial, reviewed drafts of the paper and approved the final version of the paper.
X.Z. and L.W. were involved in planning components of the research, did all the statistical analysis, reviewed drafts and approved the final version of the paper.
B.J.L., K.D.P. and N.A.G. were involved in planning components of the research, provided necessary reagents essential to the hypothesis of this paper, reviewed drafts and approved the final version of the paper.
J.C.B. and A.J.J. planned every aspect of the proposal, supervised the research, analyzed data, reviewed drafts, obtained funding for the research work and approved the final version of the paper.

REFERENCES

1. Eltom MA, Jemal A, Mbulaiteye SM, Devesa SS, Biggar RJ. Trends in Kaposi's sarcoma and non-Hodgkin's lymphoma incidence in the United States from 1973 through 1998. *Journal of the National Cancer Institute.* 2002;94(16):1204-1210.

2. Klein U, Tu Y, Stolovitzky GA, et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *The Journal of experimental medicine.* 2001;194(11):1625-1638.

3. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood.* 2000;96(8):2655-2663.

4. Diehl LF, Karnell LH, Menck HR. The American College of Surgeons Commission on Cancer and the American Cancer Society. The National Cancer Data Base report on age, gender, treatment, and outcomes of patients with chronic lymphocytic leukemia. *Cancer.* 1999;86(12):2684-2692.

5. Cantrell DA. Phosphoinositide 3-kinase signalling pathways. *J Cell Sci.* 2001;114(Pt 8):1439-1445.

6. Vanhaesebroeck B, Ali K, Bilancio A, Geering B, Foukas LC. Signalling by PI3K isoforms: insights from gene-targeted mice. *Trends Biochem Sci.* 2005;30(4):194-204.

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7. Xu GB, Lu XG, Luo LH, Zhu L. [Detection of soluble Apo-1/Fas in plasma, pleural and ascites fluid of malignant tumor patients and its clinical significance]. *Zhejiang Da Xue Xue Bao Yi Xue Ban.* 2003;32(4):335-338.

8. Jou ST, Carpino N, Takahashi Y, et al. Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex. *Mol Cell Biol.* 2002;22(24):8580-8591.

9. Fruman DA. Towards an understanding of isoform specificity in phosphoinositide 3-kinase signalling in lymphocytes. *Biochem Soc Trans.* 2004;32(Pt 2):315-319.

10. Papakonstanti EA, Zwaenepoel O, Bilancio A, et al. Distinct roles of class IA PI3K isoforms in primary and immortalised macrophages. *Journal of cell science.* 2008;121(Pt 24):4124-4133.

11. Min SH, Abrams CS. Why do phosphatidylinositol kinases have so many isoforms? *Biochem J.* 2009;423(1):e5-8.

12. Vanhaesebroeck B, Welham MJ, Kotani K, et al. P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proceedings of the National Academy of Sciences of the United States of America.* 1997;94(9):4330-4335.

13. Okkenhaug K, Bilancio A, Farjot G, et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science (New York, N.Y.* 2002;297(5583):1031-1034.

14. Kang S, Denley A, Vanhaesebroeck B, Vogt PK. Oncogenic transformation induced by the p110beta, -gamma, and -delta isoforms of class I phosphoinositide 3-kinase. *Proc Natl Acad Sci U S A.* 2006;103(5):1289-1294.

15. Sujobert P, Bardet V, Cornillet-Lefebvre P, et al. Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. *Blood.* 2005;106(3):1063-1066.

16. Billottet C, Grandage VL, Gale RE, et al. A selective inhibitor of the p110delta isoform of PI 3-kinase inhibits AML cell proliferation and survival and increases the cytotoxic effects of VP16. *Oncogene.* 2006;25(50):6648-6659.

17. Niedermeier M, Hennessy BT, Knight ZA, et al. Isoform-selective phosphoinositide 3'-kinase inhibitors inhibit CXCR4 signaling and overcome stromal cell-mediated drug resistance in chronic lymphocytic leukemia: a novel therapeutic approach. *Blood.* 2009;113(22):5549-5557.

18. Lannutti BJ, Meadows, Sarah A, Kashishian, Adam, Steiner, Bart, Pogosov, Galina, Sala-Torra, Olga, Johnson, Amy J, Byrd, John C., Radich, Jerald, Giese, Neill A. CAL-101, An Oral p110{delta} Selective Phosphatidylinositol-3-Kinase (PI3K) Inhibitor for the Treatment of B Cell Malignancies Inhibits PI3K Signaling, Cellular Viability and Protective Signals of the Microenvironment. *Blood.* 2009;114:286.

19. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood.* 1996;87(12):4990-4997.

20. Byrd JC, Shinn C, Waselenko JK, et al. Flavopiridol induces apoptosis in chronic lymphocytic leukemia cells via activation of caspase-3 without evidence of bcl-2 modulation or dependence on functional p53. *Blood.* 1998;92(10):3804-3816.

21. Lapalombella R, Yu B, Triantafillou G, et al. Lenalidomide down-regulates the CD20 antigen and antagonizes direct and antibody-dependent cellular cytotoxicity of rituximab on primary chronic lymphocytic leukemia cells. *Blood.* 2008;112(13):5180-5189.

22. Fahy RJ, Doseff AI, Wewers MD. Spontaneous human monocyte apoptosis utilizes a caspase-3-dependent pathway that is blocked by endotoxin and is independent of caspase-1. *J Immunol.* 1999;163(4):1755-1762.

23. Gilboa N, Durante D, Guggenheim S, et al. Immune deposit nephritis and single-component cryoglobulinemia associated with chronic lymphocytic leukemia. Evidence for

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a role of circulating IgG-anti-IgG immune complexes in the pathogenesis of the renal lesion. *Nephron.* 1979;24(5):223-231.

24. Ringshausen I, Schneller F, Bogner C, et al. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. *Blood.* 2002;100(10):3741-3748.

25. Byrd JC, Gribben JG, Peterson BL, et al. Select high-risk genetic features predict earlier progression following chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: justification for risk-adapted therapy. *J Clin Oncol.* 2006;24(3):437-443.

26. Montillo M, Hamblin T, Hallek M, Montserrat E, Morra E. Chronic lymphocytic leukemia: novel prognostic factors and their relevance for risk-adapted therapeutic strategies. *Haematologica.* 2005;90(3):391-399.

27. Pepper C, Thomas A, Fegan C, Hoy T, Bentley P. Flavopiridol induces apoptosis in B-cell chronic lymphocytic leukaemia cells through a p38 and ERK MAP kinase-dependent mechanism. *Leuk Lymphoma.* 2003;44(2):337-342.

28. Battle TE, Arbiser J, Frank DA. The natural product honokiol induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells. *Blood.* 2005;106(2):690-697.

29. Stanglmaier M, Reis S, Hallek M. Rituximab and alemtuzumab induce a nonclassic, caspase-independent apoptotic pathway in B-lymphoid cell lines and in chronic lymphocytic leukemia cells. *Ann Hematol.* 2004;83(10):634-645.

30. Guo H, Samarakoon A, Vanhaesebroeck B, Malarkannan S. The p110 delta of PI3K plays a critical role in NK cell terminal maturation and cytokine/chemokine generation. *The Journal of experimental medicine.* 2008;205(10):2419-2435.

31. Dil N, Marshall AJ. Role of phosphoinositide 3-kinase p110 delta in TLR4- and TLR9-mediated B cell cytokine production and differentiation. *Mol Immunol.* 2009;46(10):1970-1978.

32. Reittie JE, Yong KL, Panayiotidis P, Hoffbrand AV. Interleukin-6 inhibits apoptosis and tumour necrosis factor induced proliferation of B-chronic lymphocytic leukaemia. *Leukemia & lymphoma.* 1996;22(1-2):83-90, follow 186, color plate VI.

33. Jurlander J, Lai CF, Tan J, et al. Characterization of interleukin-10 receptor expression on B-cell chronic lymphocytic leukemia cells. *Blood.* 1997;89(11):4146-4152.

34. Digel W, Stefanic M, Schoniger W, et al. Tumor necrosis factor induces proliferation of neoplastic B cells from chronic lymphocytic leukemia. *Blood.* 1989;73(5):1242-1246.

35. Buschle M, Campana D, Carding SR, Richard C, Hoffbrand AV, Brenner MK. Interferon gamma inhibits apoptotic cell death in B cell chronic lymphocytic leukemia. *The Journal of experimental medicine.* 1993;177(1):213-218.

36. Ghia P, Chiorazzi N, Stamatopoulos K. Microenvironmental influences in chronic lymphocytic leukaemia: the role of antigen stimulation. *J Intern Med.* 2008;264(6):549-562.

37. Mone AP, Cheney C, Banks AL, et al. Alemtuzumab induces caspase-independent cell death in human chronic lymphocytic leukemia cells through a lipid raft-dependent mechanism. *Leukemia.* 2006;20(2):272-279.

38. Wierda WG, Kipps TJ, Keating MJ. Novel immune-based treatment strategies for chronic lymphocytic leukemia. *J Clin Oncol.* 2005;23(26):6325-6332.

39. Nishio M, Endo T, Tsukada N, et al. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. *Blood.* 2005;106(3):1012-1020.

40. Ferrajoli A, Keating MJ, Manshouri T, et al. The clinical significance of tumor necrosis factor-alpha plasma level in patients having chronic lymphocytic leukemia. *Blood.* 2002;100(4):1215-1219.

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41. Meads MB, Hazlehurst LA, Dalton WS. The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. *Clin Cancer Res.* 2008;14(9):2519-2526.

42. Kress CL, Konopleva M, Martinez-Garcia V, et al. Triterpenoids display single agent anti-tumor activity in a transgenic mouse model of chronic lymphocytic leukemia and small B cell lymphoma. *PLoS ONE.* 2007;2(6):e559.

43. Srinivasan L, Sasaki Y, Calado DP, et al. PI3 kinase signals BCR-dependent mature B cell survival. *Cell.* 2009;139(3):573-586.

44. Durand CA, Hartvigsen K, Fogelstrand L, et al. Phosphoinositide 3-kinase p110 delta regulates natural antibody production, marginal zone and B-1 B cell function, and autoantibody responses. *J Immunol.* 2009;183(9):5673-5684.

45. Patton DT, Garden OA, Pearce WP, et al. Cutting edge: the phosphoinositide 3-kinase p110 delta is critical for the function of CD4+CD25+Foxp3+ regulatory T cells. *J Immunol.* 2006;177(10):6598-6602.

46. Edelmann J, Klein-Hitpass L, Carpinteiro A, et al. Bone marrow fibroblasts induce expression of PI3K/NF-kappaB pathway genes and a pro-angiogenic phenotype in CLL cells. *Leuk Res.* 2008;32(10):1565-1572.

47. Friedberg JW, Sharman J, Sweetenham J, et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood.* 2010;115(13):2578-2585.

48. Decker T, Sandherr M, Goetze K, Oelsner M, Ringshausen I, Peschel C. A pilot trial of the mTOR (mammalian target of rapamycin) inhibitor RAD001 in patients with advanced B-CLL. *Ann Hematol.* 2009;88(3):221-227.

49. Zent CS, LaPlant BR, Johnston PB, et al. The treatment of recurrent/refractory chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL) with everolimus results in clinical responses and mobilization of CLL cells into the circulation. *Cancer.* 2010;116(9):2201-2207.

50. Flinn IW, Byrd JC, Furman RR, et al. Evidence of Clinical Activity in a Phase 1 Study of CAL-101, An Oral P110{Delta} Isoform-Selective Inhibitor of Phosphatidylinositol 3-Kinase, in Patients with Relapsed or Refractory B-Cell Malignancies. *ASH Annual Meeting Abstracts.* 2009;114(22):922.

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Figure Legends

Figure 1: *p110δis Expressed Abundantly in CLL Cells:* (A) CD19+ cells from CLL patients (N=20) were examined for p110δ expression by immunoblot. (B) CD19+ normal B-cells, CD3+ normal T-cells, CD56+ normal NK cells and CD19+ cells from CLL patients (N=6; each) were examined for p110δ expression by immunoblot. (C) CD19+ cells from CLL patients (N=12) and from normal donors (N=7) were examined for PI3 kinase activity. Results were calculated relative to μg of protein.

Figure 2: *CAL-101 induces selective cytotoxicity in CLL cells Independent of IgV_H mutational status or Interphase Cytogenetics:* (A) CD19+ cells from CLL patients (N=16) were incubated with or without CAL-101 (0.01 μM -100 μM) for 48 hours. Viability was determined by MTT assay and was calculated relative to time-matched untreated controls. (B) CD19+ cells from CLL patients (N=40) were incubated with or without CAL-101 (0.1 μM -10 μM) for 48 hours. Viability was determined by annexin/PI flow cytometry. (C) CD19+ cells from CLL patients (N=40) were incubated with or without 10 μM CAL-101 for 12 – 96 hours. (D) CD19+ cells from CLL patients (N=40; 10 per group) were incubated with or without 10 μM CAL-101 for 48 hours. Cytogenetics was determined independently of our lab. (E) CD19+ cells from CLL patients (N=30; 15 per group) were incubated with or without 10 μM CAL-101 for 48 hours. Mutational status was determined independently of our lab. (F) CD19+ cells from CLL patient cells (N=40) and CD19+ cells from normal B-cells (N=9) were incubated with 10 μM CAL-101 for 48 hours. In B-F viability was determined by annexin/PI flow cytometry, and was calculated relative to time-matched untreated controls.

Figure 3: *CAL-101 Cytotoxicity Against CLL Cells is Partially Dependent on Caspase Activity:* (A) CD19+ cells from CLL patients (N=4) were incubated with or without 1 or 10 μM CAL-101 or 25 μM LY294002 (pan-PI3K inhibitor) for 12 hours, and caspase-3 and PARP were assessed by immunoblot. Results are shown from one of four experiments. (B) CD19+ cells from CLL patients (N=7) were incubated with or without 1 or 10 μM CAL-101 for 12 hours. Cells were lysed and caspase activity was determined by the amino trifluoromethyl coumarin assay. Results were calculated relative to μg of protein. Each symbol represents an individual patient. (C) CD19+ cells from CLL patients (N=6) were incubated with or without 1 or 10 μM CAL-101 and 100 μM z-VAD-fmK for 48 hours. Viability was determined by annexin/PI flow cytometry, and is shown relative to time-matched untreated controls. Each symbol represents an individual patient. (D) CD19+ cells from CLL patients (N=4) were incubated with or without CAL-101 (1 μM – 10 μM) and 100 μM z-VAD-fmk for 12 hours. PARP cleavage was assessed by immunoblot. Results are shown from one of four experiments.

Figure 4: *CAL-101 Does not Demonstrate Cytotoxicity Toward Other Normal Immune Cells but Alters Cytokine Production:* (A) CD3+ T-cells and CD56+ NK cells (N=9; each) from normal volunteers were incubated with or without CAL-101 (0.1 μM -10 μM) for 48 hours. Viability was determined by annexin/PI flow cytometry, and was calculated relative to time-matched untreated controls. (B) CD3+ T-cells (N=12) from normal volunteers were incubated with or without CAL-101 (0.1 μM -10 μM) for 48 hours. Cells were stimulated using an anti-CD3 T-cell activation plate for 24 hours, and IL-6, IL-10 and TNF-α production was measured by ELISA. For CD40L mRNA assay, CD4+ T-cells from normal volunteers (N=4) were incubated with and without various doses of CAL-101 and 5 μg/mL CD28. Cells were then stimulated using an anti-CD3 T-cell activation plate for 48 hours. RT-PCR analysis was done to determine quantities of CD40L mRNA. (C) CD56+ NK cells (N=8) from normal volunteers were incubated with or without

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alemtuzumab, CAL-101 or the combination for 4 hours. IFN-γ production was determined by ELISA. (D) CD56+ NK cells (N=3) from normal volunteers were used as effector cells for a CLL cell ADCC assay. NK Cells were left untreated or treated with 10 μM CAL-101; while CLL effector cells were treated with alemtuzumab.

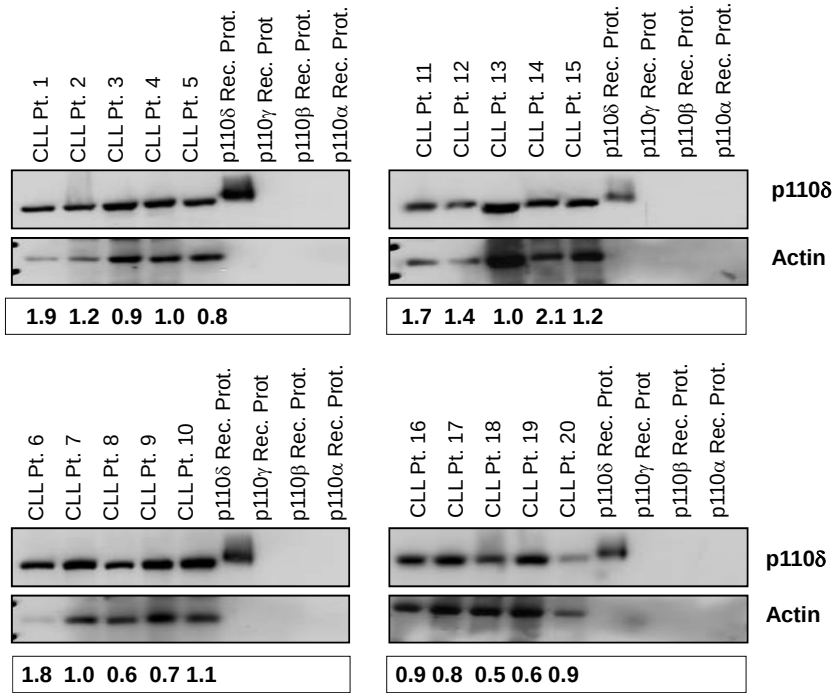
Figure 5: *CAL-101 Induces Apoptosis More Selectively than pan-PI3K Inhibitors:* (A) CD19+ cells from CLL patients (N=49) were incubated with or without 10 μM CAL-101 or 25 μM LY294002 for 48 hours. Viability was determined by annexin/PI flow cytometry and is shown relative to time-matched untreated controls. (B) CD19+ B-cells, CD3+ T-cells and CD56+ NK cells (N=10; each) were incubated with or without 10 μM CAL-101 and 25 μM LY294002 for 48 hours. Viability was determined by annexin/PI flow cytometry and is shown relative to time-matched untreated controls.

Figure 6: *CAL-101 Antagonizes CD40-CD40 Ligand Mediated CLL cell survival:* (A) CD19+ cells from CLL patients (N=4) were incubated with 10 μM CAL-101 and 1 μg/mL CD40L for 2 hours. AKT phosphorylation at ser473 was assessed by immunoblot. Results are shown from one of four experiments. (B) CD19+ cells from CLL patients (N=5-18) were incubated with or without various doses of CAL-101 and 1 μg/mL CD40L for 48 hours. Viability was determined by annexin/PI flow cytometry, and is shown relative to time-matched untreated controls. (C) CD19+ cells from CLL patients (N=3) were incubated with various concentrations of CAL-101 and 1 μg/mL CD40L for 2 hours. AKT phosphorylation at ser473 was assessed by immunoblot. Results are shown from one of three experiments. Quantification was done using the Alpha Innotech FluorChemQ MultiImage III system. (D) CD19+ cells from CLL patients (N=20) were incubated with or without 10 μM CAL-101 and 800 U/mL IL-4 for 48 hours. Viability was determined by annexin/PI flow cytometry and is shown relative to time-matched untreated controls. (E) CD19+ cells from CLL patients (N=4) were incubated with or without 10 μM CAL-101 (or 25 μM LY294002) and 800 U IL-4 for 2 hours. Western blot analysis was done to detect activation of AKT (phosphorylation at ser473) or STAT 3 (phosphorylation at Tyr705). Results are shown from one of four experiments. (F) CD19+ cells from CLL patients (N=3) were incubated with 10 μM CAL-101 and 1 μg/mL CD40L for 2 hours. GSK3β phosphorylation at ser9 was assessed by immunoblot. Results are shown from one of three experiments. (G) CD19+ cells from CLL patients (N=3) were incubated with 10 μM CAL-101 and 1 μg/mL CD40L for 2 hours. Mcl-1 expression was assessed by immunoblot. Results are shown from one of three experiments.

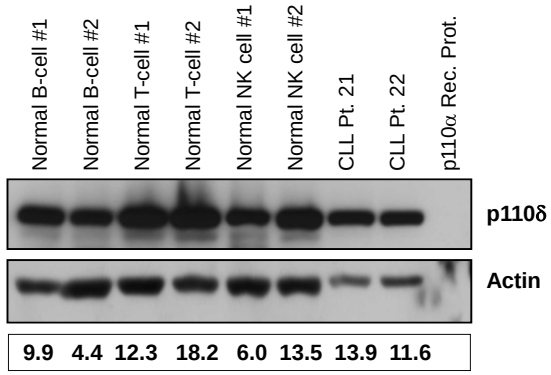
Figure 7: *CAL-101 Antagonizes Alternative Microenvironment Stimuli activated by PI3K Pathway:* (A) CD19+ cells from CLL patients (N=5-10) were incubated with or without various doses of CAL-101 and 50 ng/mL BAFF for 48 hours. (B) CD19+ cells from CLL patients (N=5) were incubated with or without various doses of CAL-101 and 20 ng/mL TNF-α for 48 hours. (C) CD19+ cells from CLL patients (N=5-10) were incubated with or without various doses of CAL-101 on and off fibronectin coated plates for 48 hours. (D) CD19+ cells from CLL patients (N=7) were isolated from peripheral blood and incubated with or without 1 or 10 μM CAL-101 in suspension or on an HS-5 cell layer for 48 hours. Viability was determined by annexin/PI flow cytometry, and is shown relative to time-matched untreated controls for each group. (E) CD19+ cells from CLL patients (N=4) were incubated with 1 μg/mL CD40L, 50ng/mL BAFF and 20ng/mL TNF-α for 2 hours. AKT phosphorylation at ser473 was assessed by immunoblot. Results are shown from one of four experiments.

Figure 1

1A



1B



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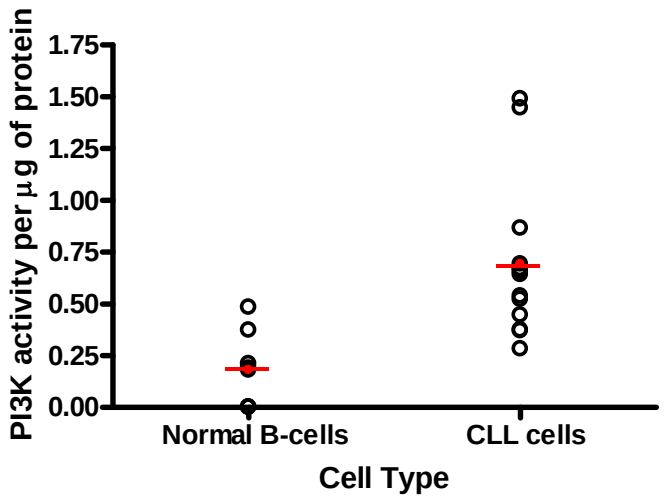

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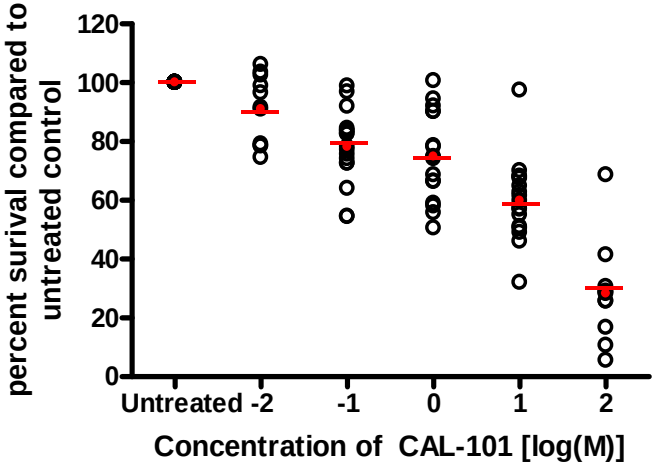
Figure 2

2A



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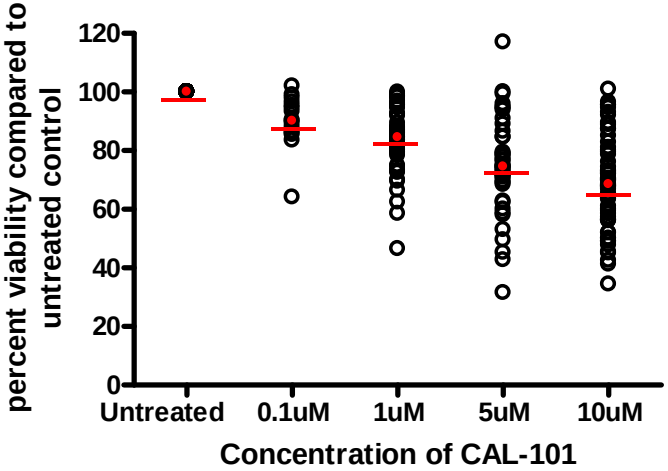

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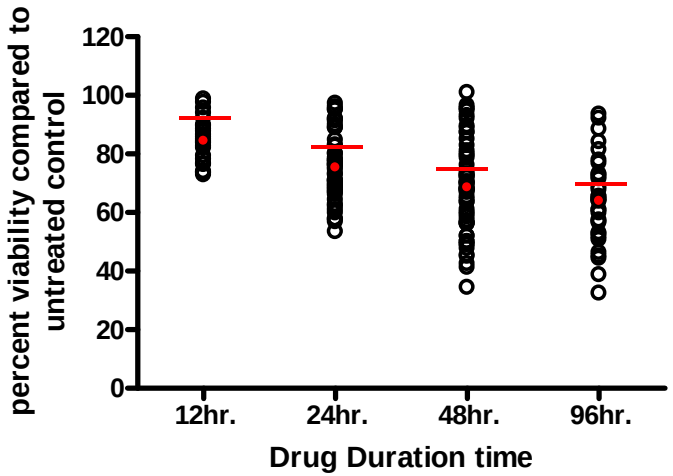
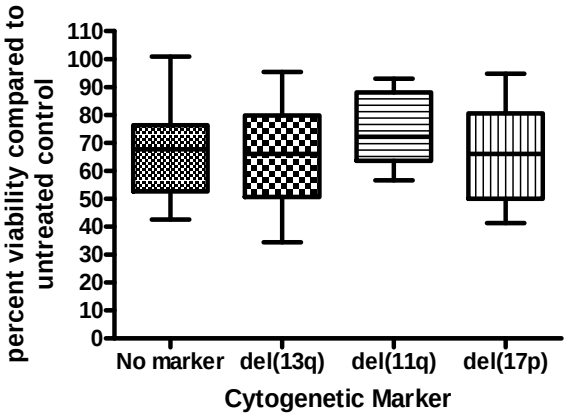
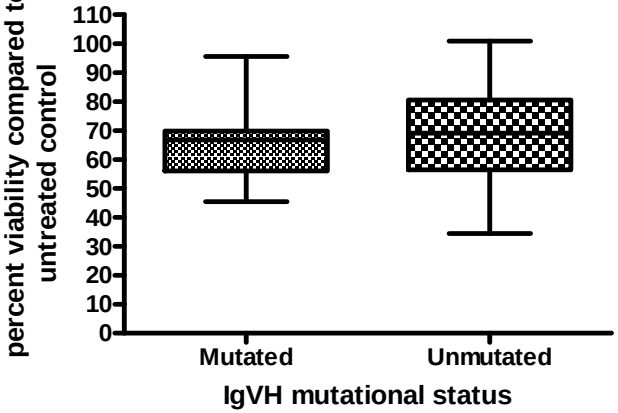


2D



2E



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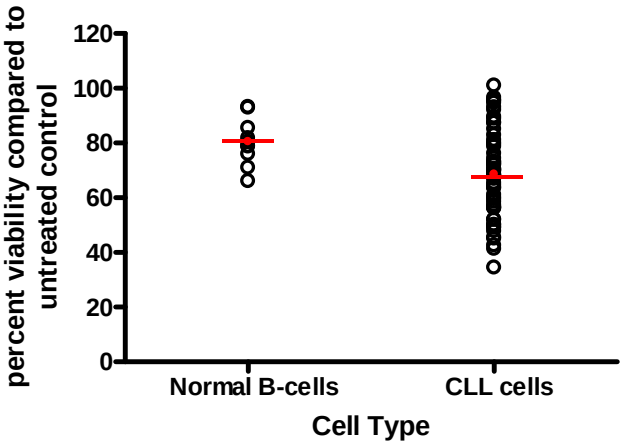

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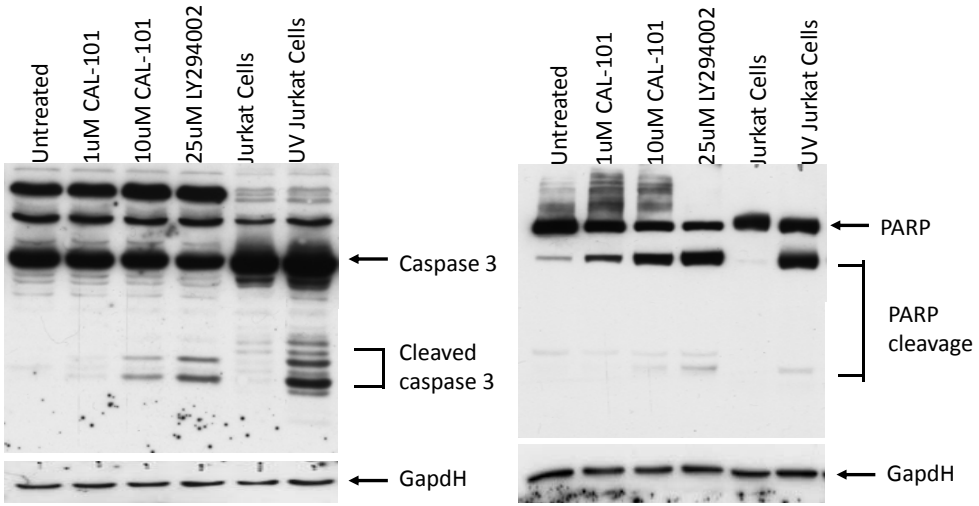
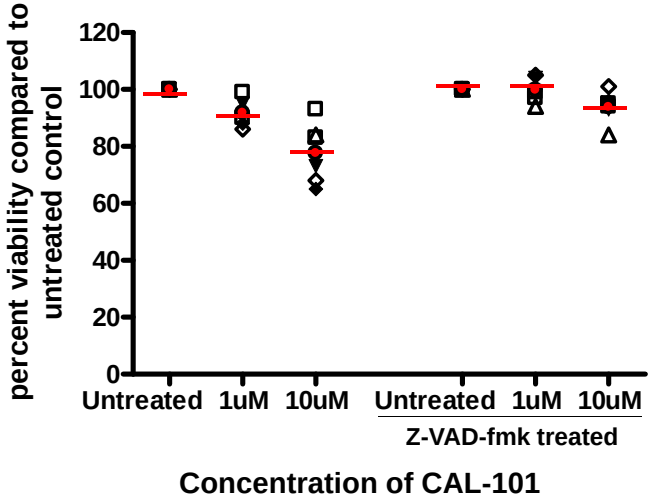
Figure 3

3A



3C



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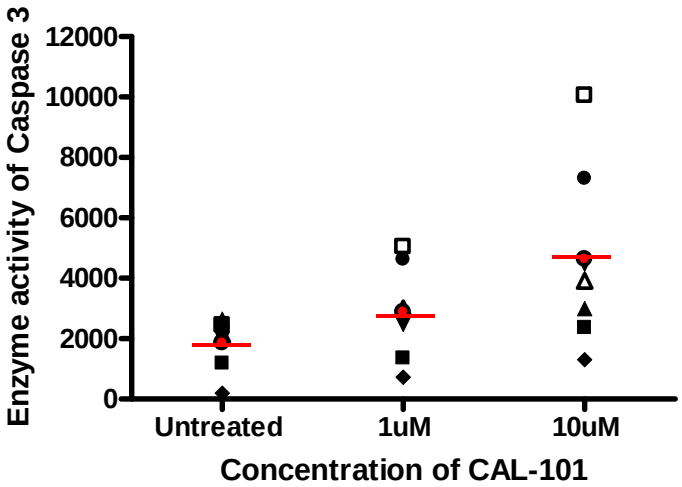

3D

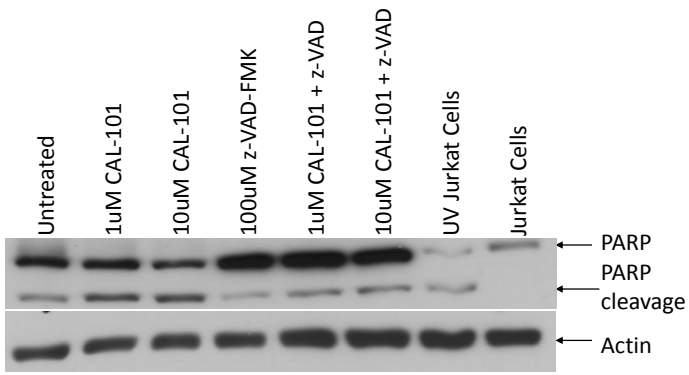

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Figure 4

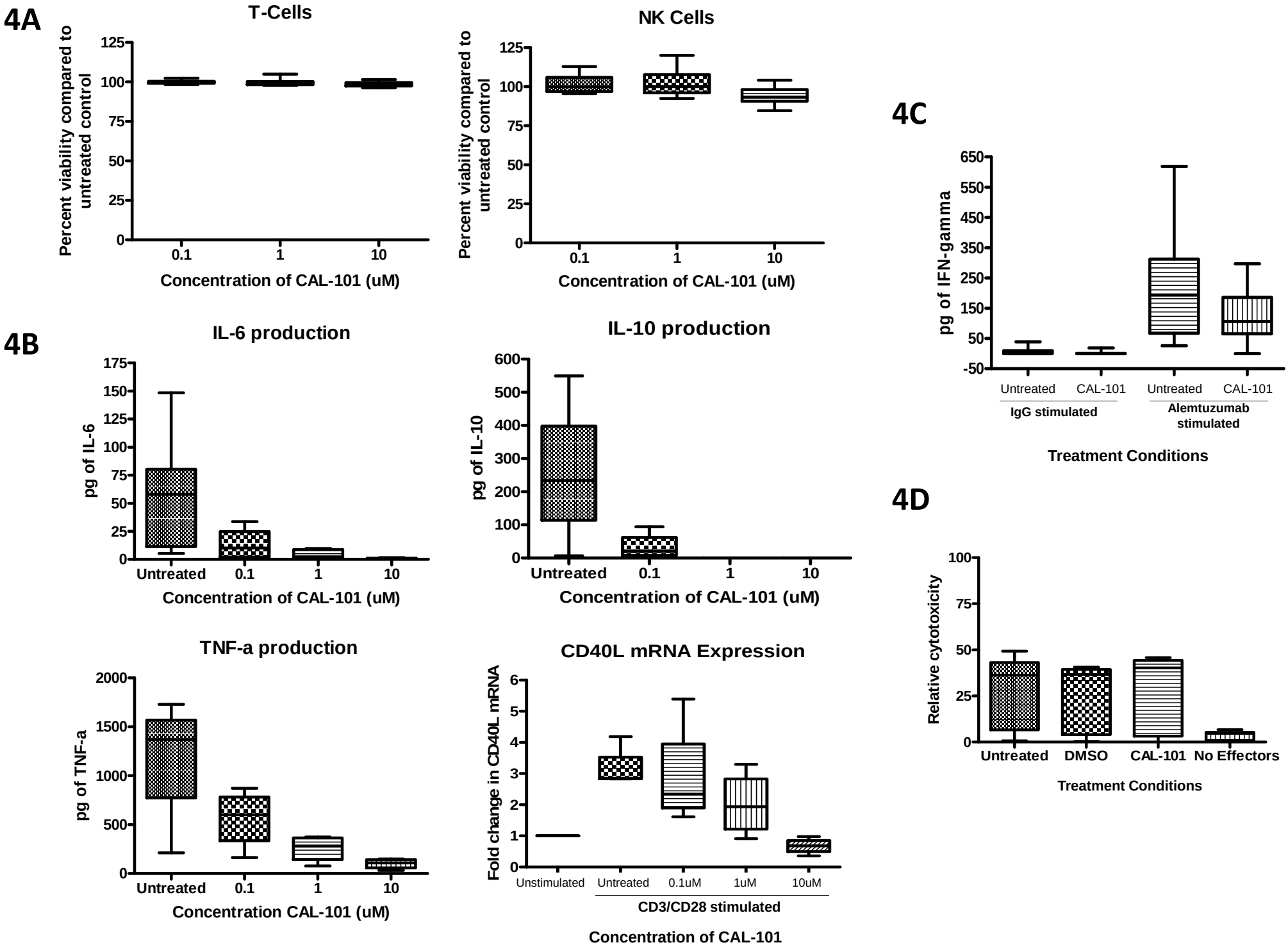

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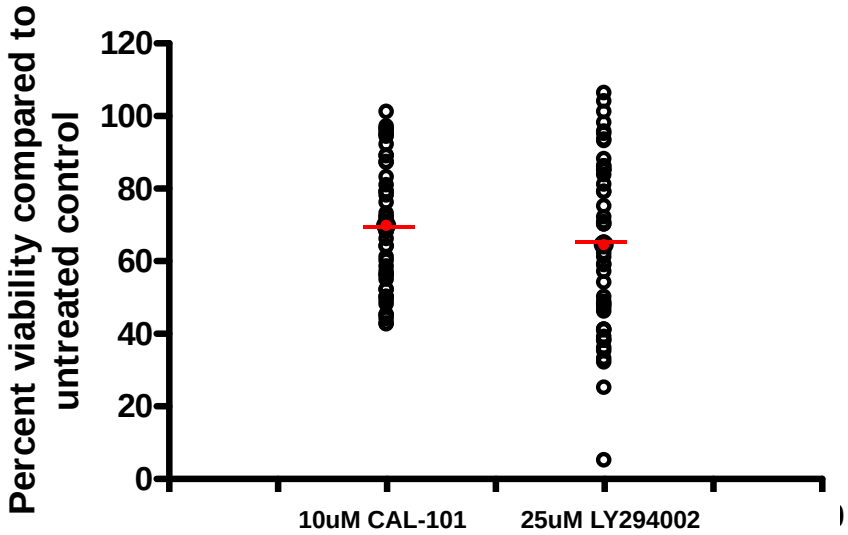
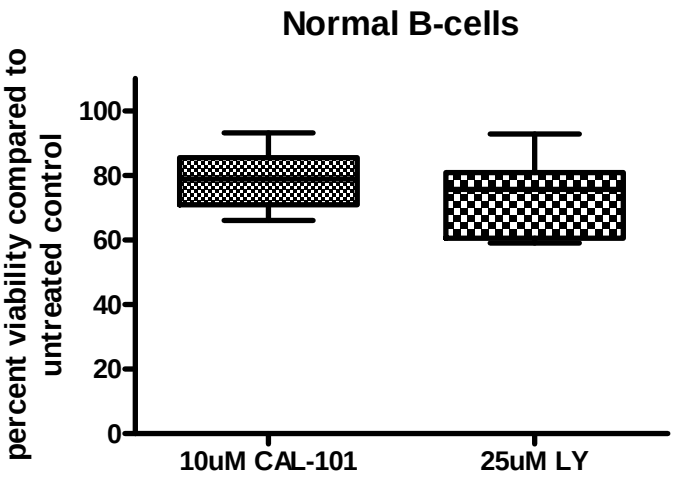
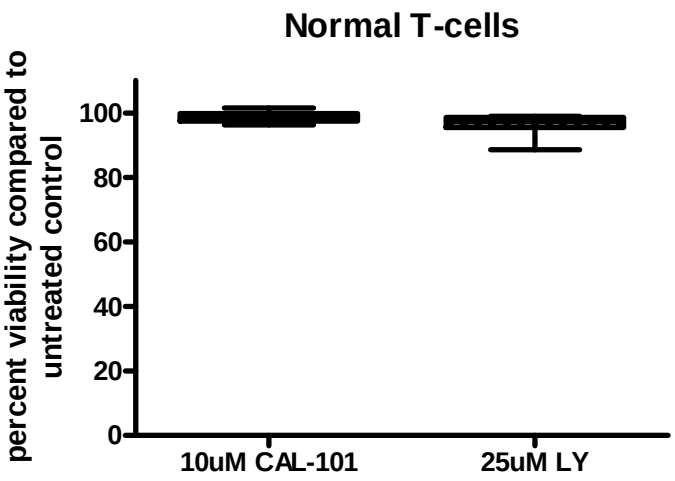
Figure 5

5A



5B





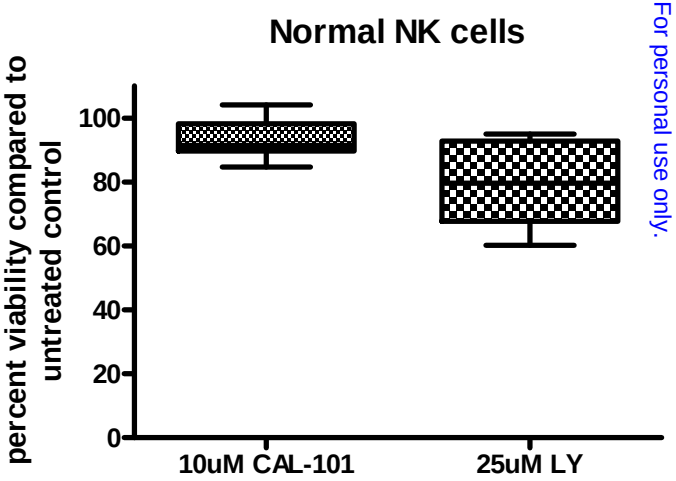


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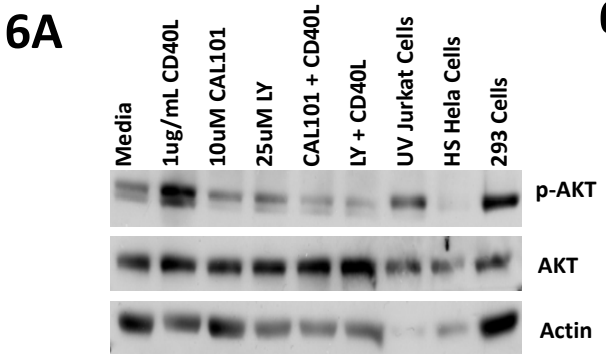
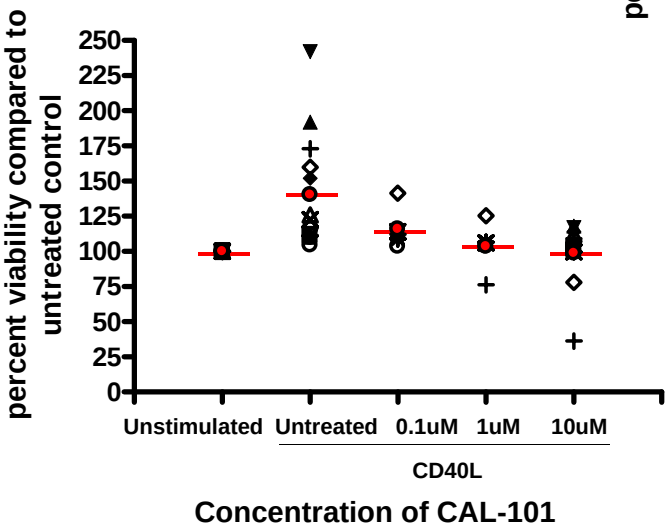
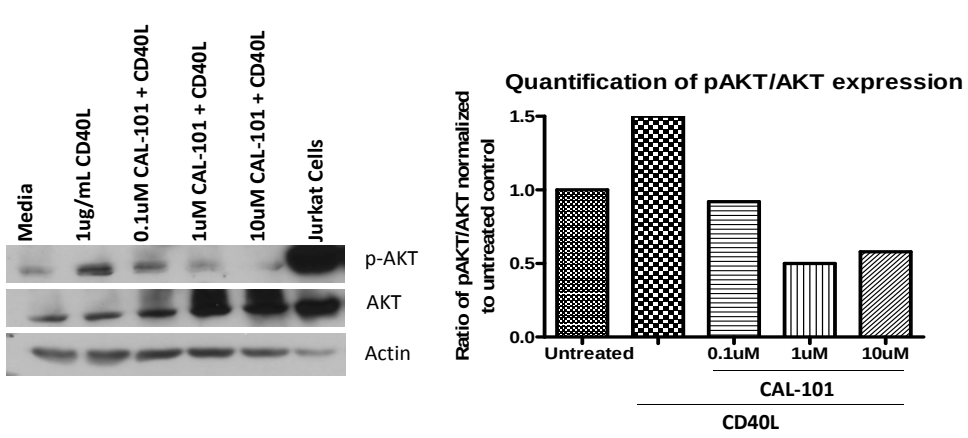
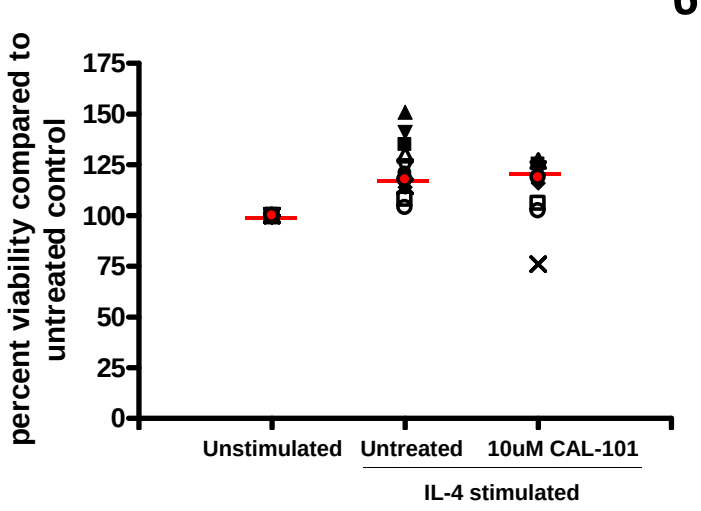
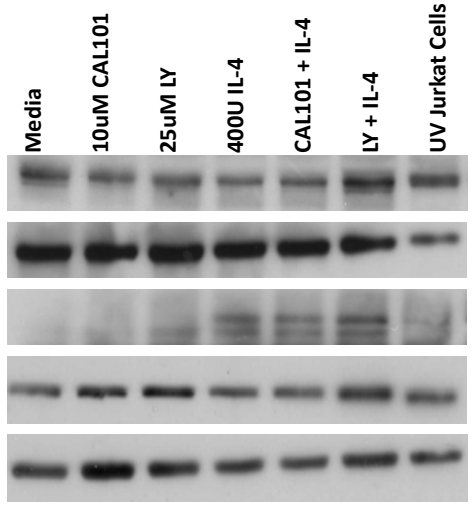
Figure 6

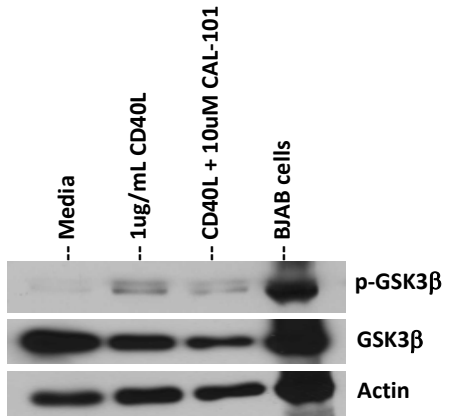












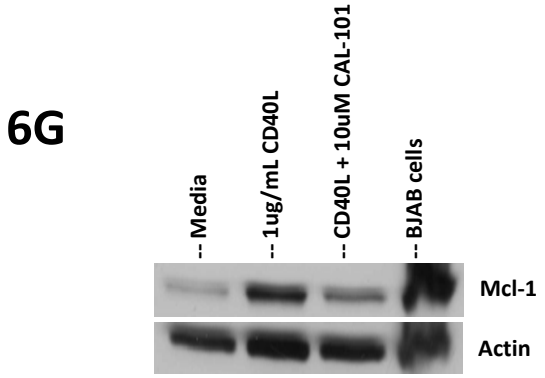


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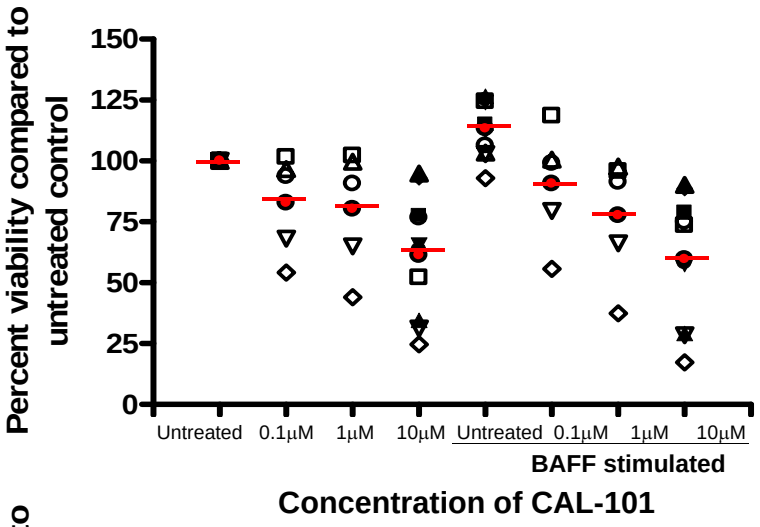
Figure 7

7A



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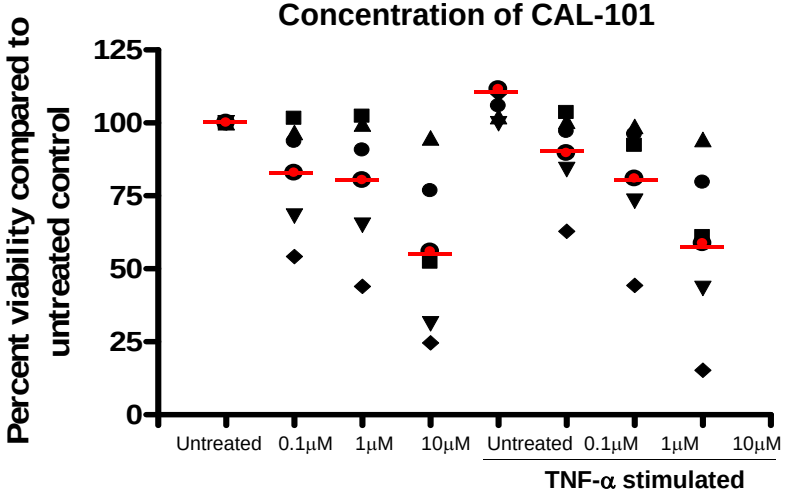

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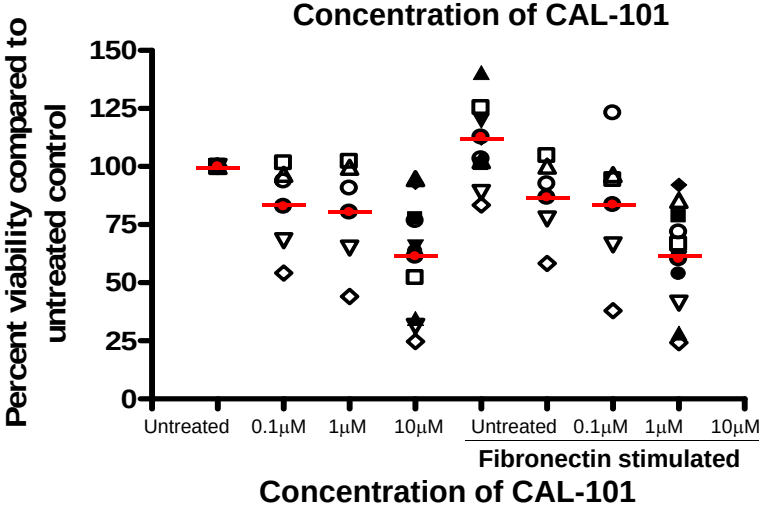
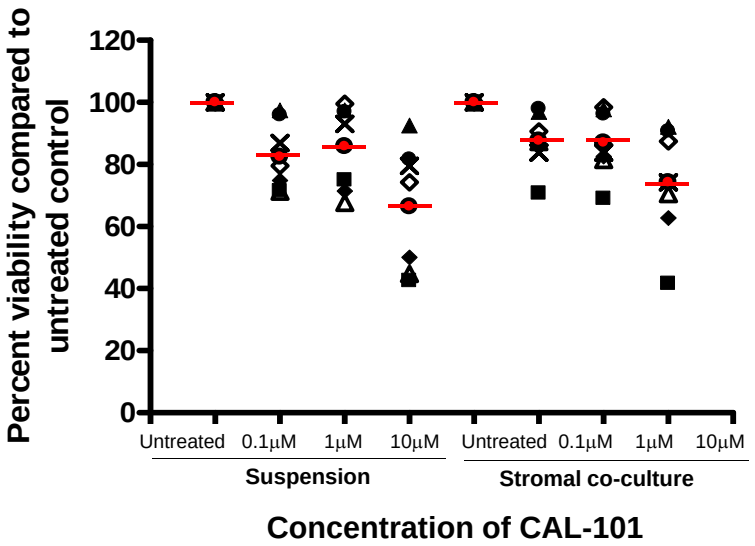


7D



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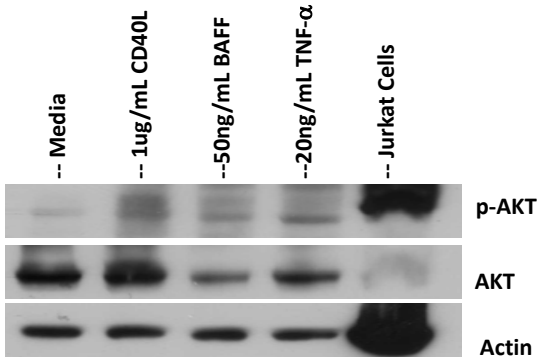

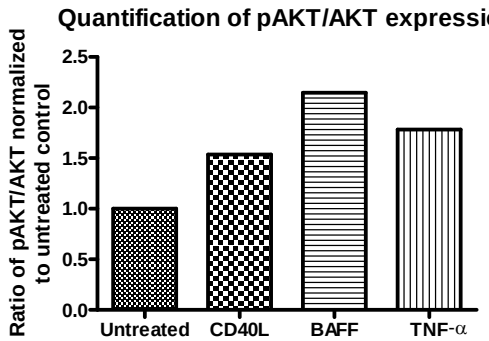

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The phosphatidylinositol 3-kinase-δ inhibitor CAL-101 demonstrates promising pre-clinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals

Sarah E. M. Herman, Amber L. Gordon, Amy J. Wagner, Nyla A. Heerema, Weiqiang Zhao, Joseph M. Flynn, Jeffrey Jones, Leslie Andritsos, Kamal D. Puri, Brian J. Lannutti, Neill A. Giese, Xiaoli Zhang, Lai Wei, John C. Byrd and Amy J. Johnson

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