Supplementary Methods and Figure

Methods

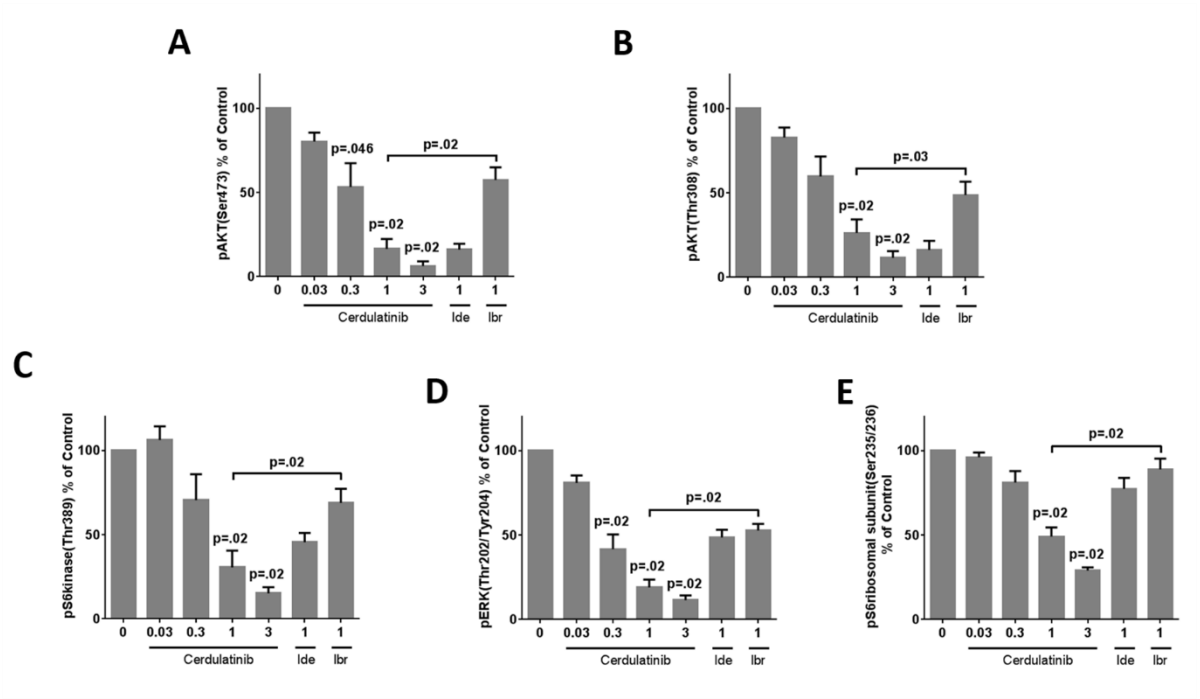
Human Whole Blood Assays

Whole blood collected into lithium heparin vacutainer tubes was used for all intracellular phospho-flow cytometry assays, as previously described[33]. Briefly, 100µl whole blood was pre-incubated for one hour in a 37°C tissue culture incubator with various concentrations of cerdulatinib, as indicated in the figures. Cells were then stimulated for 5 minutes through the B cell antigen receptor (BCR) with 5µg/ml anti-IgD and 10µg/ml anti-IgM to induce SYK auto-phosphorylation ($pSYK^{Y525/526}$ and downstream phosphorylation signaling to ERK^{Y204} and AKT^{S473}. IL-4 signaling responses were measured by induction of $pSTAT6^{Y641}$, following 20 minute stimulation of whole blood with 10ng/ml recombinant human IL-4. Signaling reactions were terminated by the addition of 3ml FACS/Lyse solution for 10 minutes at room temperature. Cells were washed twice in ice cold PBS and permeabilized for 1 hour at 4°C by re-suspending in 1ml 50 % methanol in PBS. Samples were then washed twice in PBS containing 0.5% BSA, and incubated with the phospho-specific and lineage-specific antibodies for 1 hour at room temperature in PBS/0.5% BSA prior to FACS analysis. For each assay, protein expression was calculated by normalizing to the signaling or functional response obtained with the pre-dose sample and presented as percentage (%) of control.

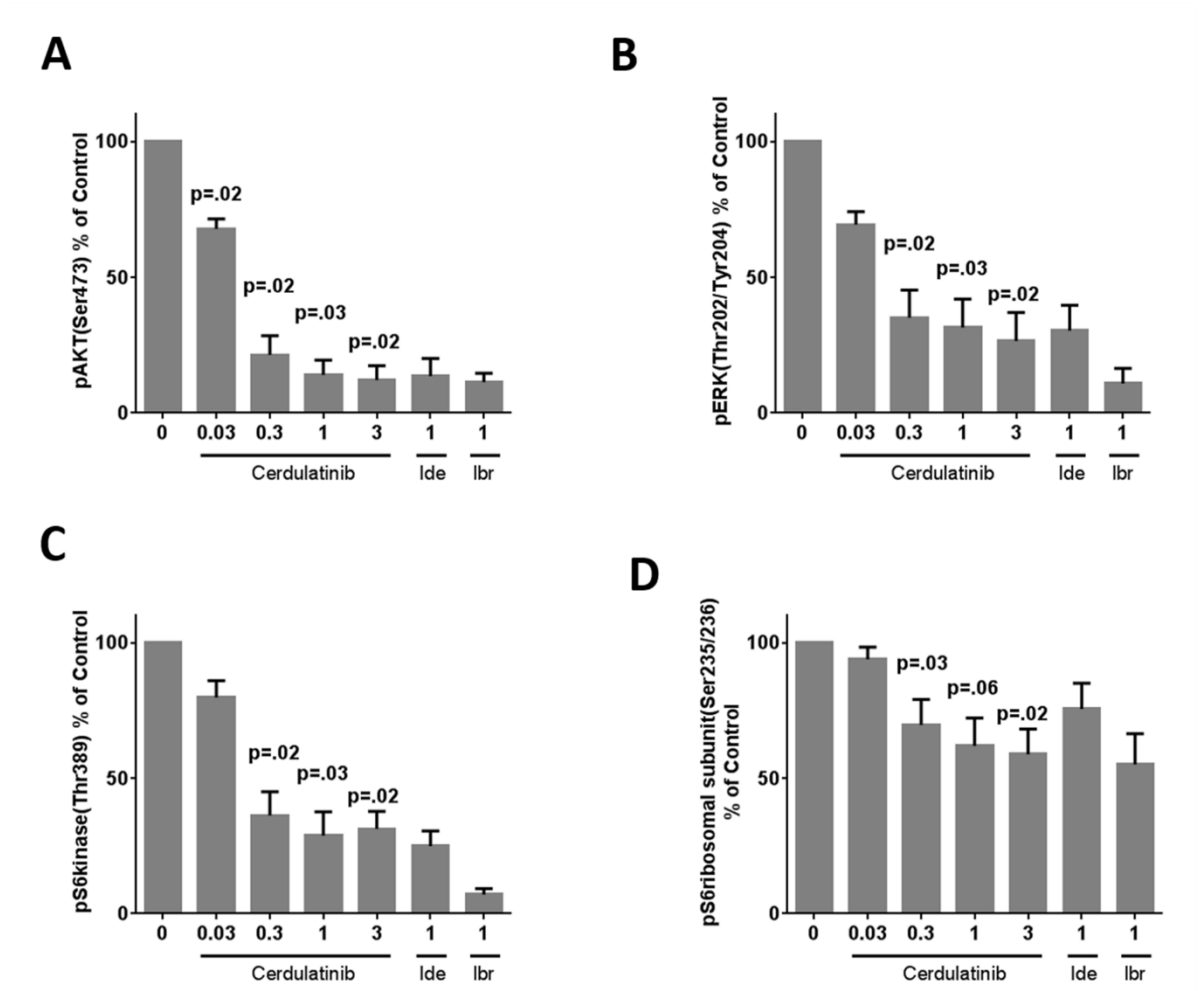
BCR stimulation and NLC co-cultures (MD Anderson)

To study the effects of cerdulatinib on B-cell receptor-dependent survival of CLL cells, freshly isolated CLL cells were incubated with 10 µg/ml soluble anti-IgM (MP Biomedicals, Santa Ana, CA) in the presence or absence of cerdulatinib for 24h and 48h hours. This was followed by the quantification of viable cells by propidium iodide (PI) and $DiOC_6$ double staining as described previously.[16] Nurse-Like Cell (NLC) co-cultures were established by culturing peripheral blood mononuclear cells (PBMC) from CLL patients in 24 well plates at a concentration of 10^7 cells/well for 14 days. PBMCs were re-suspended in RPMI1640 supplemented with 2 mmol/l L-glutamine, 100 µl/ml penicillin, 100 µg/ml streptomycin (CellGro, Manassas, VA, USA) and 10% FBS (Hyclone). To investigate whether cerdulatinib could overcome the protective effect of NLC cells, NLC co-cultures or CLL cells alone, were treated with 2µM cerdulatinib or DMSO control for 24, 48 and 72 hours followed by quantification of cell viability.

Figures

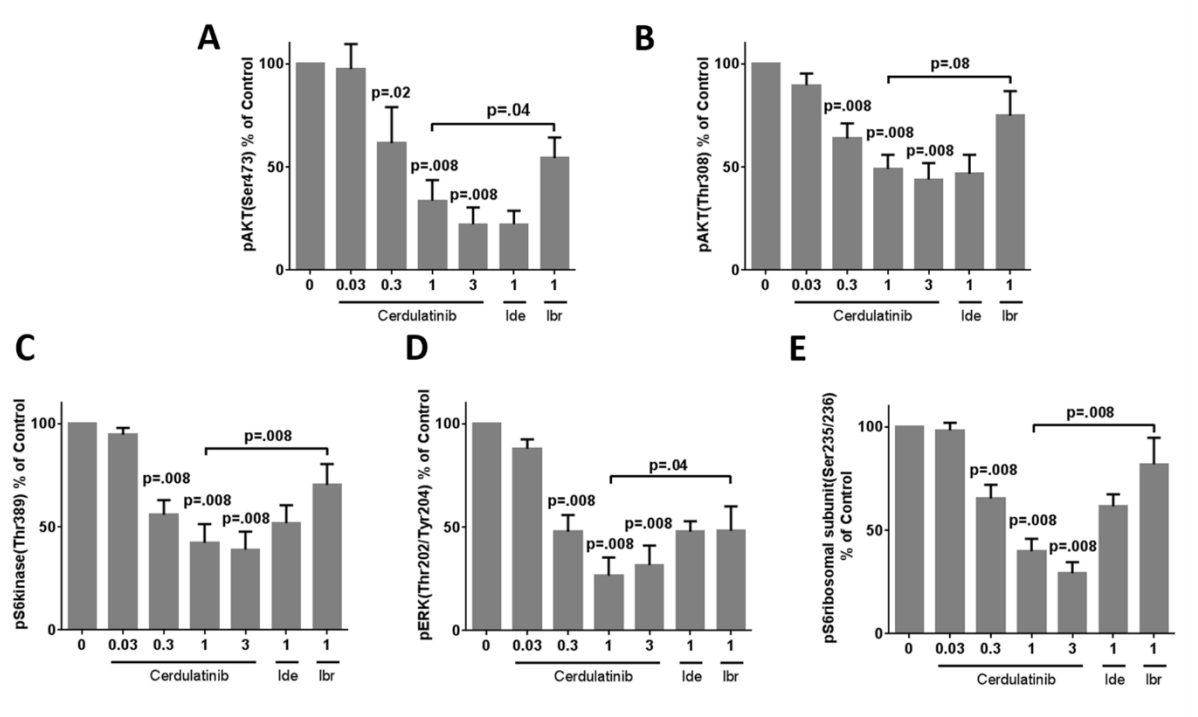


Supplementary Figure 1. Effect of cerdulatinib on Immobilised anti-IgM mediated signalling
CLL samples were treated with cerdulatinib, idelalisib (Ide) or ibrutinib (Ibr) at the stated concentrations for 1hr then stimulated with immobilised anti-IgM for a further 1.5hr. Levels of phosphorylated AKT (Ser473) (A), AKT (Thr308) (B), S6kinase (Thr389) (C), ERK (Thr202/Tyr204) (D) and S6 ribosomal subunit (Ser235/236) (E) were assessed by immunoblotting (n=8). Bar graphs depict means ± SEM

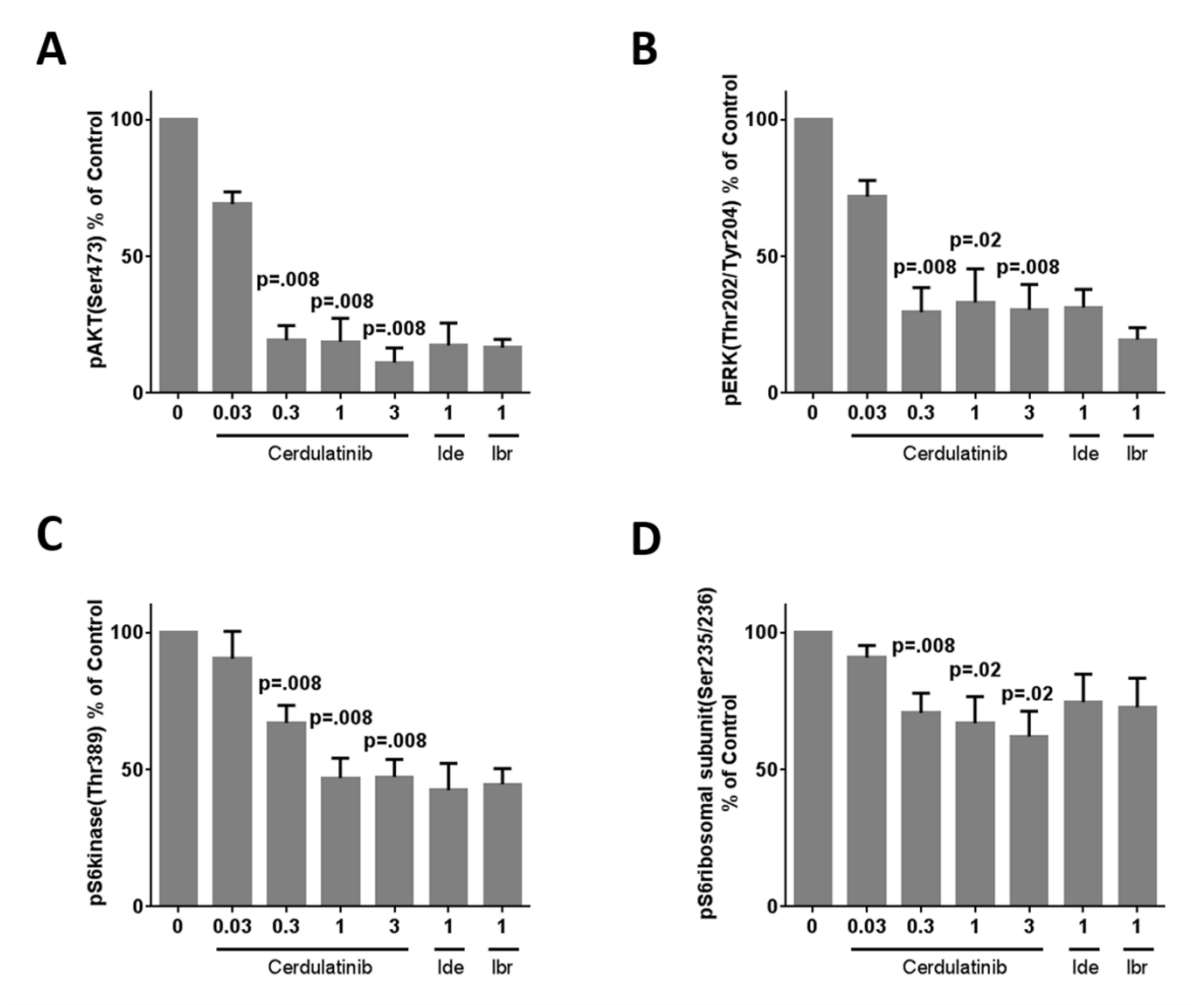


Supplementary Figure 2. Effect of cerdulatinib on soluble anti-IgM mediated signalling

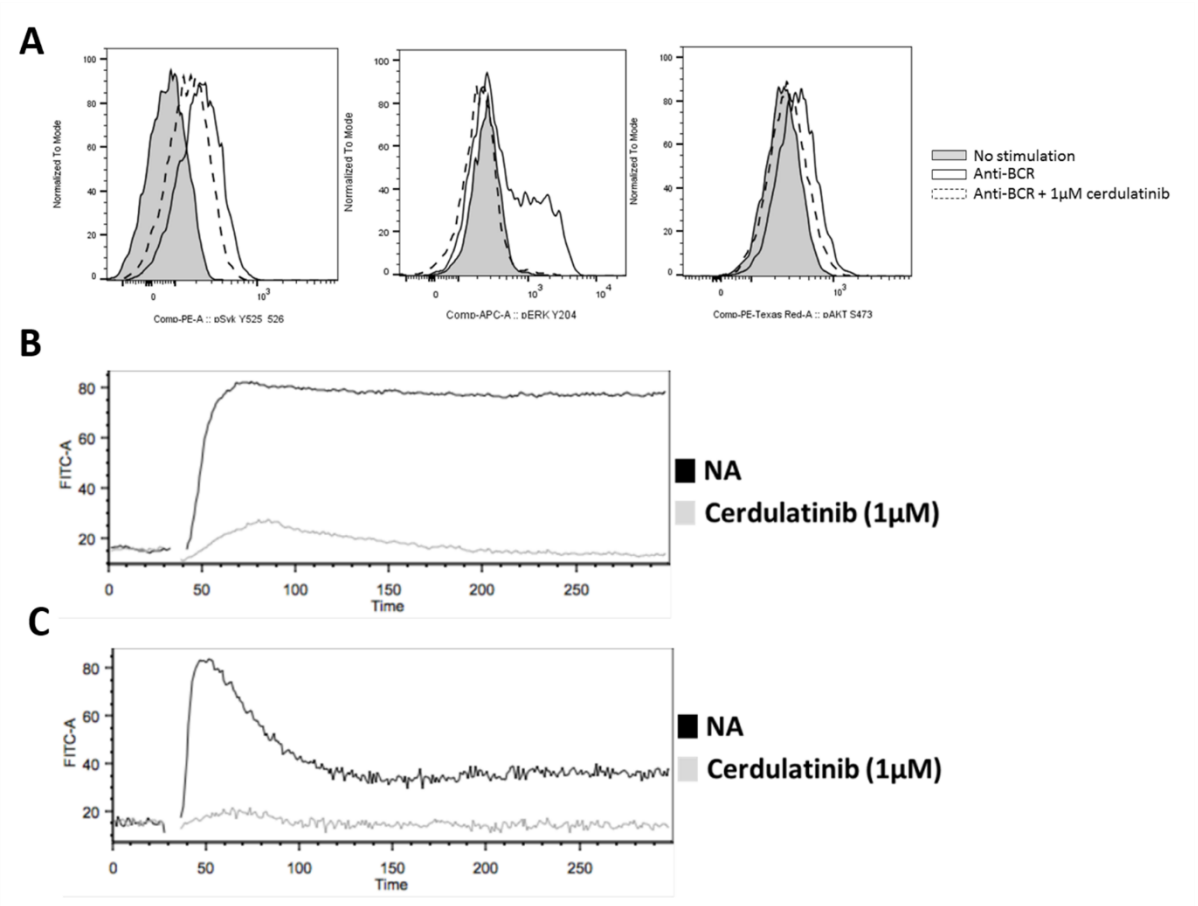
CLL samples were treated with cerdulatinib, idelalisib (Ide) or ibrutinib (Ibr) at the stated concentrations for 1hr then stimulated with soluble anti-IgM (20µg/ml) for a further 15min. Levels of phosphorylated AKT (Ser473) (A), S6kinase (Thr389) (B), ERK (Thr202/Tyr204) (C) and S6 ribosomal subunit (Ser235/236) (D) were assessed by immunoblotting (n=8). Bar graphs depict means ± SEM.



Supplementary Figure 3. Effect of cerdulatinib on Immobilised anti-IgD mediated signalling
CLL samples were treated with cerdulatinib, idelalisib (Ide) or ibrutinib (Ibr) at the stated concentrations for 1hr then stimulated with Immobilised anti-IgD for a further 1.5hr. Levels of phosphorylated AKT (Ser473) (A), AKT (Thr308) (B), S6kinase (Thr389) (C), ERK (Thr202/Tyr204) (D) and S6 ribosomal subunit (Ser235/236) (E) were assessed by immunoblotting (n=8). Bar graphs depict means ± SEM

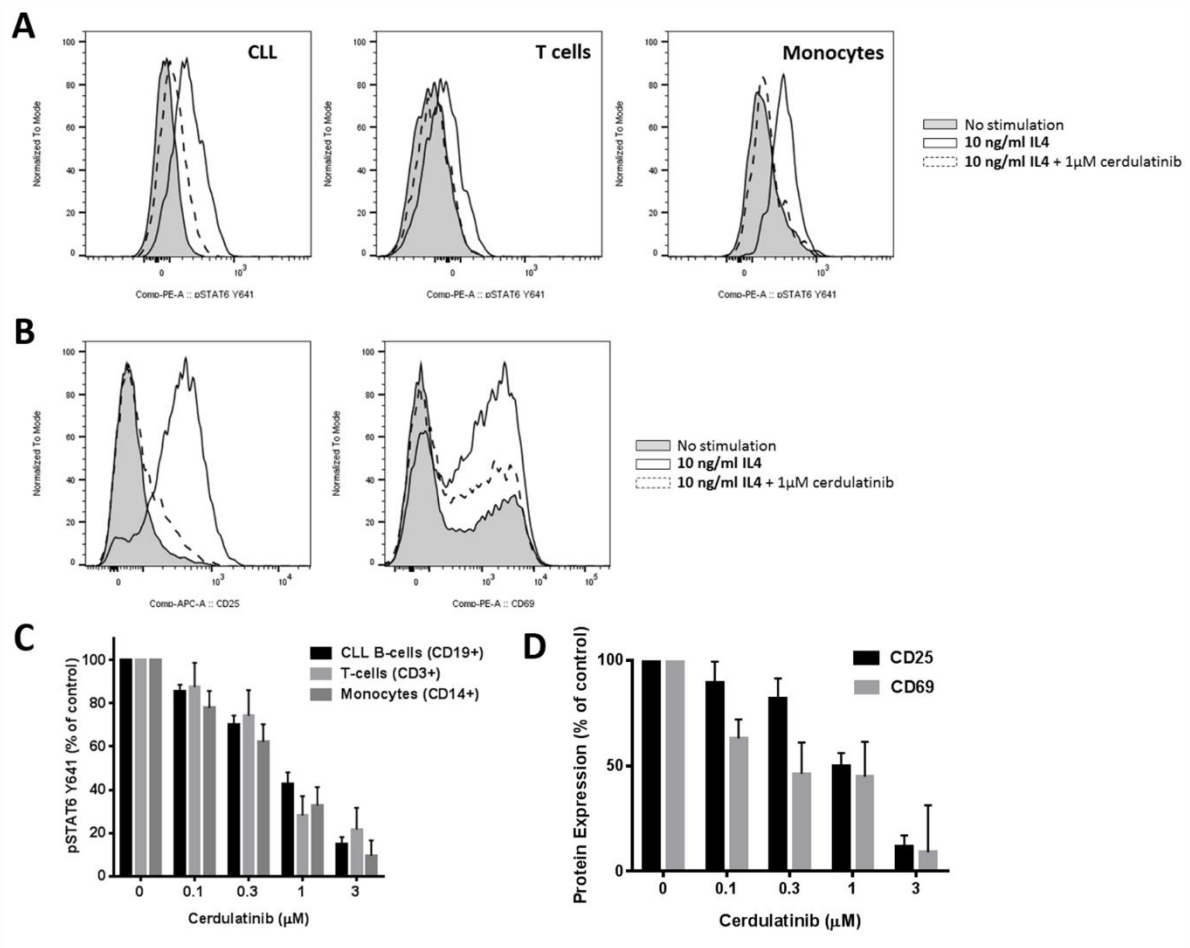


Supplementary Figure 4. Effect of cerdulatinib on soluble anti-IgD mediated signalling
CLL samples were treated with cerdulatinib, idelalisib (Ide) or ibrutinib (Ibr) at the stated concentrations for 1hr then stimulated with soluble anti-IgD (20µg/ml) for a further 5min. Levels of phosphorylated AKT (Ser473) (A), S6kinase (Thr389) (B), ERK (Thr202/Tyr204) (C) and S6 ribosomal subunit (Ser235/236) (D) were assessed by immunoblotting (n=8). Bar graphs depict means ± SEM



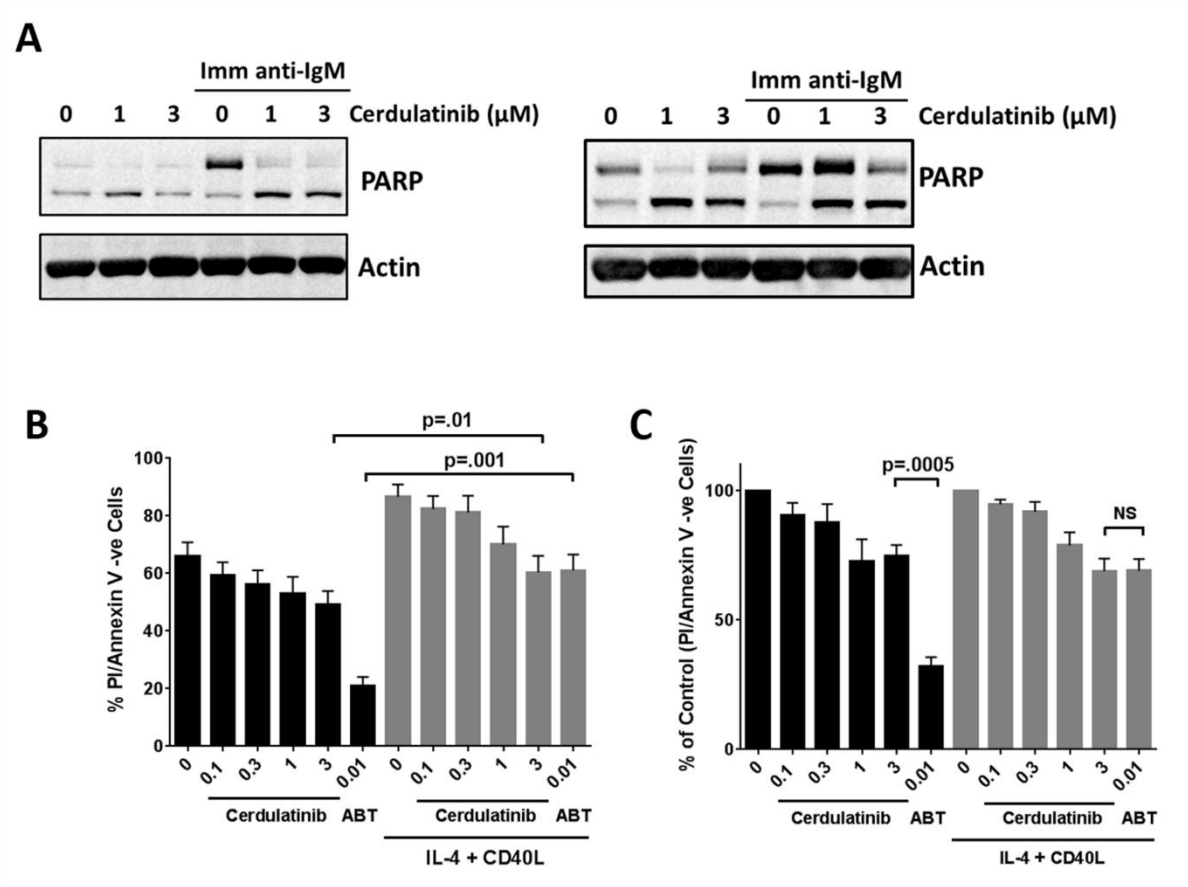
Supplementary Figure 5. Effect of cerdulatinib on anti-IgM induced calcium flux and IL-4 induced surface marker expression

Whole blood from patients with CLL were labelled with anti-CD19, permeabilised and stained for **(A)** phosphorylated (p)SYK$^{Y525/526}$, pERK^{Y204} or pAKT^{S473} following treatment with 10µg/ml soluble anti-IgM plus anti-IgD (anti-BCR). **(B,C)** CLL cells were treated with cerdulatinib for 60mins and stimulated with soluble anti-IgM **(B)** or anti-IgD **(C)** and calcium flux assessed using flow cytometry.

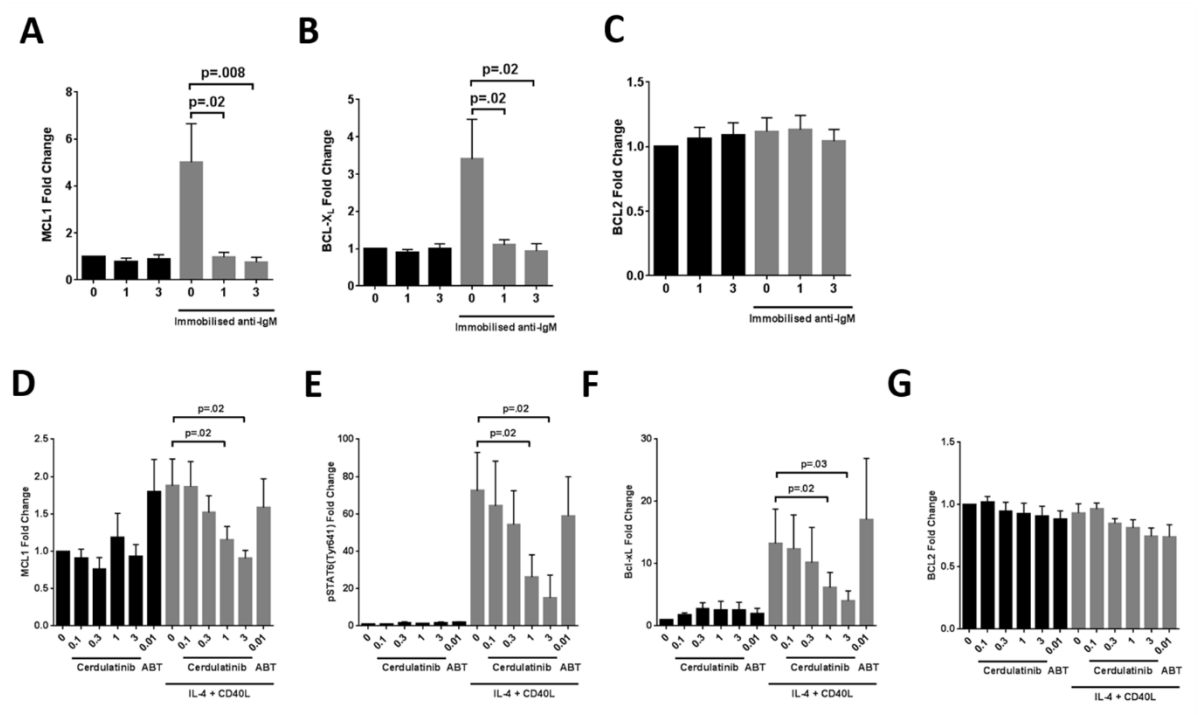


Supplementary Figure 6. Representative phosflow plots

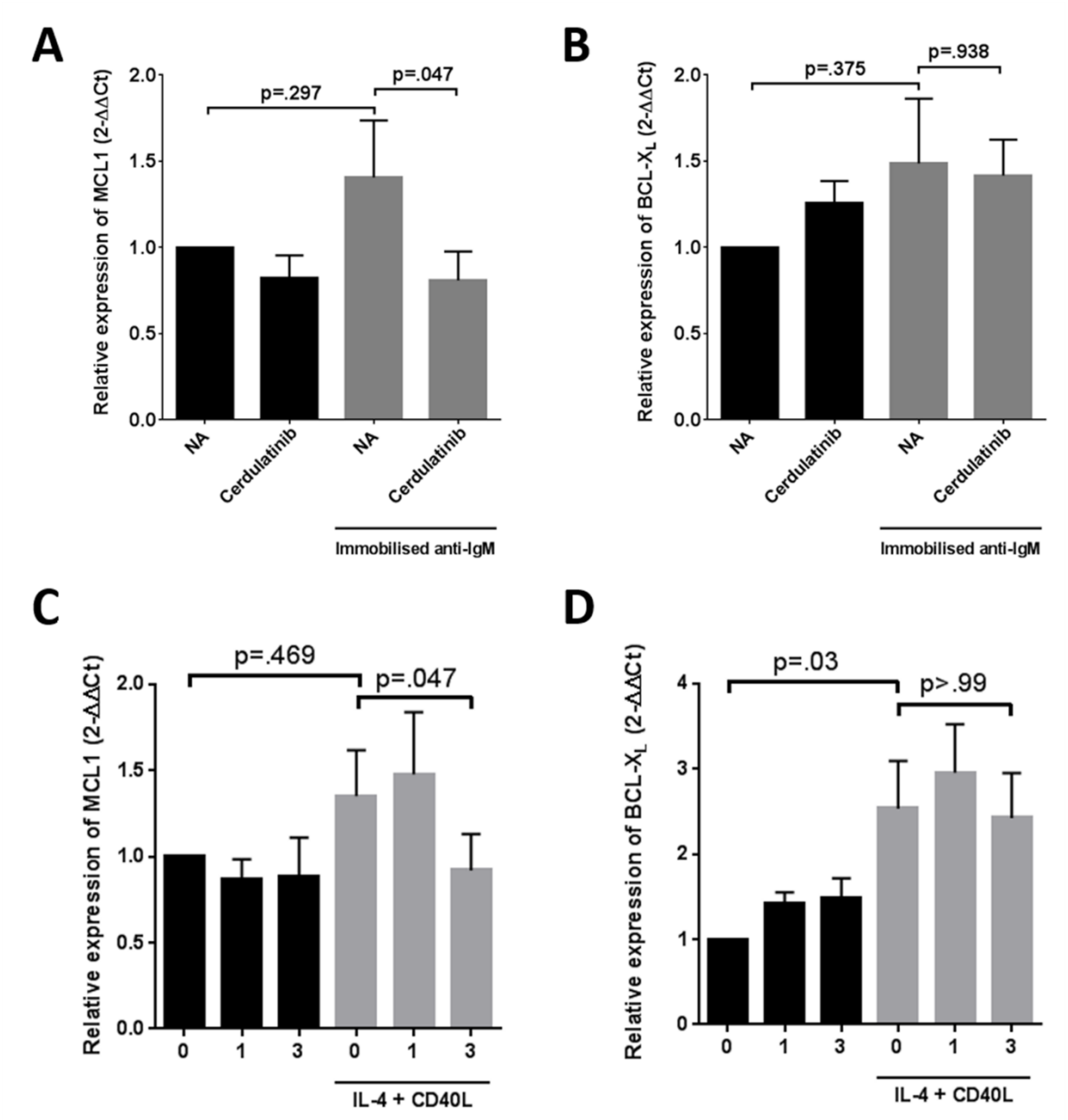
CLL whole blood, containing increasing concentrations of cerdulatinib as indicated was incubated with 10ng/ml human recombinant IL-4 for 10min prior to assessment of STAT6. The cells were **(A)** permeabilized and labelled for $pSTAT6^{Y641}$ in CLL B cells, CLL T cells or monocytes or **(B)** without permeabilization and labelled for surface markers CD19 plus CD25 and CD69 in representative samples and summarised for **(C)** pSTAT6 and **(D)** CD25 and CD69 (n=7). Bar graphs depict means ± SEM



Supplementary Figure 7. Cerdulatinib overcomes the protective effect of BCR-stimulation and IL-4/CD40L. (A) CLL samples were treated with cerdulatinib at the stated concentrations for 60min then stimulated with immobilised anti-IgM beads for a further 23hr. Apoptosis was assessed using cleavage of PARP as a marker by immunoblotting. Representative example shown of 7 separate patients. **(B,C)** CLL samples were treated with cerdulatinib or venetoclax (ABT) for 60mins then stimulated with IL-4/CD40L for a further 24hr. Apoptosis was assessed using PI/Annexin V staining and flow cytometry. **(B)** Data represented as % cells negative for PI and Annexin V (n=12). **(C)** Data represented as % of NA or IL-4+CD40L control (n=12). Bar graphs depict means ± SEM



Supplementary Figure 8. Mcl-1 and Bcl-X_L but not Bcl-2 are downregulated by cerdulatinib
CLL cells were incubated with cerdulatinib or venetoclax (ABT) at the stated concentrations for 60mins then treated with **(A, B, C)** immobilised anti-IgM (n=8) or **(D, E, F, G)** IL-4 (10ng/ml) and CD40L (300ng/ml) (n=7) for a further 23hr. Protein expression of MCL-1, BCL-X_L, BCL-2, pSTAT6 and HSC70 were assessed using immunoblotting and data expressed as Fold change from control. Bar graphs depict means ± SEM



Supplementary Figure 9. Effect of cerdulatinib on MCL-1 and BCL-X_L mRNA expression
CLL cells were incubated with cerdulatinib (1μM) for 60mins then treated with **(A,B)** immobilised anti-IgM or **(C,D)** IL-4 (10ng/ml) and CD40L (300ng/ml) for a further 23hr. mRNA expression of **(A,C)** MCL-1 and (B,D) BCL-X_L were assessed using Q-PCR. β2M was used as house-keeping gene and relative expression values normalized to untreated cells.