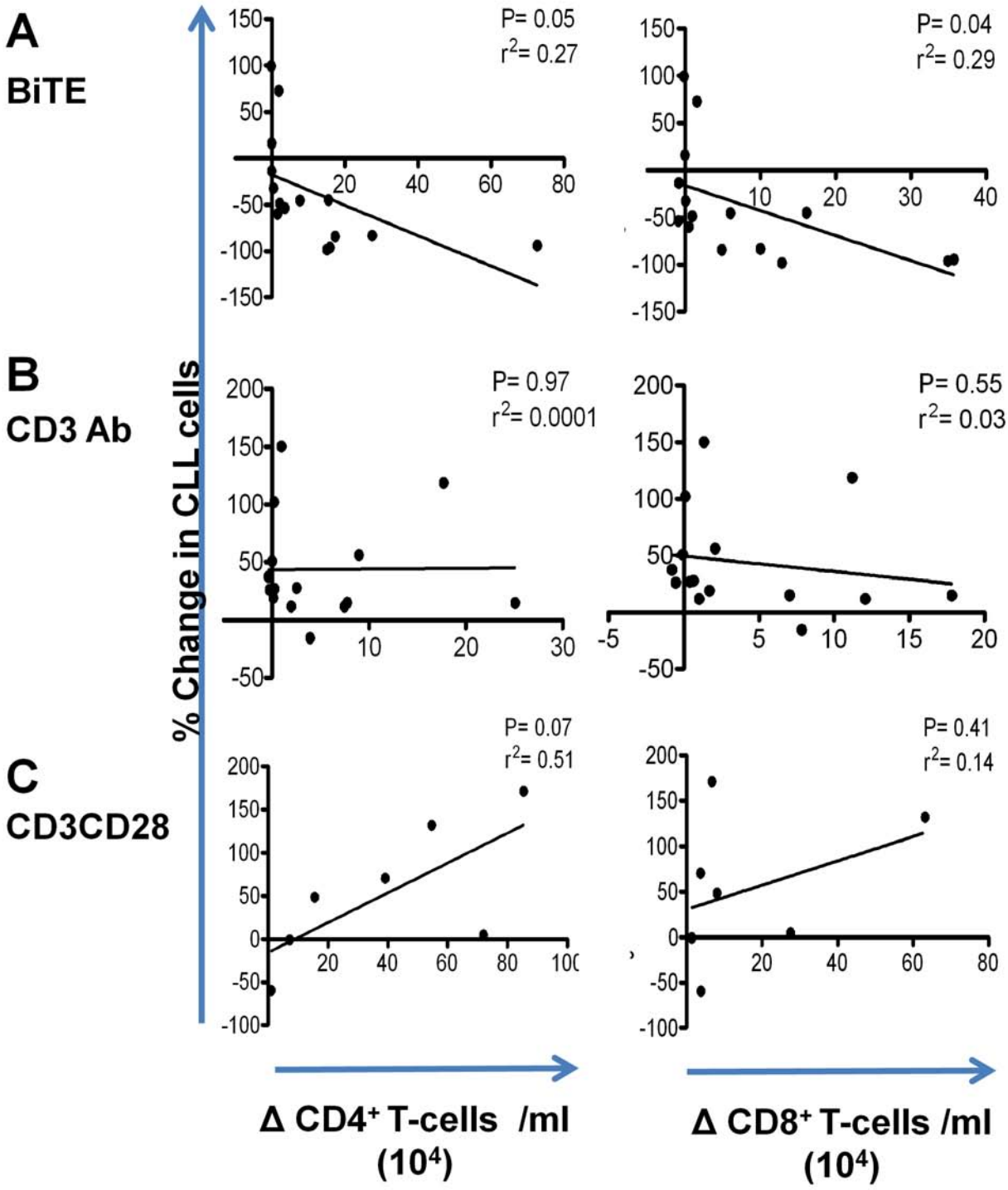
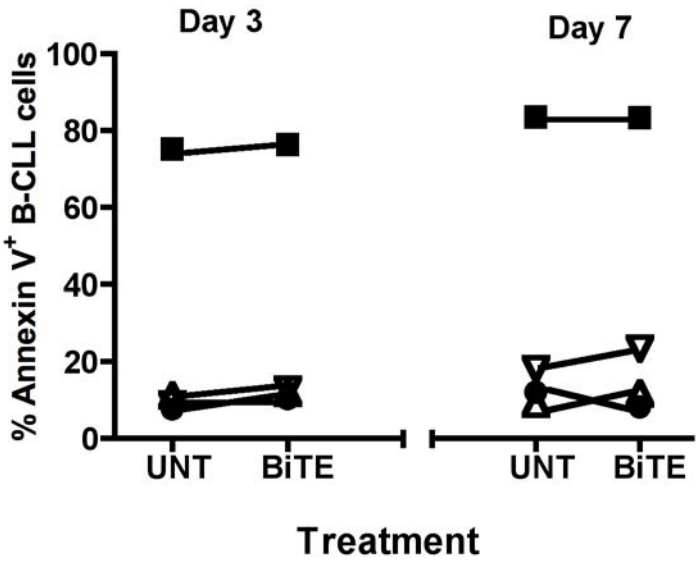
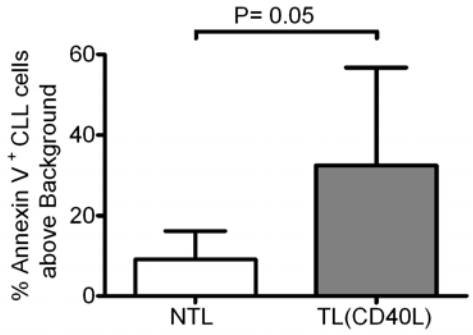
Supplementary Figure 1



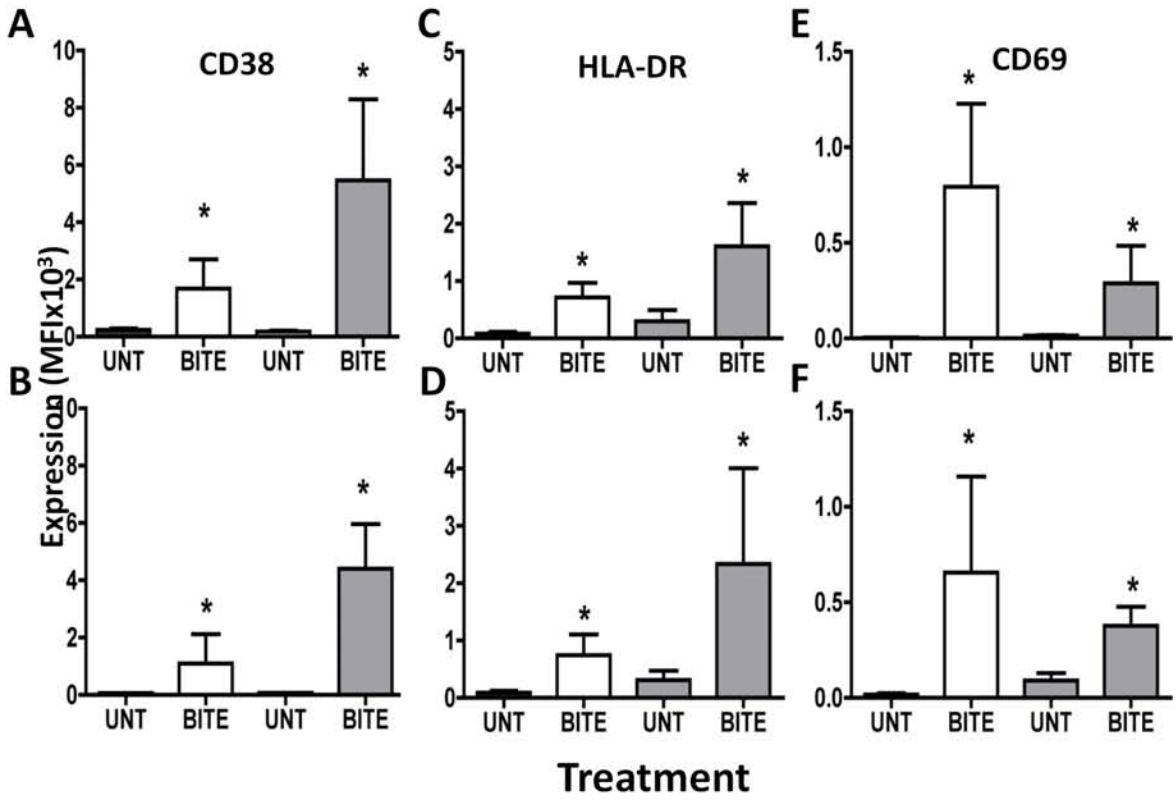
Supplementary Figure 1. Regression plots showing the percentage change in CLL cells versus the change in absolute numbers of T-cells from PBMC cultures treated with blinatumomab. Linear regression analysis was used to analyse the effect T-cell expansions in response to BiTE® (A), an agonistic anti-CD3 antibody (B) or CD3CD28 bead (C) stimulation had on the percentage change in CLL cells in experimental cultures after 7 days. Each dot represents data obtained from 1 CLL patient. N= 15 (BiTE® and CD3 antibody treated cultures) N=7 (CD3CD28 treated cultures). BiTE®= blinatumomab (10ng/ml), CD3CD28=CD3CD28 beads.



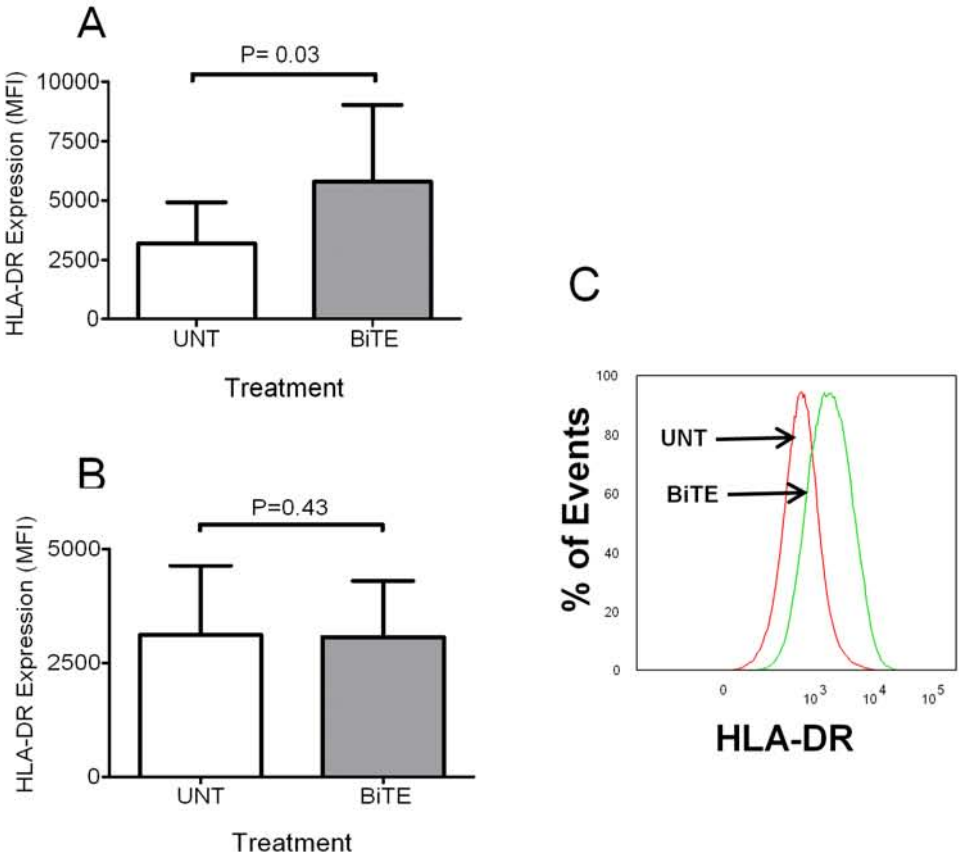
Supplementary Figure 2. Effect of blinatumomab on purified CLL cells. CLL cells were purified from PBMC by negative selection (~99% purity, Miltenyi B cell Isolation Kit), before culture with blinatumomab at 10ng/ml. The % of annexin V positive cells was assessed by flow cytometry, after 3 and 7 days of culture. Each symbol represents an individual patient (n=4). Connecting lines have been drawn to show paired cultures from each patient that were either untreated (UNT) or treated with blinatumomab (BITE).



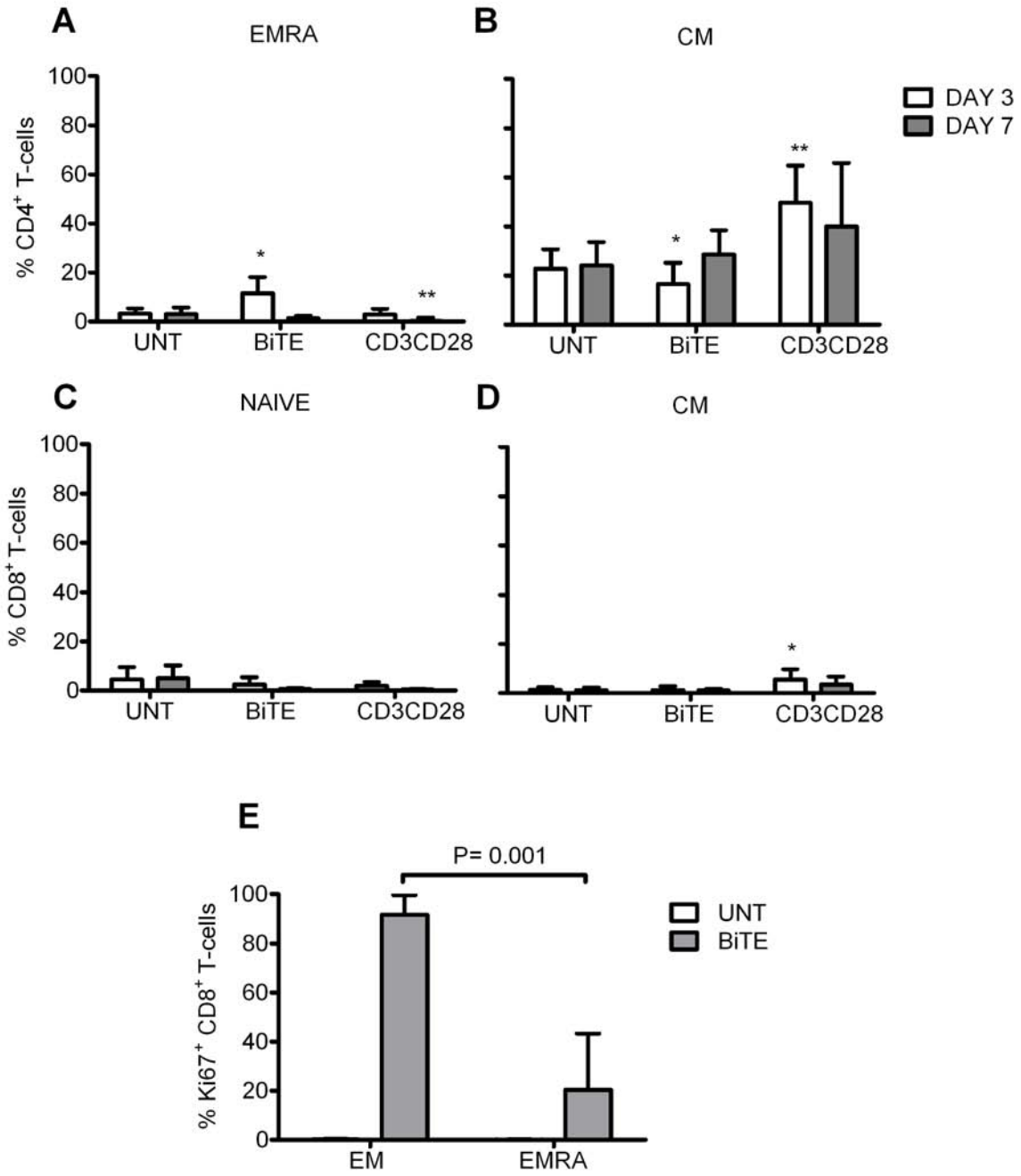
Supplementary Figure 3. Activation signals through CD40L-CD40 interactions increase CLL sensitivity to blinatumomab. CLL PBMCs were co-cultured on fibroblast cells (NTLs) or fibroblast transfected with CD40L (TL (CD40L)) in the presence or absence of blinatumomab (100ng/ml) for 7 days. The percentage of Annexin V positive CLL cells above untreated controls was measured in co-cultures with NTLS or CD40L and blinatumomab (100ng/ml).



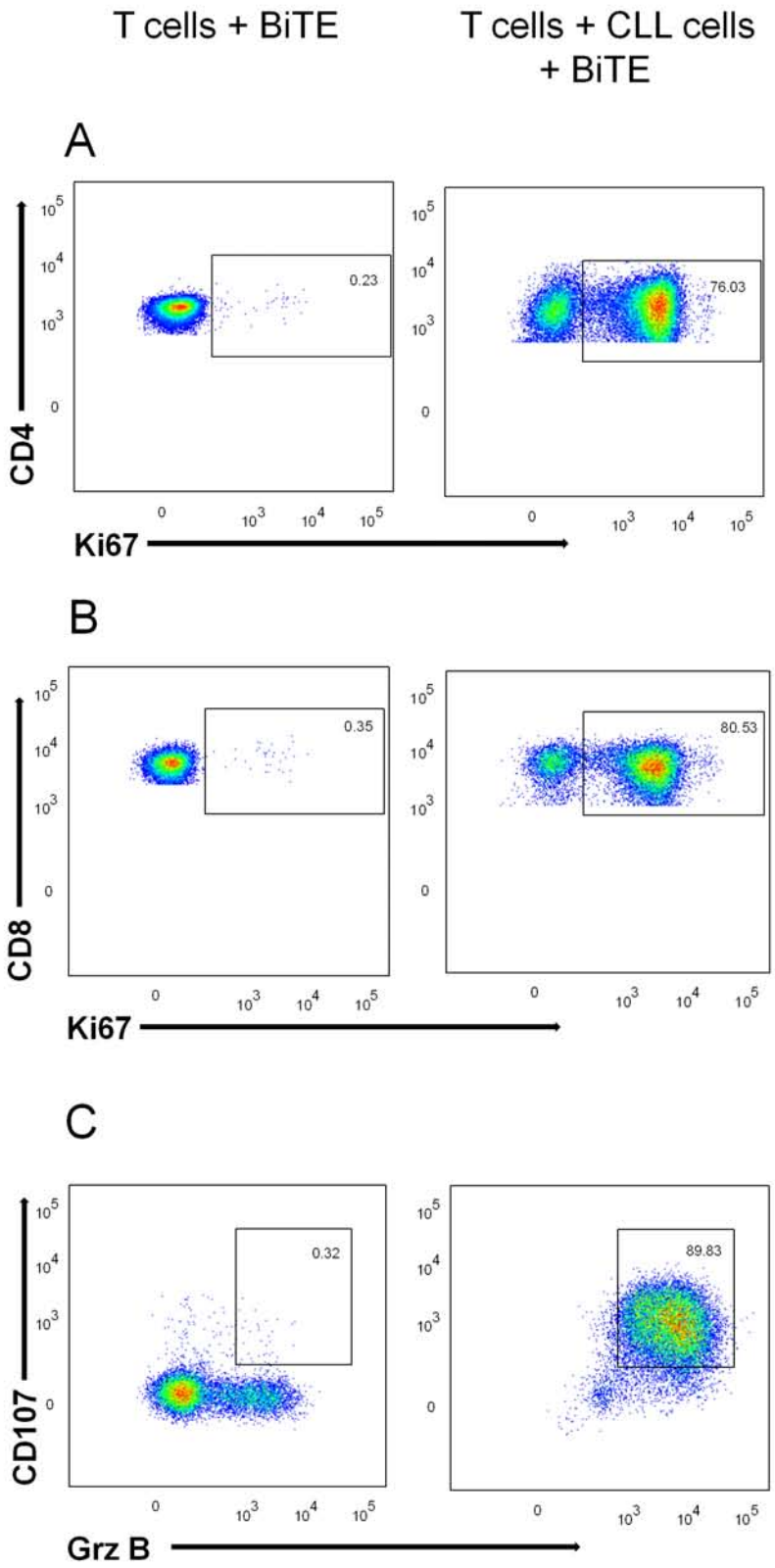
Supplementary Figure 4. T-cell activation after incubation with blinatumomab. Activation related markers CD38 (A, B), HLA-DR (C,D) and CD69 (E,F) were measured by flow cytometry for CD4+ and CD8+ T-cells after incubation of CLL PBMC with blinatumomab (10ng/ml) for 3 days.



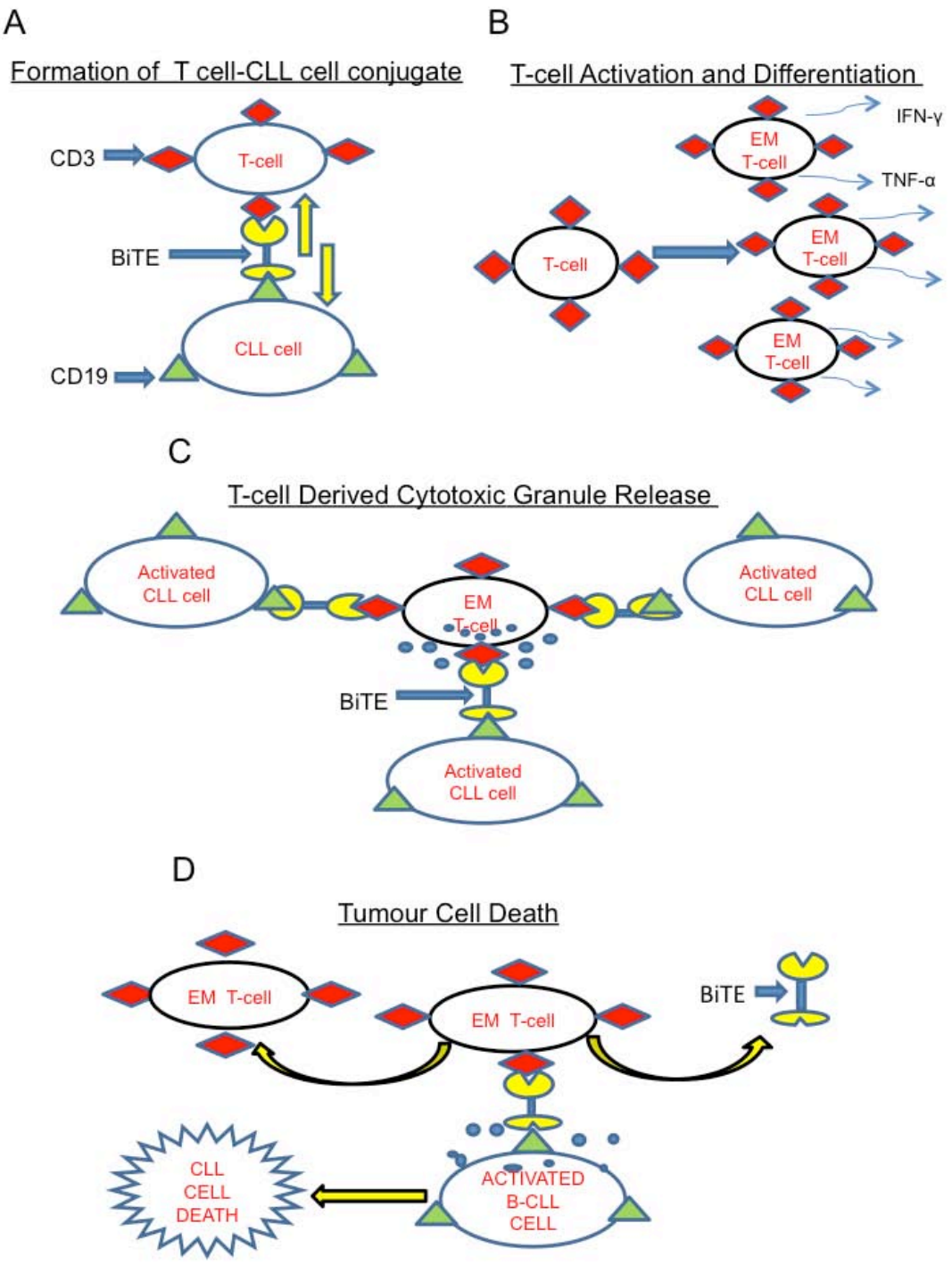
Supplementary Figure 5. Activation of CLL cells in PBMC cultures treated with blinatumomab. Expression of HLA-DR on CLL cells taken from PBMC (A) or Pure B cell (B) cultures treated for 3 days with blinatumomab. (C) Histogram showing HLA-DR expression on CLL cells taken from a PBMC culture that was treated with or without blinatumomab for 3 days. UNT=untreated, BiTE®= blinatumomab (10ng/ml).



Supplementary Figure 6. Changes in Naive and central memory T-cell subsets after treatment with blinatumomab. The percentage of naive and central memory (CM) $CD4^+$ (A-B) and $CD8^+$ (C-D) T-cells were measured in CLL PBMC cultures treated with blinatumomab for 3 or 7 days. (E) % of Ki67 expressing EM and EMRA $CD8^+$ T-cells after 7 days in culture. UNT=untreated, BiTE®= blinatumomab (10ng/ml), CD3CD28=CD3CD28 beads.



Supplementary Figure 7. Blinatumomab induced activation of T-cells is CLL cell dependent. T-cells were treated with blinatumomab (10ng/ml) in the presence or absence of CLL cells. Flow cytometric analysis showing the levels of intracellular Ki67 expression in $CD4^+$ (A) and $CD8^+$ (B) T-cells after 3 days in culture with blinatumomab. Intracellular granzyme B (grz B) and surface CD107 expression measured in $CD8^+$ T-cells at day 3 (C). Culture conditions: left panels T cells only, right panel T cells plus CLL cells.



Supplementary Figure 8. Mechanism of blinatumomab directed T-cell killing of Tumour CLL cells. (A) Blinatumomab engages both CD19 on CLL cells and CD3 on T-cells bringing both cells into close proximity. Activation signals are delivered to both cells. (B) Activated T-cells proliferate and release pro-inflammatory cytokines including IFN-γ and TNF-α whilst also differentiating into an effector memory phenotype. (C) Blinatumomab brings effector memory T-cells and CLL cells into close proximity, allowing targeted release of cytotoxic granules from both $CD4^+$ and $CD8^+$ T-cells towards the CLL cell surface. (D) Cytotoxic granules enter the CLL cell inducing death by apoptosis. Blinatumomab disengages and is free to bind other T-cells and CLL cells.

Supplemental data

Design and Methods

Blood samples from CLL patients

28 CLL patients were recruited from clinics at the University Hospital of Wales and Llandough Hospital, Cardiff (age ranging from 53-83 years). This study was approved by the South East Wales Research Ethics Committee (02/4806), and all patient blood samples obtained with informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Axis-shield, Dundee, UK) density gradient centrifugation. The majority of the assays were performed on fresh samples from either treatment naïve patients, with the exception of Figure 1 where samples from patients who had received standard fludarabine or chlorambucil treatment were also tested.

Antibodies and flow cytometry

The following pre-titrated antibodies were used in this study: CD8-APC-Cy7, CD4-Pacific blue, CD3-AmCyan, CD45RA-APC, CD107-FITC, IFN-γ-PE, Granzyme B-PE, TNF-α-APC, IgG1 k isotype control-PE-Cy7 (BD Biosciences, Oxford, UK), CD5-PE-Cy5.5 (Biolegend, Cambridge, UK), CD5-PE, Human CD3 purified Ab (Invitrogen, Paisley, UK), CCR7-PE, Ki67-FITC (Dako, Ely, UK), Annexin V-FITC (eBioscience, Hatfield, UK), IgG1-FITC IgG1-PE isotype controls (Serotec, Kidlington, UK). Blinatumomab (MT103, AMG103) was provided by Amgen Inc. (Munich, Germany). Analysis of T-cell and CLL cells was performed by Flow cytometry (FACSCanto II cytometer, Becton Dickinson, CA, USA) and Flowjo software (Tree Star inc., OR, USA). To avoid loss of detection due to competitive binding of CD3 and CD19 epitopes with BiTE®, T-cells were identified using CD4 and CD8 markers. CLL cells were gated based on $CD5^+$ $CD4^-$ $CD8^-$ cells. Analysis of this population showed that it was >99% CD19 positive.

Cell culture

PBMCs from CLL patients were incubated in RPMI 1640 supplemented with 5% AB serum (AB media). Blinatumomab was added to PBMC cultures at 10 or 100ng/ml. Human T-cell activator CD3/CD28 dynabeads (Invitrogen) were added at $1x10^5$ beads per $1x10^6$ PBMCs as a positive control for T-cell activation. For absolute counts of T-cells and CLL cells, an anti-CD3 antibody (Invitrogen) at 27.7 or 277ng/ml was used as a positive control representing the same molar concentration as 10 or 100ng/ml blinatumomab. Cultures were incubated at 37°C, 5% CO_2 for 3 and 7 days.

Intracellular Ki67 staining

PBMC were labeled with optimal concentrations of antibodies against CD4, CD8, CCR7 and CD45RA. Cells were then fixed and permeabilized (Fix and Perm, Caltag-Medsystems, Buckingham, UK) using the manufacturer's protocol. 1% nonyl phenoxypolyethoxylethanol (NP40) was added to the

permeabilization buffer to enhance the permeability of the nuclear membranes. The cells were then stained with a Ki67-FITC antibody and analyzed by flow cytometry.

Cytokine secretion assay

Cytokine release was measured using a Human Th1/Th2 11plex RTU Flowcytomix kit (eBioscience) for simultaneous detection of 11 cytokines (IL-6, IL-8, IL-10, IL-2, IFN-γ, IL-12 p70, TNF-β, TNF-α, IL-1β, IL-5 and IL-4) in supernatants taken from PBMC cultures treated with or without 10ng/ml blinatumomab. Cytokine concentrations were calculated from a standard curve generated with known human cytokine concentrations using flow cytomix Pro 2.2 software (eBioscience).

Intracellular cytokine staining and the determination of CD107 and Granzyme B expression

PBMC were cultured for 3 days in the presence or absence of 10ng/ml blinatumomab in AB media. Cultured cells were then treated with golgi plug and golgi stop (BD biosciences) and incubated with an anti-CD107 antibody for 5 hours at 37°C. After incubation cells were labeled with antibodies against CD4 and CD8 before being fixed and permeabilized (Cytofix/ Cytoperm Kit, BD biosciences). Intracellular staining was carried out using antibodies against granzyme B or IFN-γ and TNF-α. The cells were analyzed by flow cytometry and the percentage of T-cells expressing IFN-γ and TNF-α or granzyme B and surface CD107 determined.

Absolute counts of T-cells and CLL cells

For absolute cell counts, fluorescent Cytocount^{TM} Beads (Dako, Stockport, UK) of a defined concentration were used. PBMC samples that had been treated with or without blinatumomab for 7 days were suspended in 200 µl of binding buffer (Invitrogen) and surface stained with antibodies against Annexin V, CD5, CD8 and CD4. One hundred l of cytocount beads were added to each sample and the cells analyzed by flow cytometry. In total $3x10^5$ events were recorded and the absolute number of T-cells and CLL cells were calculated based on the known bead concentrations using the equation:-

Flow cytometry-based cytotoxicity assay

Mouse fibroblast cells transfected with CD40L (TL(CD40L)) and non-transfected cells (NTL) were kindly provided by Dr Aneela Majid (University of Leicester), and cultured as previously described.[21] For co-cultures, $5x10^4$ irradiated (80 Gy) NTL or TL(CD40L) cells were seeded into a 48-well plate. The plate was then incubated for 24 hours at 37°C to allow the fibroblast cells to adhere to the well surface. One million $1x10^6$ PBMCs from CLL patients were subsequently added to the surface of the irradiated NTL and TL (CD40L) cells. The CLL cells were then treated with 10 or 100 ng/ml blinatumomab and incubated at 37°C for 7 days. CLL PBMCs were then carefully removed from the surface of the fibroblast monolayer, washed

twice in PBS and resuspended in 200µl of binding buffer and labeled with antibodies against Annexin V, CD5, CD8 and CD4 for 15 mins before analysis by flow cytometry. Twenty to forty thousand events were recorded and CLL cell death was determined by measuring the percentage of Annexin V positive CLL cells in each sample.

Immunofluorescence labeling

CLL B-cells were purified from PBMC by negative selection using the B-cell isolation kit (Miltenyi, Bisley, UK) following the manufacturer's instructions. T-cells were isolated from CLL patient PBMC by negative selection, using a Pan T-cell isolation kit (Miltenyi). Purified T-cells were labeled with 15mM red CFSE (Invitrogen) and purified CLL cells (B-CLL isolation kit, Mitlenyi) labelled with 10 mM Green CFSE (Invitrogen) in 1ml of RPMI supplemented with 1% fetal calf serum (FCS). Cells were incubated at 37°C for 10 minutes before being washed twice in RPMI supplemented with 10% FCS. Labeled cells were put into culture at a ratio of 1 effector T-cell to every 10 target CLL cells for 12 hours.

Phase contrast and confocal microscopy

Phase contrast and confocal microscopy were performed using a Leica RS2 microscope and analyzed using Leica confocal software (Leica Microsystems, Milton Keynes, UK) and ImageJ software (National institutes of Health). For 3-D image reconstruction of clusters, 50 confocal sections were taken and assembled using Imaris imaging software (BITPLANE scientific software).

Statistical analysis

Data were analyzed using standard non-parametric tests using Graphpad Prism 5.0 software (Graphpad Software Inc., CA, USA). For statistical analysis between 1 group measuring two nominal variables, a paired *t*-test was used when the data were shown to conform to a Gaussian distribution.

Supplementary Information

Starting cell numbers for patients studied in Figure 1A,B

Table S1. CD4 T cells/ml

Patient	Starting	Ending			
		Untreated	*BiTE*	*CD3*	*CD3 CD28*
1	41381	26605	302020	104054	ND
2	10744	7733	84937	17255	ND
3	21770	17694	37202	19996	ND
4	24054	23624	175283	24910	ND
5	26028	36795	210812	62170	ND
6	31994	15973	38387	15992	ND
7	22680	16506	52837	12615	407722
8	207072	5350	5535	2667	15900
9	110700	44131	43345	133928	898876
10	206280	26236	753379	276904	97713
11	38646	16199	16339	90712	736165
12	42066	21929	37978	24251	177473
13	116946	64932	220680	241856	612150

Table S2. CD8 T cells/ml

Patient	Starting	Ending			
		Untreated	*BITE*	*CD3*	*CD3 CD28*
1	20580	7749	108185	78041	ND
2	20660	9601	70230	23192	ND
3	8280	2720	18861	3732	ND
4	23028	12057	140999	29040	ND
5	19440	8086	57299	14504	ND
6	61215	13585	23363	12988	ND
7	42998	16762	8225	8908	53286
8	20736	14505	6325	9100	52903
9	37454	6228	4813	27148	73550
10	75240	4537	361967	182755	18496
11	22686	3876	3988	124765	279515
12	52029	13038	18109	17165	93684
13	131729	63724	225330	175715	695710

Absolute CLL cell numbers for Figure 1C

Table S3. CLL cells/ml

Patient	Starting	Ending			
		Untreated	*BiTE*	*CD3*	*CD3 CD28*
1	1426500	1148932	192030	1326041	ND
2	1455750	307157	167851	768947	ND
3	1465500	183019	316348	369746	ND
4	1443000	1202200	20295	1434613	ND
5	1446000	1360410	211087	1739583	ND
6	1384500	433248	221372	653518	ND
7	1405500	974821	452533	1338940	1661018
8	1212000	702645	607013	888328	285440
9	1315500	258348	515427	403322	700794
10	1140000	628804	35114	723719	622587
11	1386000	682502	792160	766156	714380
12	1377000	905341	362393	1149857	1344138
13	1171500	302025	166650	660677	700289

PBMC were cultured with various treatments for 7 days before determining cell counts. These included no treatment (untreated), blinatumomab (BiTE), anti-CD3 antibody and CD3-CD28 beads. Starting cell counts for CD4,CD8 and CLL cells were determined for 13 of the 15 patients. ND=not determined.

Table S4. Clinical details of treated patients (Figure 1).

Patient	Treatment	Time of sample post treatment
7	Fludarabine/Cyclophosphamide x1	2 years 2 months
8	Chrorambucil x2	2 years 1 month
11	Chlorambucil/Fludarabine x1	11 years
12	Steroids (continuous)	1 week
13	Fludarabine/Cyclophosphamide x6	3 years 10 months