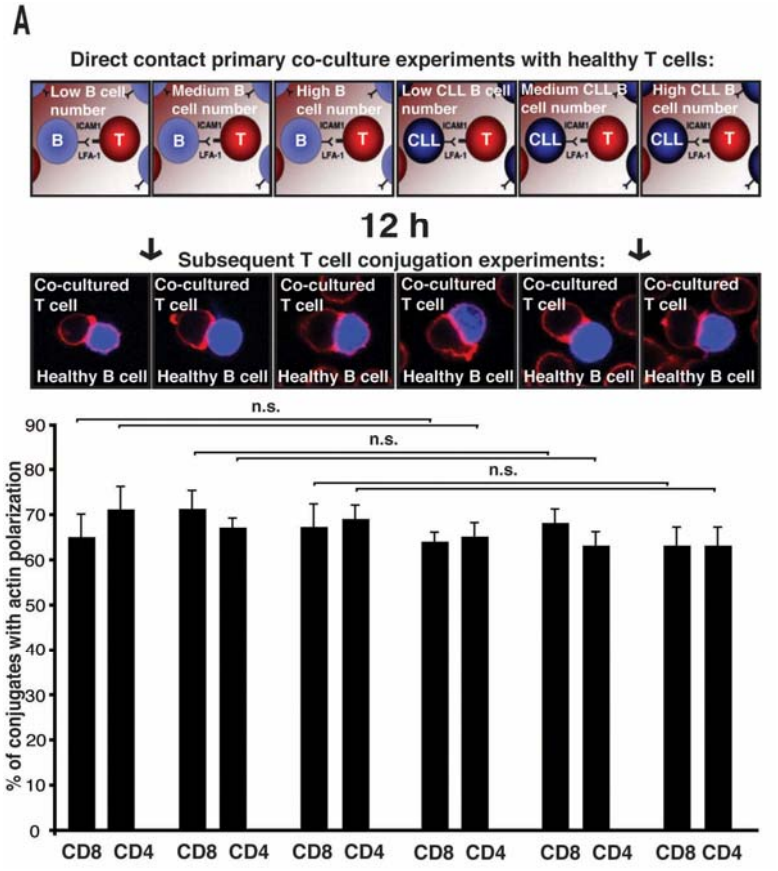
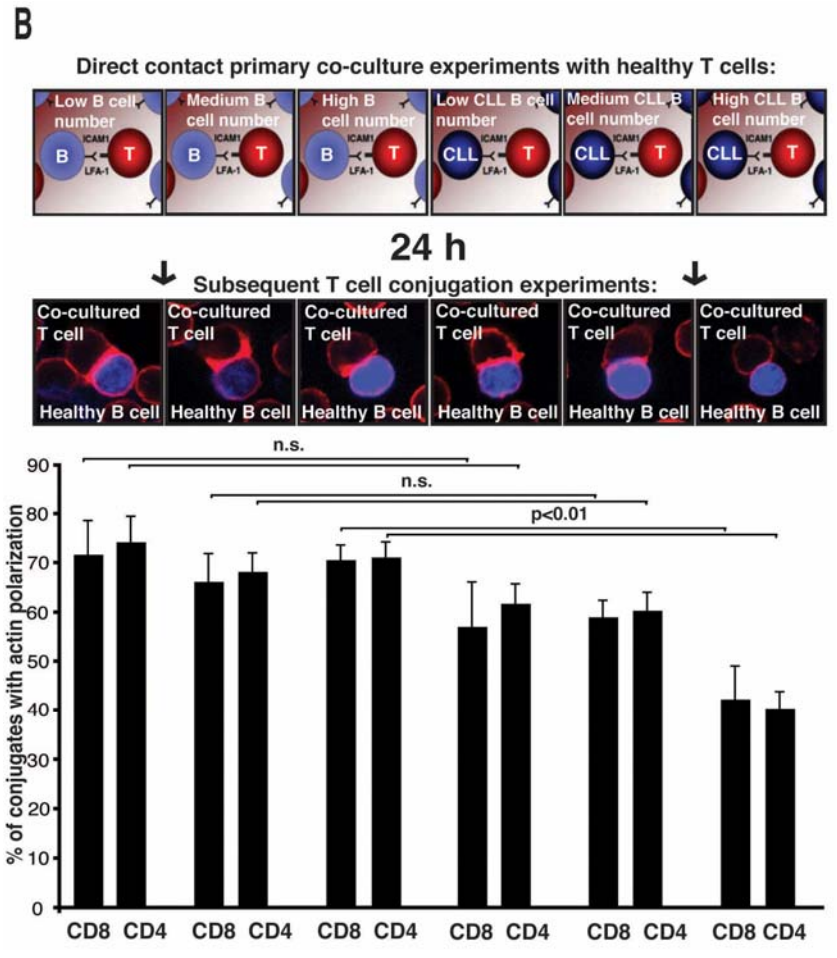
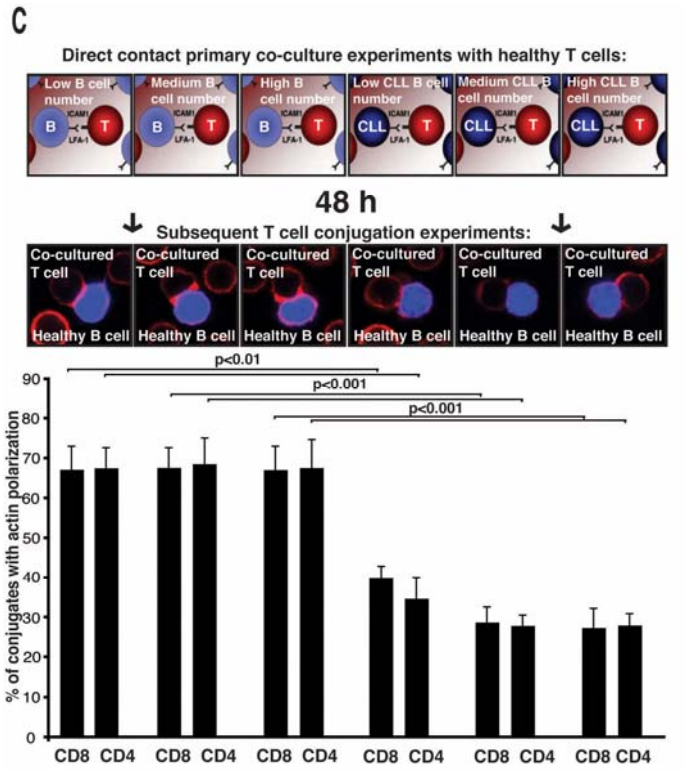
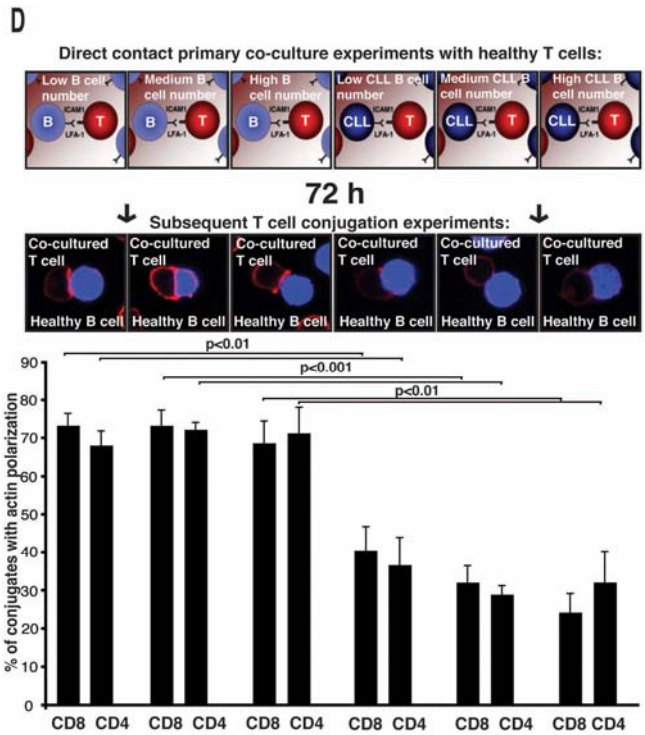

A
Direct contact primary co-culture experiments with healthy T cells:
Low B cell number
Medium B cell number
High B cell number
Low CLL B cell number
Medium CLL B cell number
High CLL B cell number
B
CLL
T
ICAM1
LFA-1
12 h
Subsequent T cell conjugation experiments:
Co-cultured T cell
Healthy B cell
n.s.
n.s.
n.s.
% of conjugates with actin polarization
0
10
20
30
40
50
60
70
80
90
CD8 CD4 CD8 CD4 CD8 CD4 CD8 CD4 CD8 CD4 CD8 CD4


B
Direct contact primary co-culture experiments with healthy T cells:
Low B cell number
Medium B cell number
High B cell number
Low CLL B cell number
Medium CLL B cell number
High CLL B cell number
B
CLL
T
ICAM1
LFA-1
24 h
Subsequent T cell conjugation experiments:
Co-cultured T cell
Healthy B cell
n.s.
n.s.
p<0.01
% of conjugates with actin polarization
0
10
20
30
40
50
60
70
80
90
CD8 CD4 CD8 CD4 CD8 CD4 CD8 CD4 CD8 CD4 CD8 CD4

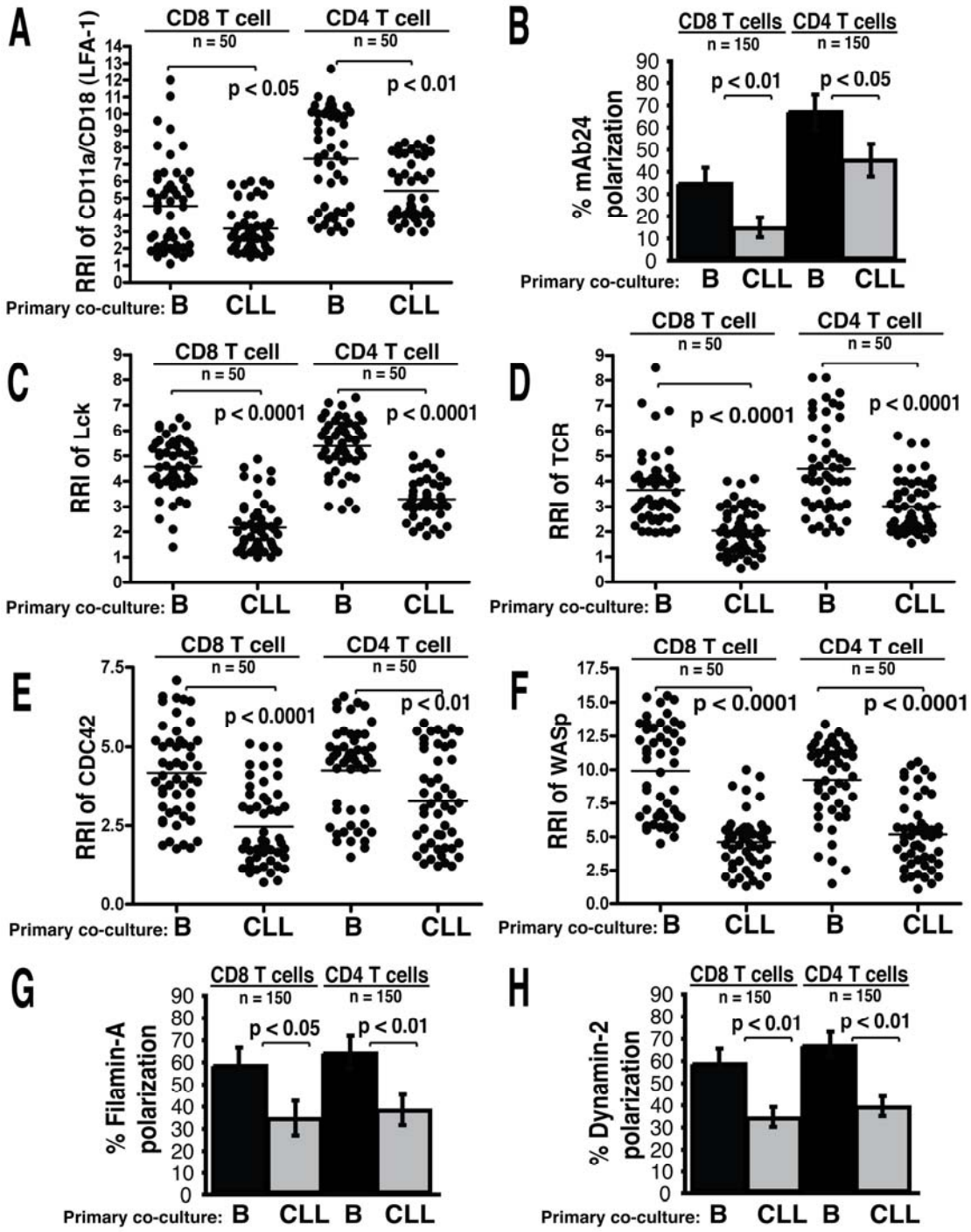




Supplemental Figure 1

CLL cells induce defective immunological synapse formation in healthy allogeneic T cells by direct cell contact. Healthy T cells (3×10^6) co-cultured for 12 h (**A**), 24 h (**B**), 48 h (**C**), or 72 h (**D**) with low (1.5×10^5) , medium (1.5×10^6) or high (15 x

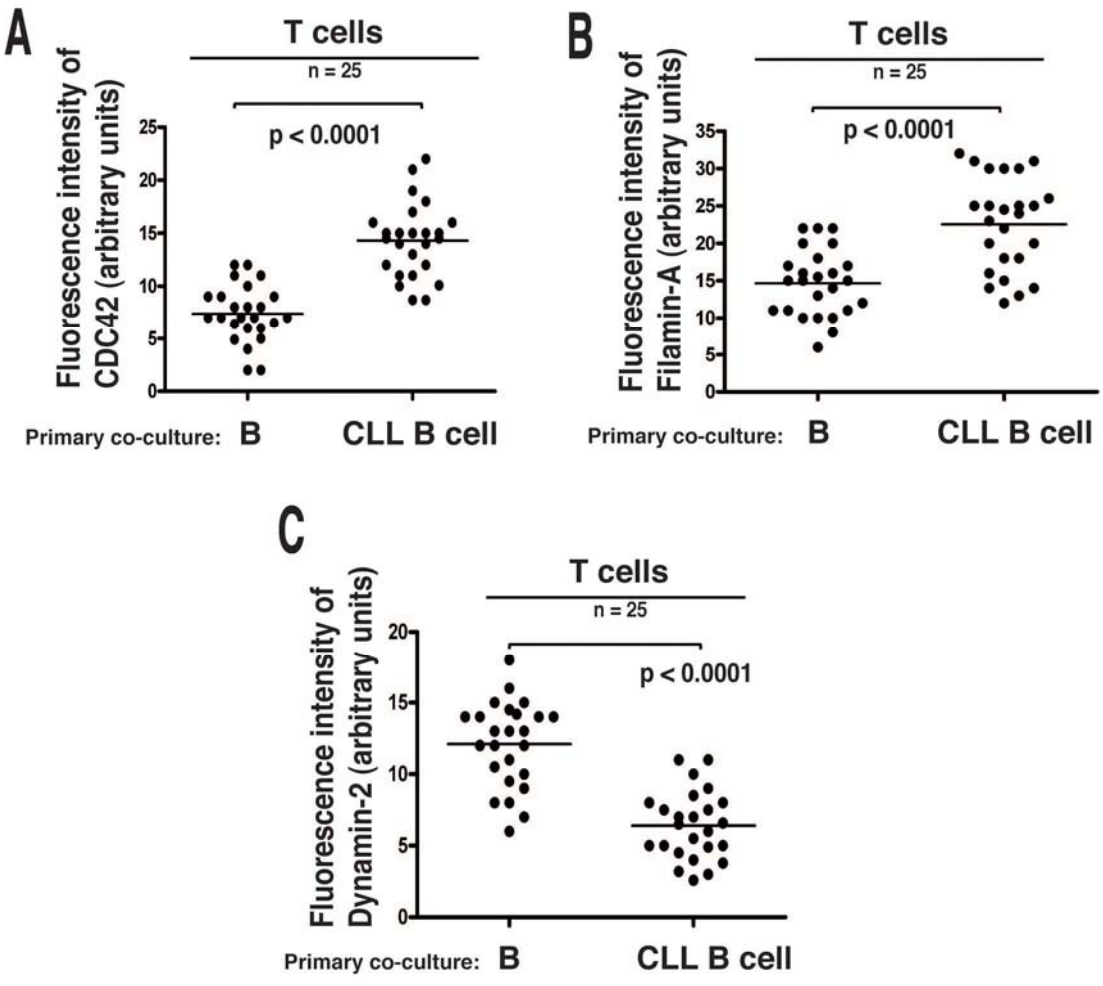
10^6) cell numbers of healthy allogeneic B cells (B) or allogeneic CLL B cells (CLL), and subsequently used in conjugation assays with sAg-pulsed third-party allogeneic healthy donor B cells (APCs, blue). Conjugates were selected at random for imaging and were scored for accumulation of F-actin (red) at the immune synapse. Data are the mean ± SD from 3 independent experiments with 50 conjugates analyzed per experiment.



Supplemental Figure 2

Quantitative image analysis of protein accumulation at the immunological synapse in conjugate experiments performed in Figure 4. (**A**, **C**-**F**) Quantification results shown are representative of evaluation of 150 conjugates from 3 independent experiments. Quantitative image analysis (relative recruitment index, RRI) of (**A**) CD11a/CD18,

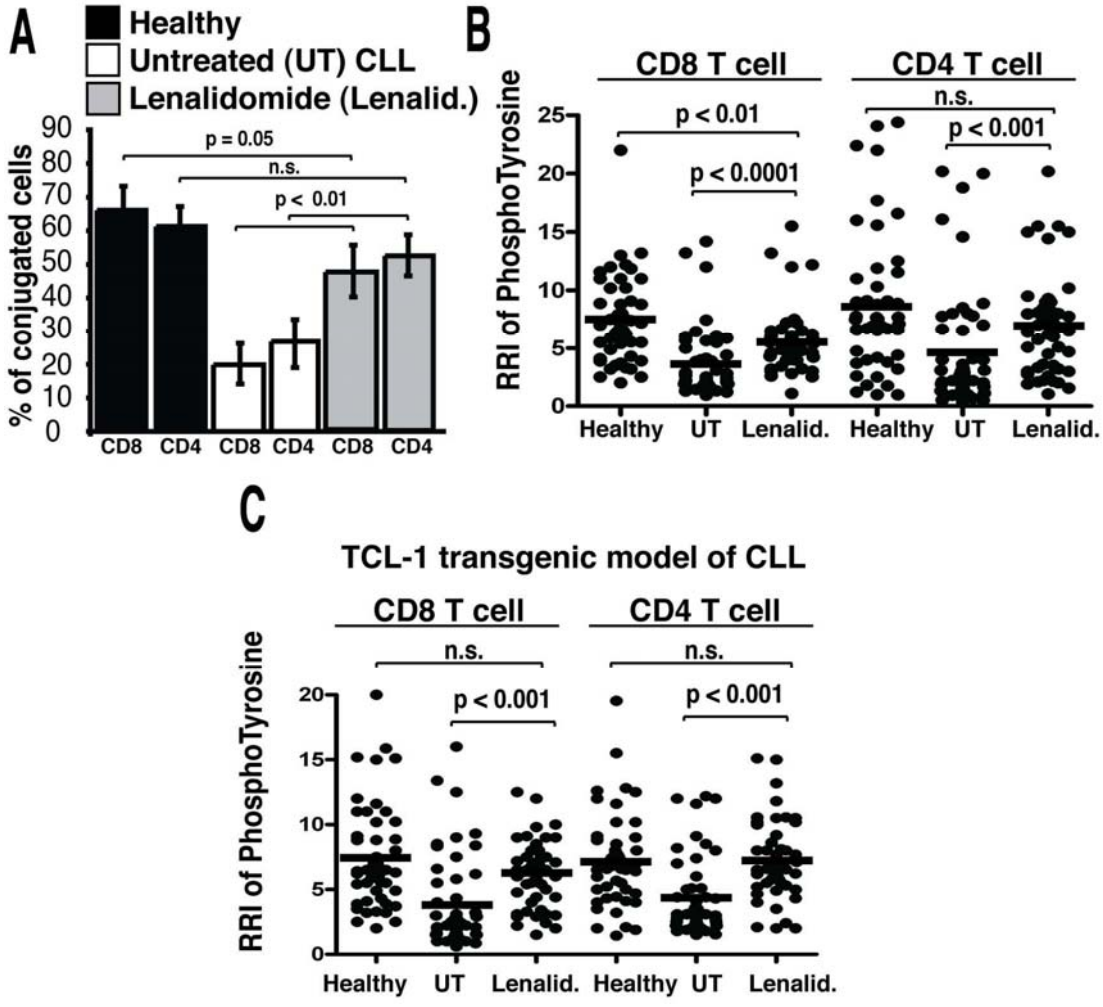
(**C**) Lck, (**D**) TCR, (**E**) Cdc42, (**F**) WASp, at the immunological synapse is shown with each dot representing a T cell conjugate (50 conjugates analyzed per experiment). The black bar shows the mean value. (**B**, **G** and **H**) Randomly chosen conjugates were scored by an expert histopathologist (A.M.L) for accumulation of (**B**) high affinity CD11a/CD18 (mAb24), (**G**) Filamin-A, and (**H**) Dynamin-2, at the immune synapse. Data represent means from 3 independent experiments (50 conjugates analyzed per experiment) ± SD.



Supplemental Figure 3

(**A**-**C**) Quantitative image analysis of protein expression (**A**) Cdc42, (**B**) Filamin-A, and (**C**) Dynamin-2, in non-conjugated T cells following primary co-culture with

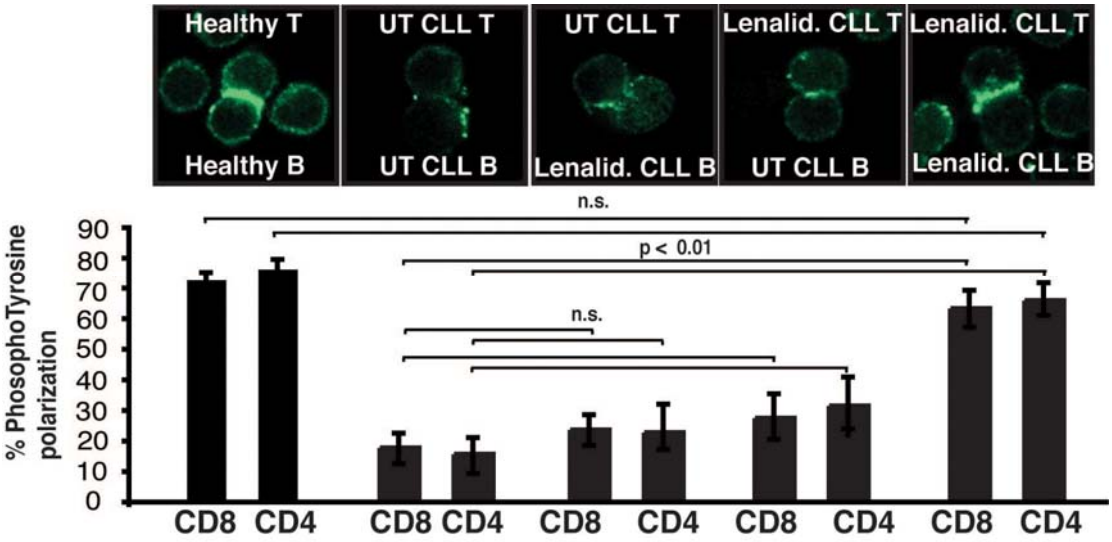
allogeneic CLL B cells or allogeneic healthy B cells (B) for 48 h. These T cells were then subsequently analyzed by immunofluorescence, confocal microscopy and quantitative analysis (fluorescence intensity was calculated using ImageJ software). Results are representative of at least 3 independent experiments with $CD8^+$ and $CD4^+$ T cells. Image sets to be compared were acquired during the same session and using the same acquisition settings as described in methods. Statistical analyses were carried out by the nonparametrical Mann-Whitney test using PRISM software.



Supplemental Figure 4

(**A**) Autologous T cell-sAg-pulsed CLL cells conjugates from untreated (UT) CLL (white bars) or lenalidomide (Lenalid.) treated (grey bars) patient cells were scored by

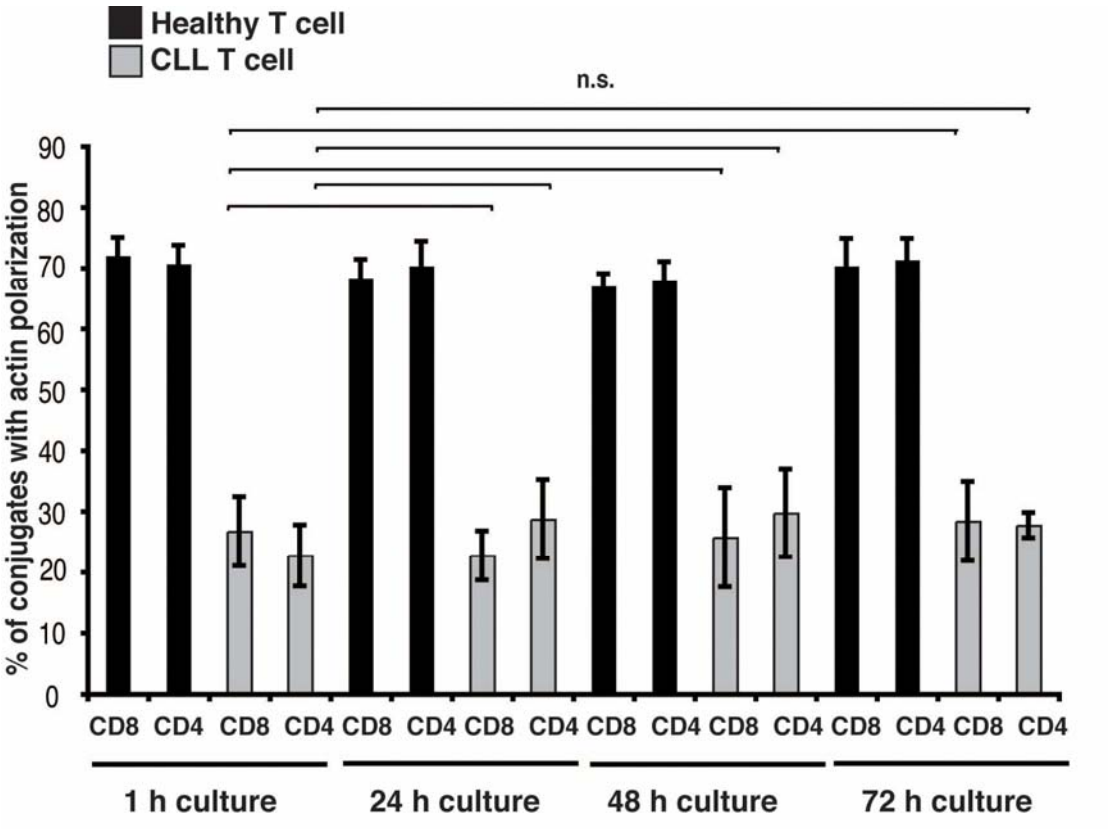
visual counting using a confocal microscope. As controls (Healthy), autologous age-matched healthy donor cells were used (black bars). Each dataset are the mean ± SD from 3 independent experiments with 50 random T cells analyzed per experiment. (**B and C**) UT CLL or Lenalid. treated autologous CLL patient (**B**) and Eμ-TCL1 mouse cells (**C**) were stained for phosphotyrosine using immunofluorescence and confocal microscopy (sAg-pulsed CLL B cells). As controls (Healthy), autologous age-matched healthy donor or wild-type mice cells were used. Quantification results shown are representative of evaluation of 150 conjugates from 3 independent experiments. Relative recruitment index (RRI) of phosphotyrosine accumulation at the immunological synapse is shown with each dot representing a T cell conjugate (50 conjugates analyzed per experiment). The black bar shows the mean value.



Supplemental Figure 5

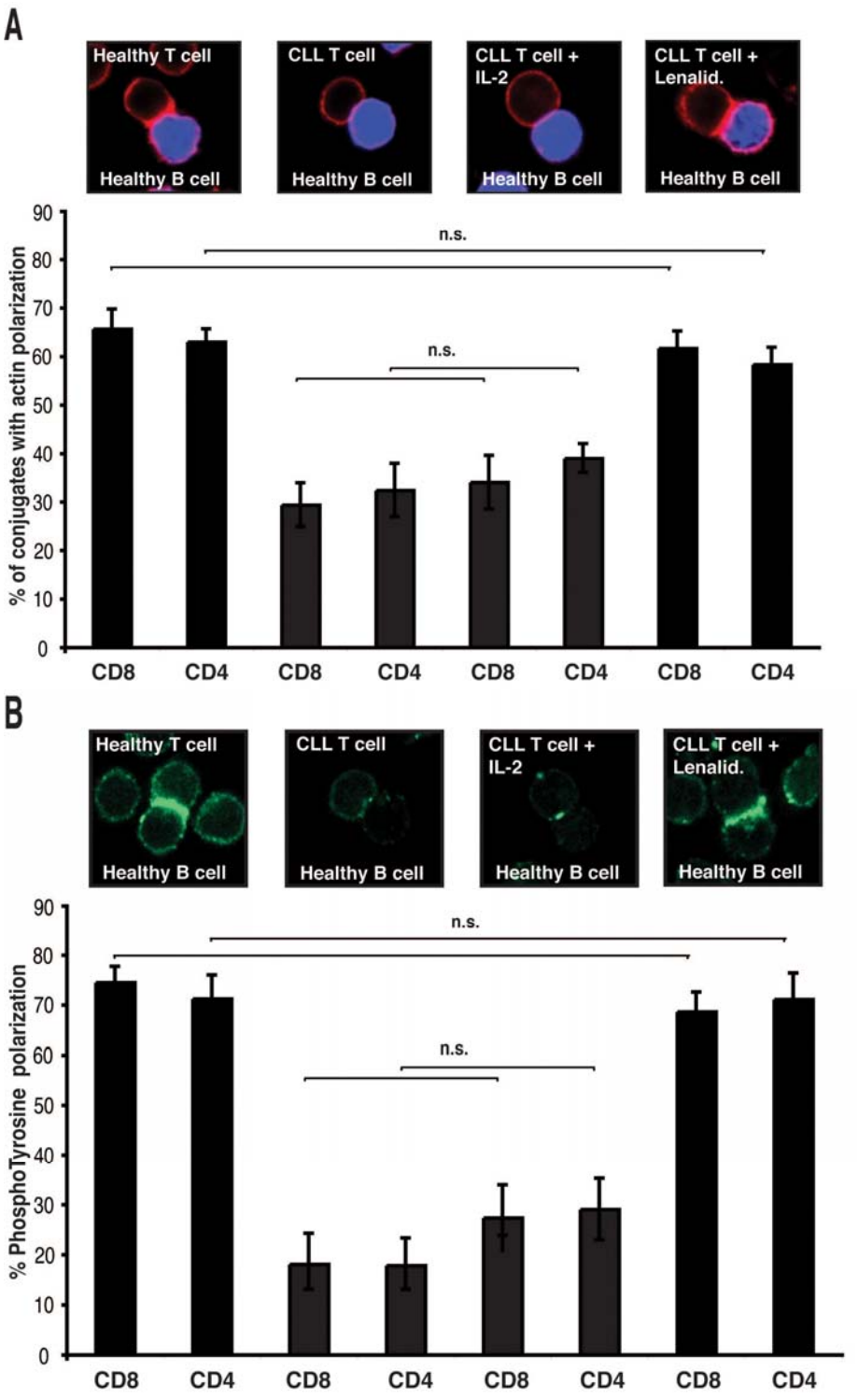
Conjugates from Figure 6A were scored by an expert histopathologist (A.M.L) for accumulation of phosphotyrosine (green) at the immune synapse. Data represent

means from 3 independent experiments (50 conjugates analyzed per experiment) ± SD.



Supplemental Figure 6

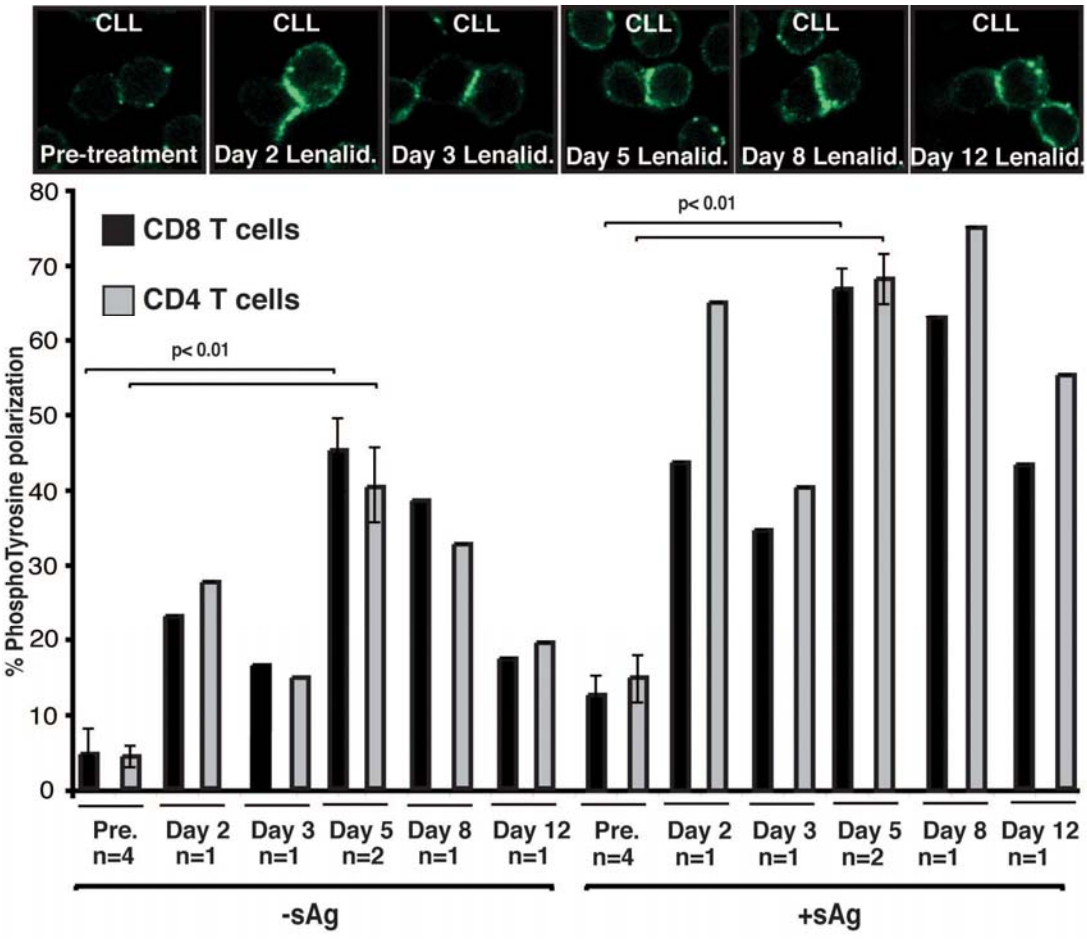
Conjugates from experiments using cultured (1h, 24h, 48h and 72h in full medium) CLL T cells (grey bars) with healthy allogeneic sAg-pulsed B cells were selected at random for imaging and scored for accumulation of F-actin at the immune synapse. As controls, healthy T cells (black bars) were cultured in full medium for the same time points and conjugated with healthy allogeneic sAg-pulsed B cells. Data are the mean ± SD from 3 independent experiments with 50 conjugates analyzed per experiment.



Supplemental Figure 7

Allogeneic T cell-sAg-pulsed B cell (blue) conjugates using healthy T cells, CLL T cells, exongenous IL-2 (100 U/ml, 24h) treated CLL T cells, or lenalidomide (Lenalid.) treated CLL T cells were selected at random for imaging and scored for

accumulation of (**A**) F-actin (red) or (**B**) phosophotyrosine (green) at the immune synapse. Data are the mean ± SD from 3 independent experiments with 50 conjugates analyzed per experiment.



Supplemental Figure 8

Autologous T cell-CLL B cell conjugates (± sAg) from four patients before (Pre.) and after treatment with lenalidomide (Day 2,3,5,8 and 12) were selected at random for imaging and scored for accumulation of phosophotyrosine (green) at the immune synapse. 50 conjugates were analyzed per experiment.

Methods

Cell isolation. Healthy lymphocytes for the co-culture assays were obtained from buffy coats prepared by the National Blood transfusion service (UK). CLL patient or healthy donor mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation and $CD4^+$ and $CD8^+$ T cells were negatively selected using the $CD4^+$ and $CD8^+$ T cell Isolation Kit II and MACs separation columns (Miltenyi Biotec). $CD3^+$ T cells were negatively selected using the Pan T cell Isolation Kit II (Miltenyi Biotec). Normal and malignant B cells (CLL cells) were positively selected using CD19 Microbeads (Miltenyi Biotec). We used healthy allogeneic B cells rather than $CD5^+$ B cells as controls based upon previous data demonstrating that the gene expression profile of CLL cells was more similar to B cells than $CD5^+$ B cells (39). The purity of the isolated T cells and B cells (higher than 95%) was determined using anti-CD4, CD8 and CD20 antibodies with flow cytometry.

Mice. CLL development was detected by examination for lymphadenopathy and splenomegaly. Transgenic mice that had developed CLL or age-matched wild-type mice were sacrificed and the spleen extracted. Splenic mononuclear cells were separated by ficoll-hypaque centrifugation. Cells were stained with antibodies specific for murine CD5, CD19, Igκ, Igλ, and IgM (BD Biosciences) for flow cytometry analysis to confirm both surface antigen expression and clonality. $CD4^+$ and $CD8^+$ T cells were negatively selected using the mouse $CD4^+$ and $CD8^+$ T cell Isolation Kits and MACs separation columns (Miltenyi Biotec). Normal and malignant B cells (CLL cells) were positively selected using mouse CD19 Microbeads (Miltenyi Biotec). The

purity of the isolated lymphocytes (higher than 95%) was detected using anti-CD4, CD8 and CD19 antibodies with flow cytometry.

Antibodies and reagents. RPMI 1640 (GlutaMax), pooled AB human serum, gentamicin, Rhodamine Phalloidin, CellTracker Blue CMAC, and Alexa Fluor 488-labeled goat anti-mouse and anti-rabbit IgG antibodies were all purchased from Invitrogen. Dynamin-2 rabbit polyclonal antibody was a generious gift from M.A. McNiven. β2 integrin activation reporter mAb24 was a generous gift from N. Hogg. Anti-integrin αL/LFA-1α (mAb) was from BD Biosciences. Anti-Filamin-A (mAb) and anti-CD3 (clone UCHT1, mAb) were from Chemicon. Anti-WASP (D-1, mAb), anti-Cdc42 (B-8, mAb), and anti-Lck (3A5, mAb) were from Santa Cruz Biotechnology. Anti-phosphotyrosine, clone 4G10 (mAb and FITC conjugate) was purchased from Millipore. All other reagents were purchased from Sigma unless otherwise stated.

Immunofluorescence and confocal microscopy image acquisition. After fixation, coverslips were permeabilized in 0.3% Triton-X-100 for 5 min. Blocking and washing was performed in PBS/0.05% saponin/0.25% fish skin gelatin. Primary and secondary antibodies were applied sequentially for 45 min at 4 ^{o}C in 5% goat serum (Sigma) in PBS. F-actin was stained with rhodamine phalloidin (Invitrogen) applied alone or with the secondary antibody. After labeling specimens were mounted on Prolong Gold anti-fade reagent (Invitrogen). The specificity of staining was optimized and controlled by using appropriate dilutions of isotype-control primary antibodies and subsequent fluorescent secondary antibodies. Background staining with control antibodies was compared with positively stained cells and was not visible using

identical acquisition settings. Medial optical section images were captured with a Zeiss 510 Meta confocal laser-scanning microscope using a 63x objective. Fluorescence was acquired sequentially to prevent passage of fluorescence from other channels. Detectors were set to detect an optimal signal below the saturation limits. Image sets to be compared were acquired during the same session and using the same acquisition settings.

Quantitative image analysis of conjugate formation and F-actin polymerization. Conjugation was scored by visual counting under a confocal microscope with random selection of at least 50 T cells scored as conjugates only if they contacted no more than one blue stained healthy or CLL B cell, and if there was no other T cells touching the T cell being scored. Polarization of proteins at the T cell contact site was scored independently by A.G.R and an expert histopathologist (A.M.L). Those conjugates showing a distinct polymerized protein band at the T cell contact site were considered polarized (score=1). Conjugates lacking protein polymerization (score=0) or showing weak protein polymerization (score=0.5) were included in this analysis. Statistical significance was calculated using a Student's *t*-test (two-tailed). A difference between values was considered significant when a $p<0.05$ was obtained. These findings were verified using ImageJ software to calculate the relative recruitment index of proteins to the T cell-B cell contact site.

Quantitative image analysis of fluorescence. To quantitate recruitment of F-actin, CD11a/CD18 (LFA-1), Lck, TCR, Cdc42, WASp, and Phospho-tyrosine to the immunological synapse, the relative recruitment index (RRI) was calculated using the ImageJ programme (multi-measure tool analysis of tiff images). Boxes were drawn

around the immunological synapse site, the regions of the T cell not in contact with B cells, and a background area. The RRI was calculated as: (mean fluorescence intensity (MFI) at synapse – background) / (MFI at the T cell regions not in contact with B cells – background). Fifty conjugates were examined quantitatively for each experiment. Statistical analyses were carried out by the nonparametrical Mann-Whitney test using PRISM software. A difference between values was considered significant when a $p<0.05$ was obtained.

CLL cell-T cell co-culture assays. To analyze the impact on T cells of soluble-derived tumor factors, 3 x 10^6 per millilitre (ml) healthy $CD8^+$ or $CD4^+$ T cells (lower well) were incubated with 1.5 x 10^6/ml healthy B cells or CLL cells (upper transwell insert) in full culture medium (10% human serum) in 24-well transwell plates (0.4-μm pore) for 48 h. To analyze the impact on T cells of tumor cell direct contact, T and B cells (2:1 ratio) were co-cultured together in full culture medium for 48 h (24-well plate). For cell adhesion blocking co-culture experiments, T cells were co-cultured with CLL cells that had been pre-treated with blocking antibody (anti-CD54, ICAM-1, 10 μg/ml) for 1 h and subsequently washed to remove any unbound antibody. All experiments were performed using isotype matched IgG as controls. After transwell or direct contact co-culture, cells were harvested and T cells isolated by magnetic cell separation (negative selection, Miltenyi Biotec).

Cytotoxicity. Target cells were labeled with 100 μCi ^{51}Cr (3.7 MBq; NEN) and seeded in 96-well U-bottom microtiter plates at a concentration of 2.5×10^3 in triplicates. Effector cells were added in a ratio of 1:3, 1:10, and 1:30 and co-cultured for 4 h at 37°C in a 5% CO_2 humidified atmosphere. After 4 h the supernatants were harvested

and the released ^{51}Cr was measured in a γ-Counter (Wallac). Spontaneous release was determined by incubation of treated target cells in medium alone and maximum release was determined by resuspending the wells with 2% Triton X-100. Specific lysis was determined for each individual experiment as follows: specific lysis (%) = (experimental Cr^{51} release - spontaneous ^{51}Cr release)/maximum ^{51}Cr release spontaneous - ^{51}Cr release) × 100.

Allogeneic Mixed Lymphocyte Reaction (MLR). Healthy $CD3^+$ T cells (repeated for 5 healthy donors) were co-cultured in direct contact for 48 h with either unstimulated or CD40-activated allogeneic healthy B cells or allogeneic CLL cells (3 previously untreated patients). The healthy T cells were then negatively isolated (magnetic bead depletion of B cells) and rested in medium for 24 h. Secondary mixed lymphocyte reactions (MLR) were set-up using the isolated T cells (1×10^5) with third party allogeneic irradiated PBMCs (25 Gy) as stimulators (10^5). [^{3}H]Thymidine incorporation was assessed for the last 16 h of a 3 day culture. The stimulation index was calculated as cpm of $CD3^+$ T cells with stimulator cells / cpm of $CD3^+$ T cells alone.