Supplemental materials for Ramsay et al. Blood/2012/411687

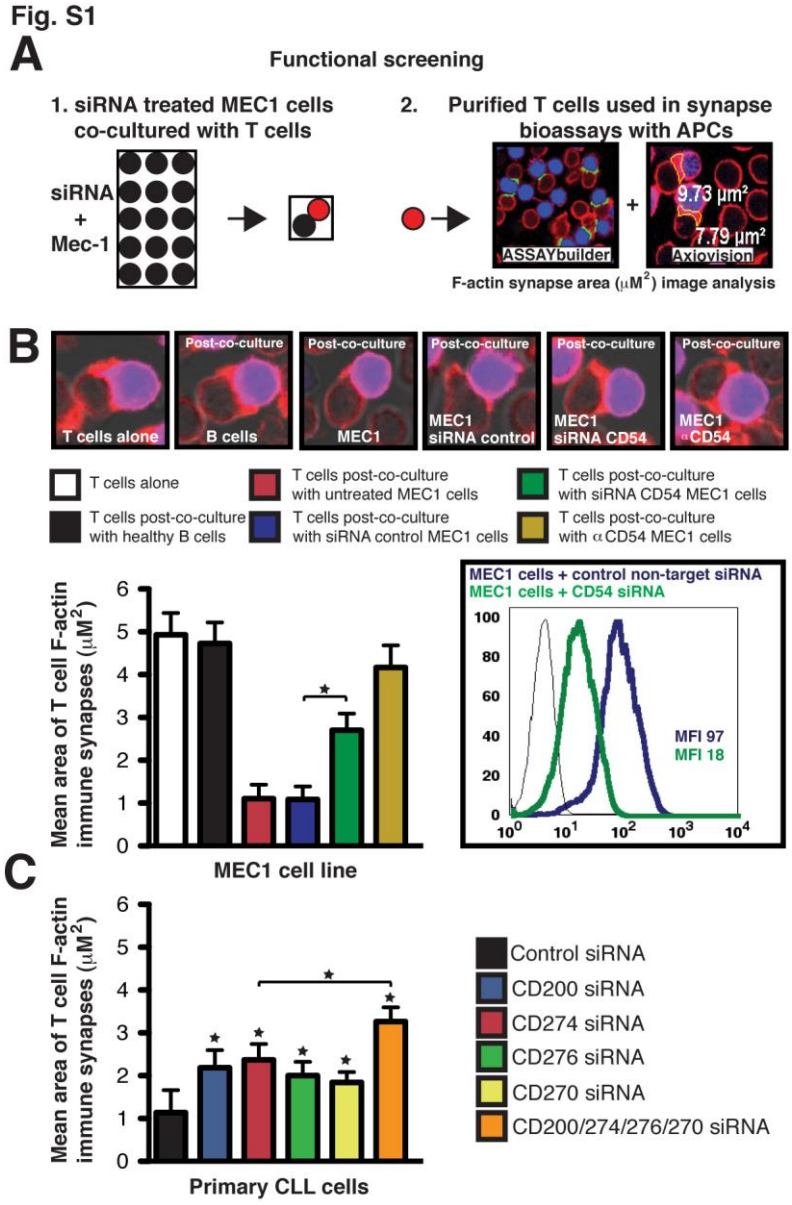

Figure S1. B cell leukemia cell line MEC1 induces defective actin polymerization at the immunological synapse in previously healthy T cells and is suitable for siRNA functional screening synapse bioassays. (**A**) Schematic of the functional screening strategy. siRNA treated MEC1 cells were co-cultured (48 h) with healthy donor allogeneic T cells. T cells were then negatively selected and used in conjugation assays with sAg-pulsed third-party healthy donor allogeneic B cells as APCs (CMAC dyed, blue). Image acquisition of confocal microscopy allowed

quantitative area measurement (μM^2) of F-actin polymerization (rhodamine phalloidin, red) at each T cell-APC conjugate (minimum n=100) per experiment. (**B**) Mean synapse area ± SD from 6 healthy donor allogeneic T cell functional screens with MEC1 cells that were untreated, siRNA treated, or pre-treated with anti-CD54 neutralizing antibody (α). Targeting CD54 and non-targeting siRNA (control) treated cells acted as positive and negative controls. T cells alone without primary co-culture were included as control conjugation experiments with APCs (third-party healthy donor allogeneic B cells + sAg). Primary co-culture using healthy B cells showed no effect on the ability of T cells to form synapses with APCs. Right bottom, Expression (MFI) of CD54 on siRNA treated MEC1 cells as determined by flow cytometry (non-targeting control compared to CD54 siRNA). Anti-human CD54 antibody (clone BBIG-I1, FITC) was used. (**C**) Mean T cell synapse area ± SD from 6 CLL patients treated with siRNA targeting inhibitory ligands before primary co-culture with healthy donor allogeneic T cells (n=6). Purified T cells were then used in conjugation assays with sAg-pulsed third-party healthy donor allogeneic B cells as APCs. The confocal images show T cell-APC conjugates post-primary co-culture. Original magnification x63. * $P < 0.05$.

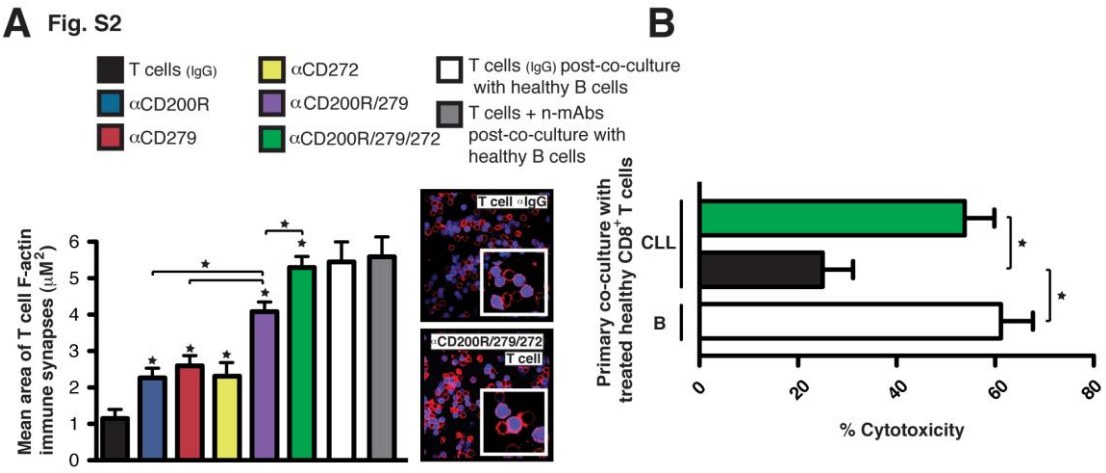

Figure S2. Blockade of the T cell inhibitory receptors CD200R, CD272 (BTLA), and CD279 (PD-1) prevented CLL inhibitory signaling. (**A**) Mean T cell F-actin synapse area and (**B**) % cytotoxicity ± SD from 6 healthy donor allogeneic T cell functional screens with primary CLL patient cells (n=6), pre-treating T cells with neutralizing antibodies (n-mAbs, α) before primary co-culture (48 h) with CLL cells. T cells were then purified from co-culture (negative selection) and used in subsequent conjugation assays or cytotoxicity assays with third-party sAg-pulsed healthy donor allogeneic B cells as APCs. T cells post-co-culture with healthy donor allogeneic B cells were included in control conjugation experiments with APCs. The confocal images show T cell-APC conjugate populations post-primary co-culture. Original magnification x63. * $P < 0.05$.

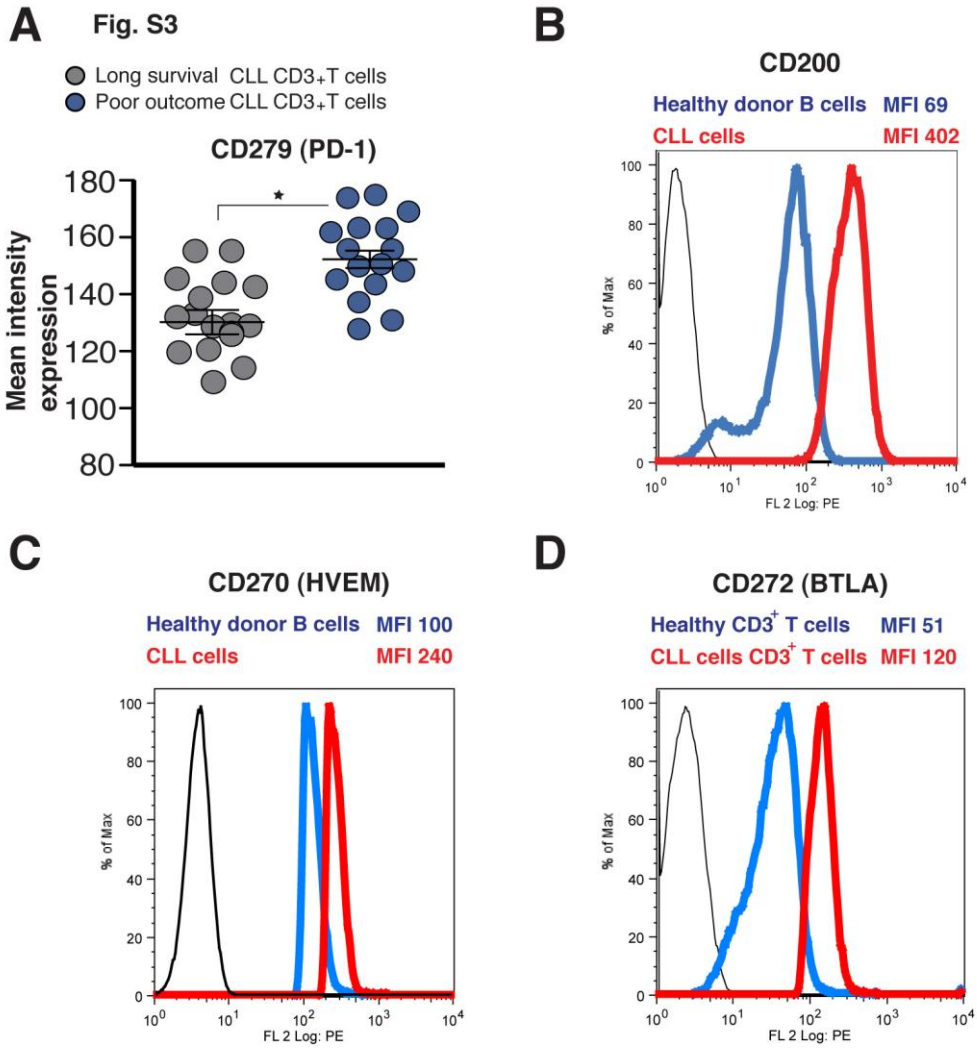

Figure S3. Increased expression of inhibitory receptor CD279 (PD-1) on CD3$^+$ T cells in poor prognosis compared to good prognosis CLL. (A) Immunohistochemistry mean intensity expression analysis ± SEM of CD279 on CD3$^+$ T cells using an extreme of survival diagnostic CLL TMA (> 10 year long median survival group compared to a short-survival 38 month median survival patient group). * $P < 0.05$. (**B-D**) Representative flow cytometry histograms (FlowJo analysis software) used to plot mean fluorescence intensity (MFI). Inhibitory ligands CD200 and CD270 and inhibitory receptor CD272 were expressed on all cells examined but had markedly up-regulated MFI expression in CLL patients compared to age-matched healthy donors.

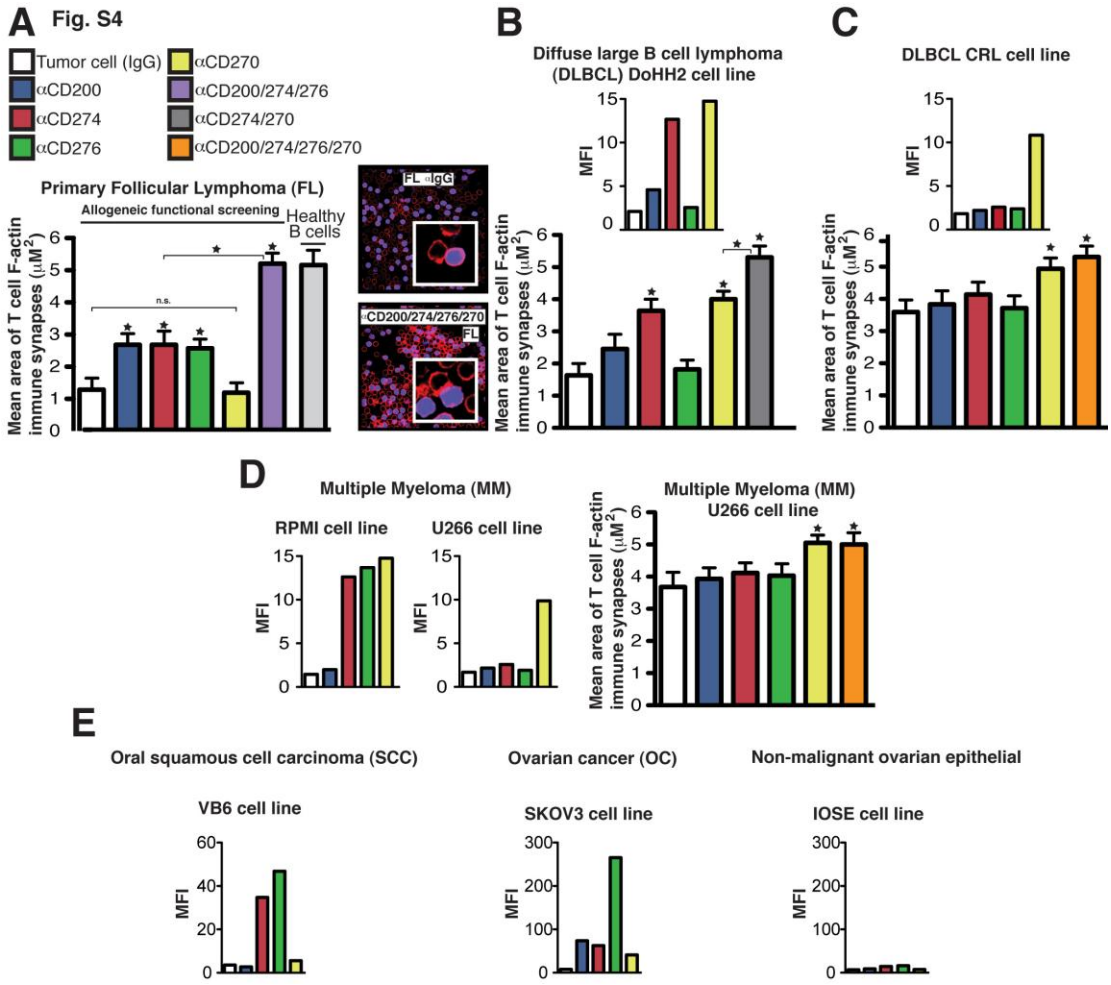

Figure S4. Follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL) cells, multiple myeloma (MM), squamous cell carcinoma (SCC) and ovarian cancer (OC) cell lines that express inhibitory ligands induce T cell F-actin dysfunction in previously healthy T cells. Healthy donor allogeneic T cell F-actin synapse function with third-party healthy donor allogeneic B cells (+sAg) as APCs post-primary co-culture (24 h) with (**A**) primary tumor-infiltrated FL cells, (**B**) DLBCL cell line DoHH2, (**C**) DLBCL cell line CRL, or (**D**) MM cell line U266 pre-treated with neutralizing antibodies (α). Colored columns show the mean T cell synapse area ± SD from 6 donor functional screens. (**E**) MFI expression data of cell surface inhibitory ligands on all cell lines (B-E) is also shown. * $P < 0.05$.

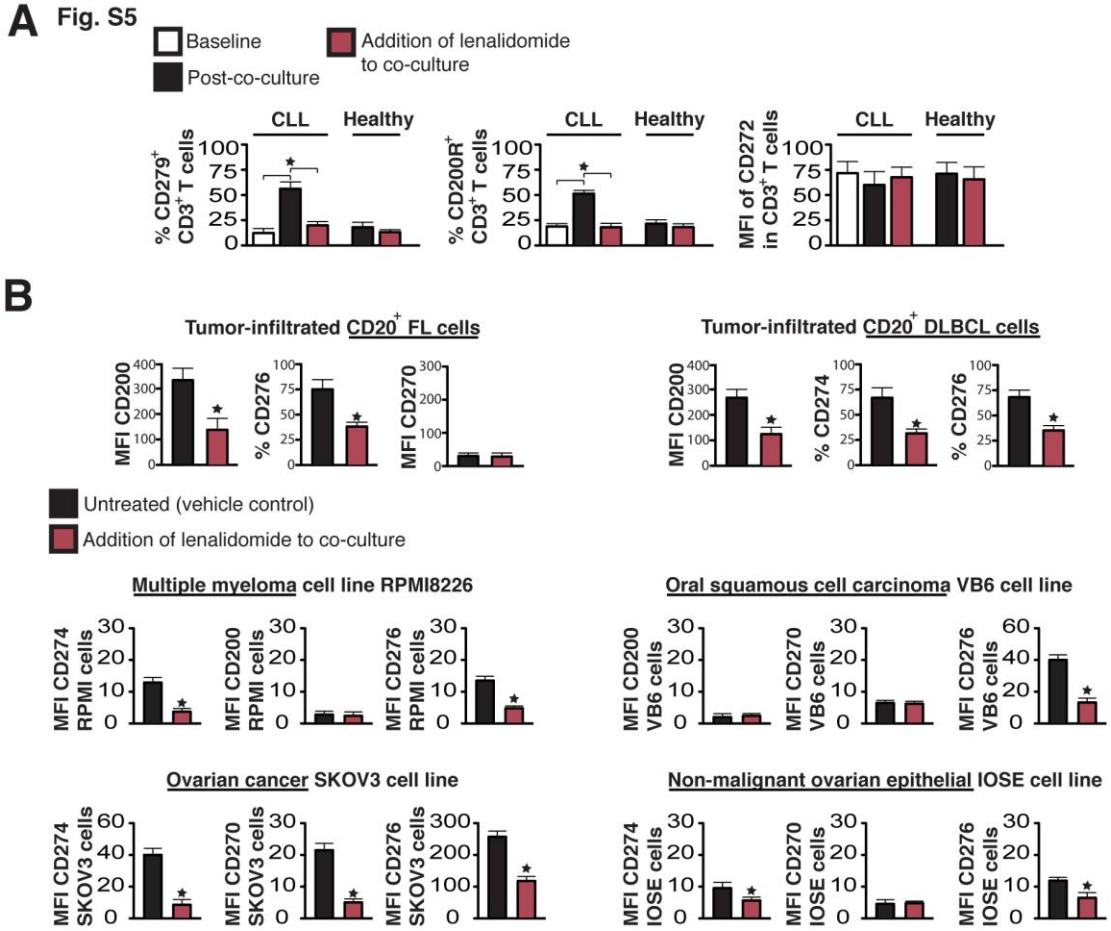

Figure S5. Lenalidomide treatment down-regulates expression of inhibitory molecules during tumor immunosuppressive co-culture assays. (A) FACs MFI or % positive expression of CD279, CD200R, and CD272 on healthy donor $CD3^+$ T cells before (baseline) and post-co-culture (48 h) with CLL cells (CLL) or third-party age-matched healthy donor allogeneic B cells (Healthy) in the presence of vehicle control or lenalidomide (1μM). Columns show the mean ± SEM from 6 co-culture experiments. (**B**) FACs MFI or % positive expression of inhibitory ligand expression on hematologic and solid cancer cells ± SEM from 6 independent experiments before and after 24 h exposure to lenalidomide. * $P < 0.05$.

Fig. S6

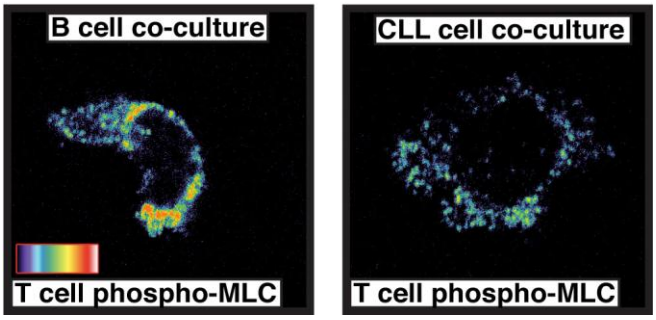

Figure S6. CLL immunosuppressive signaling down-regulates the expression of T cell phosphorylated-myosin light chain (MLC) compared to control healthy B cell experiments. T cells from co-culture (Figure 6) were analyzed for their expression of phosphorylated (phospho)-MLC. The rainbow scale is applied with yellow/red representing the strongest expression.