Supplemental materials for Ramsay et al.

Chronic lymphocytic leukemia cells induce defective LFA-1-directed T cell motility by altering Rho GTPase signaling that is reversible with lenalidomide

Fig. S1

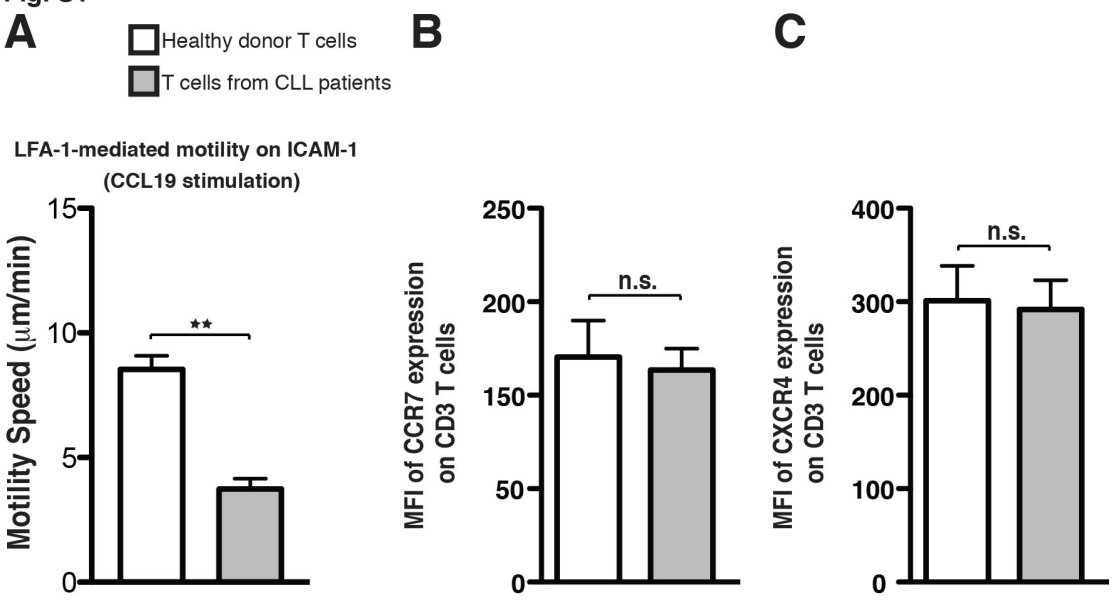

Figure S1. T cells from CLL patients exhibit impaired LFA-1-mediated migration following exposure to arrest chemokines CCL19 (or CXCL12) compared to healthy donor T cells, despite these cells expressing similar levels of chemokine receptor CCR7 or CXCR4. (A) Primary CD3 T cells from age-matched healthy donors (white bar charts) and CLL patients (grey bar charts) were allowed to adhere to immobilized CD54 ligand for 10 min following exposure to chemokine CCL19 (CXCL12 data is shown in Figure 1). Video microscopy was used to record migration on CD54 for 20 min before tracking and analysis. Bar-chart data show the mean speed of migration (μm/min) ± SEM of 6 patient samples. ** *P*<.01. (**B** and **C**) Median Fluorescence Intensity (MFI) analysis of CCR7 and CXCR4 receptors on peripheral blood CD3 T cells. Columns show the mean ± SEM from 14 patients. Non-significant findings are denoted by ns.

Fig. S2

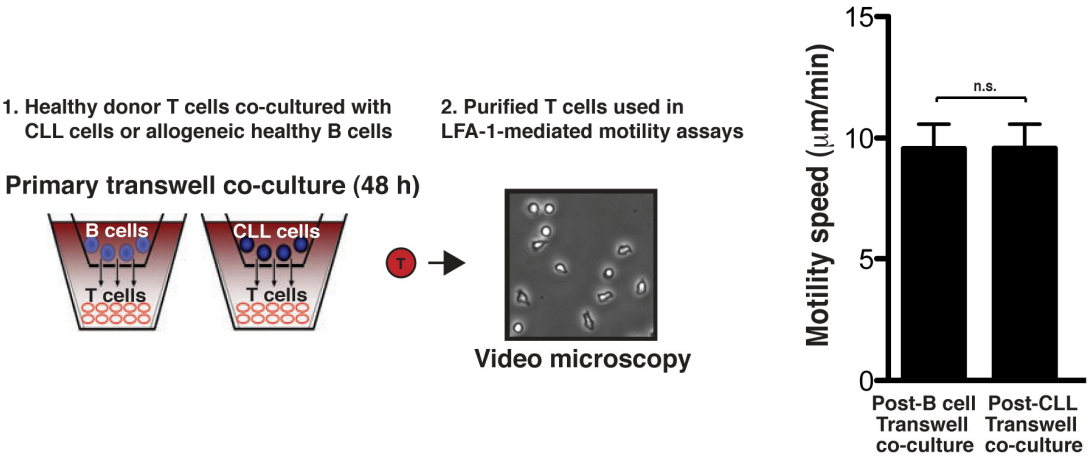

Figure S2. Primary transwell co-culture assays showed that tumor soluble factors alone did not induce the T cell LFA-1 motility defect. Schematic summary of two-part transwell co-culture functional assays. Healthy donor CD3 T cells were co-cultured (48 hours) with either CLL cells or third-party healthy B cells (control) in transwell culture plates and then subsequently used in migration assays. Video microscopy recorded migration on CD54 for 20 min before tracking and analysis. Bar-chart data are the mean motility speed (μm/min) ± SEM of 14 independent CLL patient experiments. Nonsignificant findings are denoted by ns. Original magnification x20.

Fig. S3

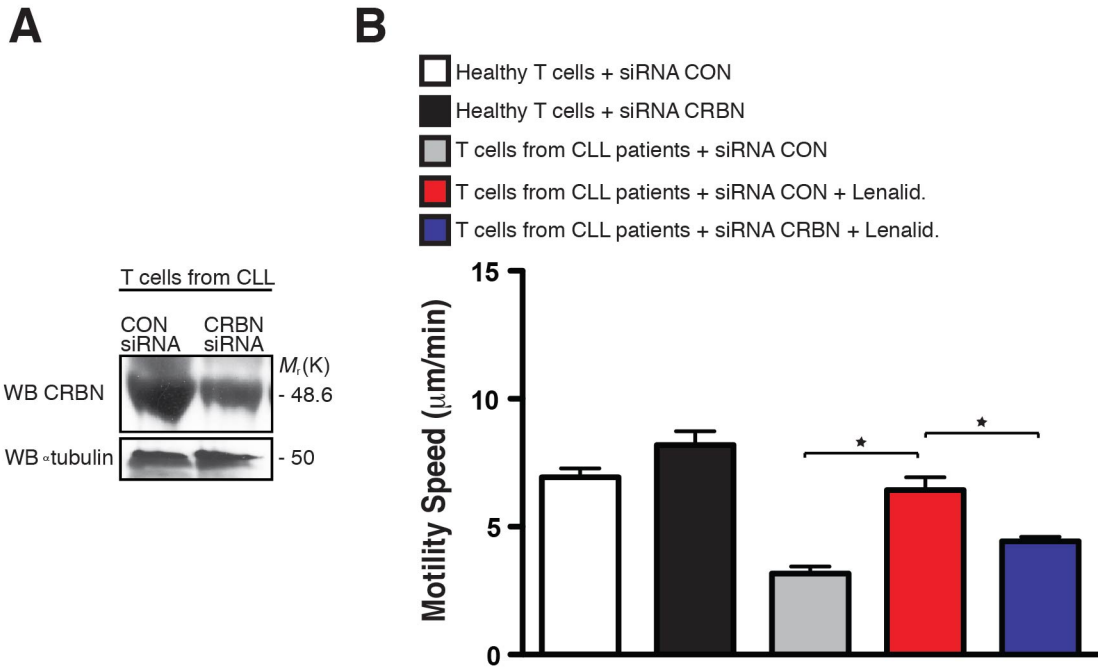

Figure S3. Cereblon is required for lenalidomide to repair the LFA-1 mediated motility defect in CLL patient T cells. Primary CD3 T cells (from CLL patients and age-matched healthy donors) were transfected (Amaxa) as indicated with control (CON) or cereblon (CRBN) siRNA SMARTpool-targeted reagents (Dharmacon) according to the manufacturer's protocol. (**A**) T cells from CLL patients transfected (24 hours) with CON or CRBN siRNA were analyzed by Western blotting (WB) with the indicated antibodies (anti-CRBN mAb, Sigma and anti-α-tubulin rabbit polyclonal, Abcam). Levels of CRBN expression were reduced by ~50% in primary T cells following 24 hour treatment with CRBN siRNA. (**B**) Transfected T cells were allowed to adhere to immobilized CD54 ligand for 10 min following exposure to chemokine CXCL12. Video microscopy was used to record migration on CD54 for 20 min before tracking and analysis. Bar-chart data show the mean speed of migration (μm/min) ± SEM of 3 donor or patient samples. * P<.05.

Fig. S4

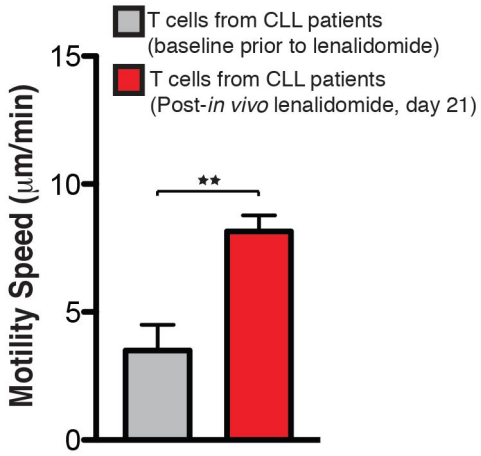

Figure S4. Lenalidomide in vivo treatment enhances patient T cell LFA-1-mediated motility. Primary CD3 T cells isolated from CLL patients receiving lenalidomide during a clinical trial were purified by negative selection and allowed to adhere to immobilized CD54 ligand for 10 min following exposure to chemokine CXCL12. Video microscopy was used to record migration on CD54 for 20 min before tracking and analysis. Bar-chart data are the mean motility speed (μm/min) ± SEM of 3 CLL patient samples. ** *P*<.01.

Fig. S5

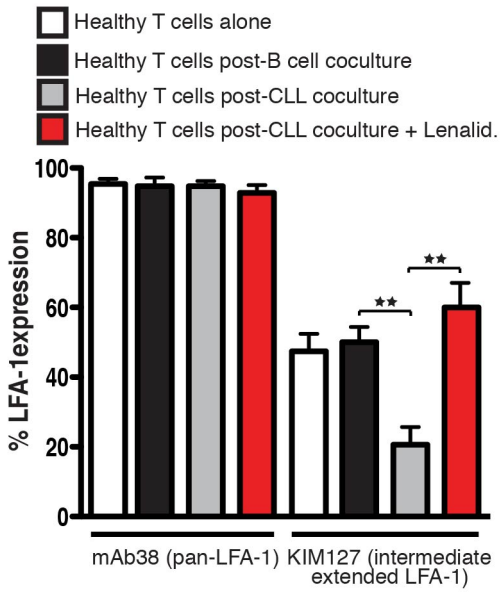

Figure S5. Direct CLL tumor contact causes defective expression of intermediate affinity LFA-1 on previously healthy T cells following chemokine stimulation. Percent positive flow cytometric expression analysis of pan-LFA-1 (mAb 38) and extended intermediate affinity LFA-1 (mAb KIM127) on chemokine CXCL12-treated healthy T cells alone or purified following co-culture (48 hours) with third-party healthy B cells, CLL cells or CLL cells and lenalidomide (columns show the mean ± SEM from 6 patients).

Fig. S6

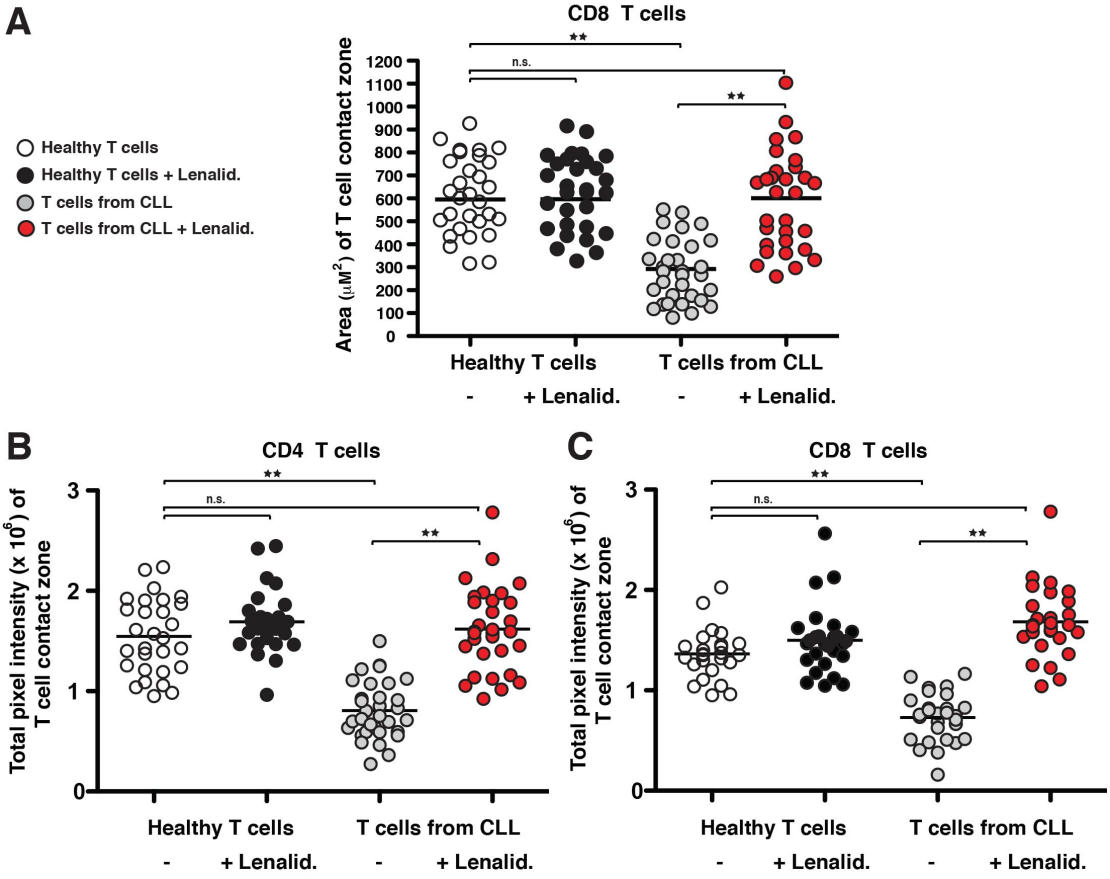

Figure S6. Lenalidomide treatment of T cells from CLL rescues their firm adhesion to CD54. IRM quantification of the (**A**) area (μM^2) and (**B** and **C**) strength (pixel intensity) of close cell contact points during LFA-1 directed motility on CD54 for n=30 T cells per experimental population as indicated (dot-plots are representative of 3 independent CLL patient experiments). T cells from CLL patients were compared to age-matched healthy donor cells treated with or without lenalidomide (Lenalid.). Nonsignificant findings are denoted by ns. ** *P*<.01.

Fig. S7

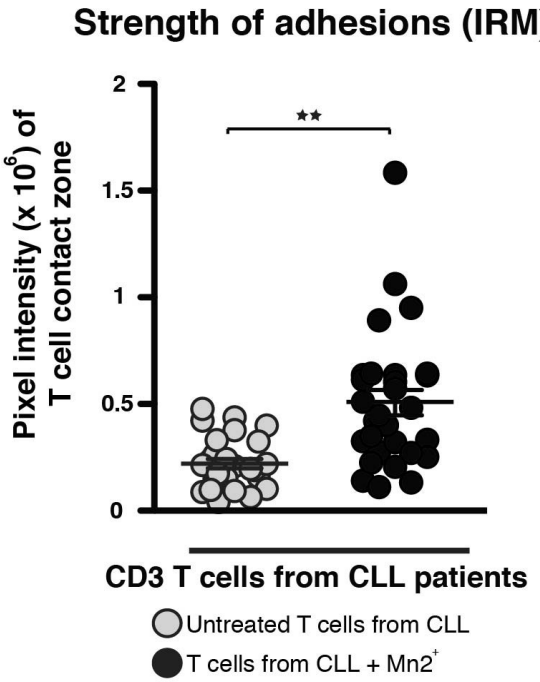

Figure S7. LFA-1 expressed on T cells from CLL patients has the potential to become activated if inside-out signaling is by-passed with manganese ($Mn2^+$) treatment. IRM quantification of the strength (pixel intensity) of T cell adhesions (contact points) on CD54 for n=30 T cells per experimental population as indicated (representative of 3 independent CLL patient experiments). Primary T cells from CLL patients were washed three times into HEPES buffer (20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose, pH 7.4) either with (+ $Mn2^+$) or without (Untreated) 20mM $MnCl_2$. T cells were then added to Ibidi µ-Slides VI coated with 3 µg/ml CD54-Fc and left for 10 minutes at 37 °C to allow adherence prior to imaging. ** *P*<.01.

Fig. S8

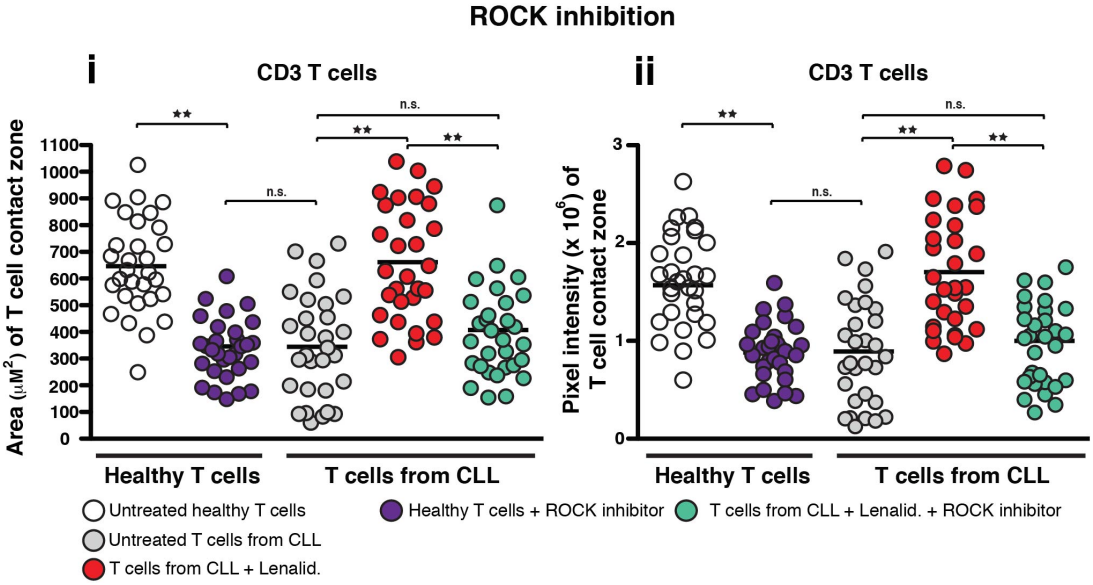

Figure S8. Lenalidomide targets Rho GTPase signaling in T cells from CLL patients. IRM quantification of the (**i**) area (μM^2) and (**ii**) strength (pixel intensity) of close cell contact points during LFA-1 directed motility on CD54 for n=30 T cells per experimental population as indicated. T cells from CLL patients were compared to age-matched healthy donors treated with or without a pharmacologic inhibitor targeting RhoA effector protein Rho kinase (ROCK) and co-treatment with or without lenalidomide (Lenalid.). Dot-plots are representative of 3 independent CLL patient experiments. Nonsignificant findings are denoted by ns. ** *P*<.01.

Fig. S9

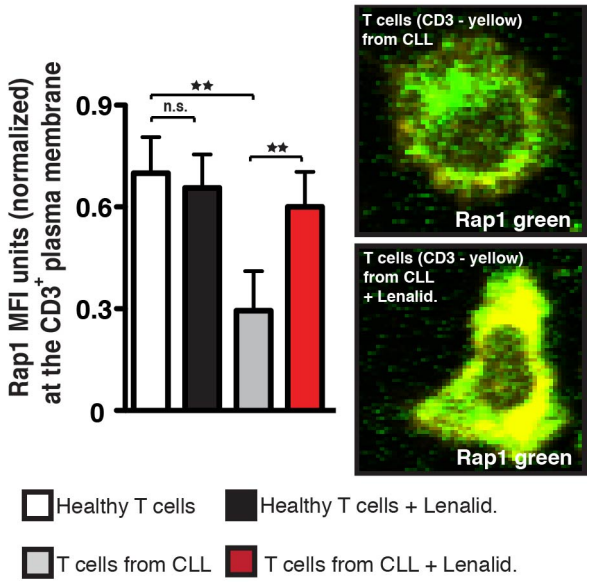

Figure S9. Lenalidomide rescues small GTPase Rap1 trafficking (localization) to the plasma membrane on mobile T cells. Normalized mean fluorescence expression (MFI) of Rap1 immunofluorescent staining at the $CD3^+$ T cell plasma membrane from 2 independent experiments (mean ± SD) examining untreated or lenalidomide (Lenalid.) treated T cells from CLL patients compared to age-matched healthy donors cells migrating on immobilized CD54. Representative images of Rap1 (green) and CD3 (yellow) staining visualized using confocal microscopy (with Alexa Fluor 488 or 647 secondary antibodies respectively) for untreated and lenalidiomide treated T cells from CLL patients are shown as indicated. Nonsignificant findings are denoted by ns. ** *P*<.01. Original magnification x63.