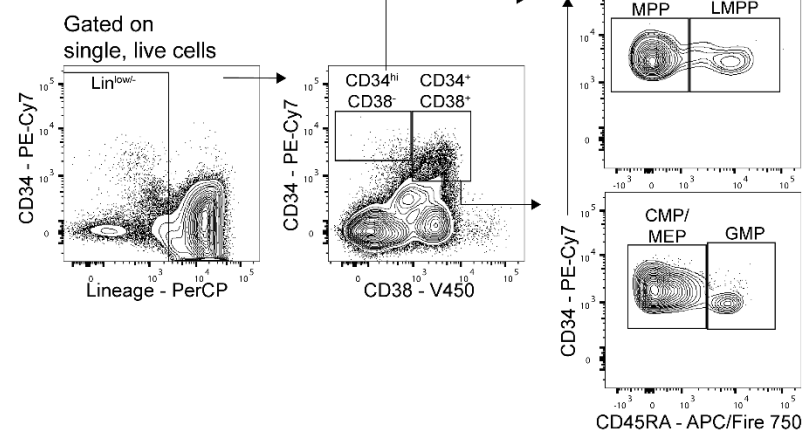**Supplemental Information**

Chronic lymphocytic leukemia

B-cell-derived TNFα impairs

bone marrow myelopoiesis

Bryce A. Manso, Jordan E. Krull, Kimberly A. Gwin, Petra K. Lothert, Baustin M. Welch, Anne J. Novak, Sameer A. Parikh, Neil E. Kay, and Kay L. Medina
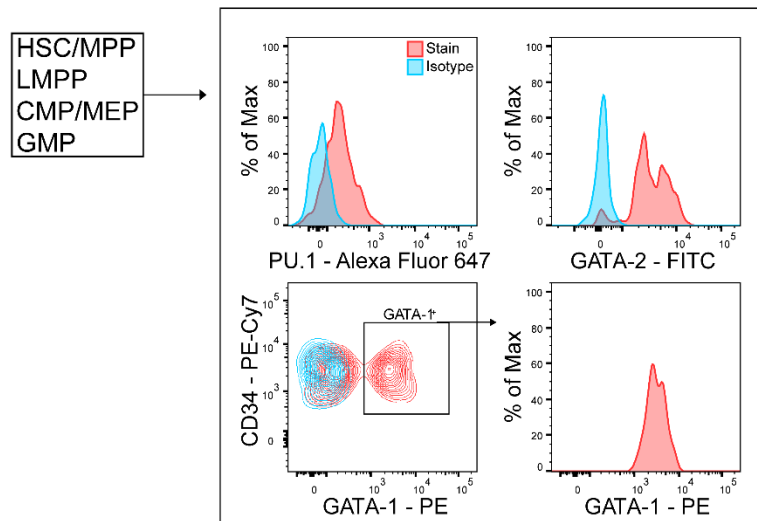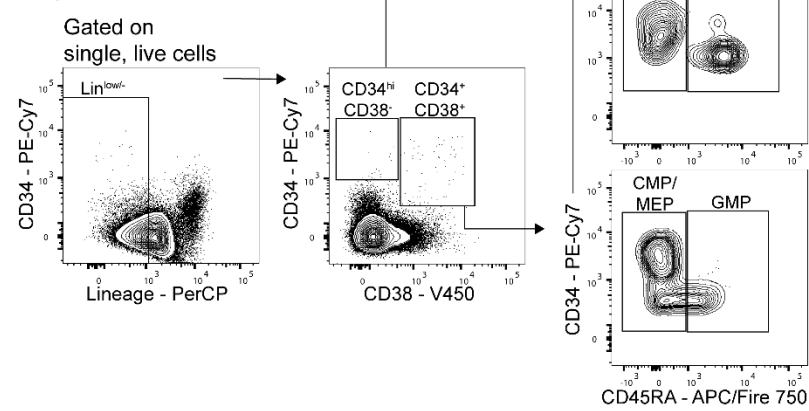
Figure S1. Flow cytometry gating strategy for *ex vivo* and *in vitro* HSPCs and transcription factors. Related to Figures 1-3. The sequential gating strategy used for analysis is illustrated using representative control and CLL *ex vivo* bone marrow samples. Following gating, each population was assessed for expression of PU.1, GATA-2, and GATA-1 as shown.

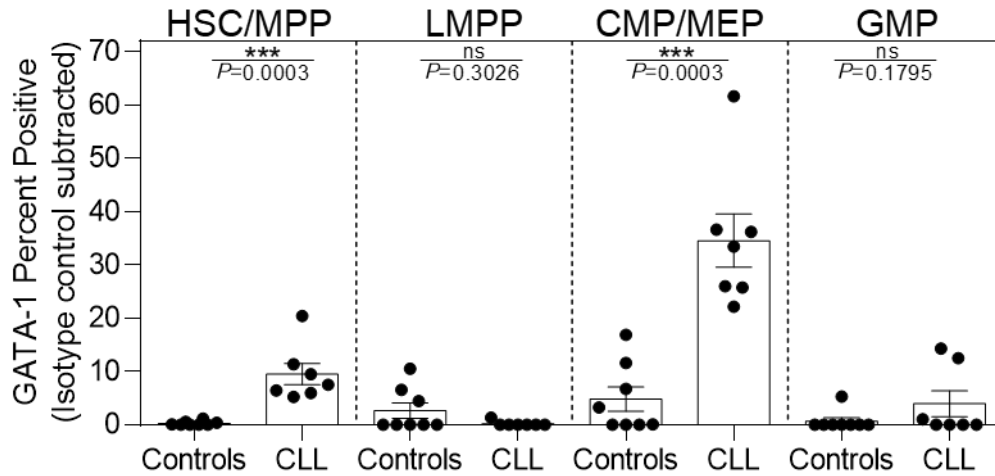**Figure S2. Frequency of GATA-1^{+} cells among HSPCs from control and CLL patient bone marrow.**
Related to Figure 1. Freshly isolated BM was evaluated by intranuclear flow cytometry for the frequency of GATA-1 positive cells among HSC/MPP, LMPP, CMP/MEP, and GMP populations. Each point represents individual donors and Data is presented as mean +/- SEM. Control n=8 and CLL n=7.
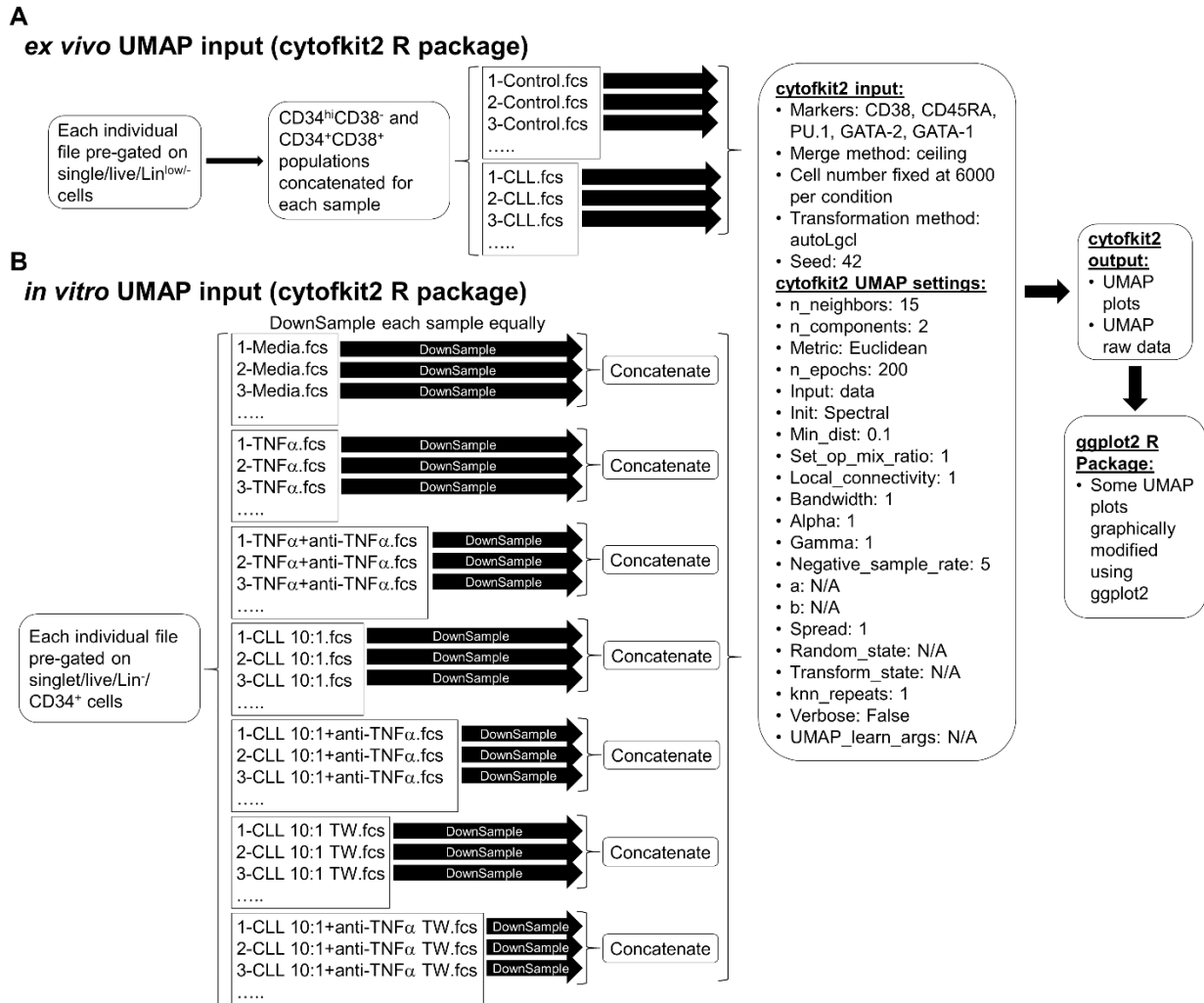***P<0.001 and ns by Mann-Whitney U test.

Figure S3. Experimental design and input for *ex vivo* and *in vitro* UMAP analysis. Related to Figures 1 and 4. (**A**) CD34^{hi}CD38^{-} and CD34^{+}CD38^{+} populations among the Lin^{low/-} compartment were concatenated in FlowJo and used as the raw data input for the *ex vivo* UMAP analysis. Data from the *ex vivo* flow cytometry analysis was exported from FlowJo and imported directly into the cytofkit2 R package with the settings and pipeline outlined. (**B**) Lin^{-}CD34^{+} cells were used as the raw data input for the *in vitro* UMAP analysis. Each individual *in vitro* data file was pre-processed in FlowJo by using the DownSample plugin to obtain files of 1000 Lin^{-}CD34^{+} cells. All data files from each individual *in vitro* condition was then concatenated and DownSampled to 6000 total cells prior to inputting into R using the cytofkit2 package and pipeline outlined. All UMAP graphical plots and raw data was generated and the raw UMAP data was further graphed using the ggplot2 R package or inputting the analyzed data back into FlowJo. Each UMAP figure is indicative of the combination (or specific subsets) of the total *ex vivo* or *in vitro* data.
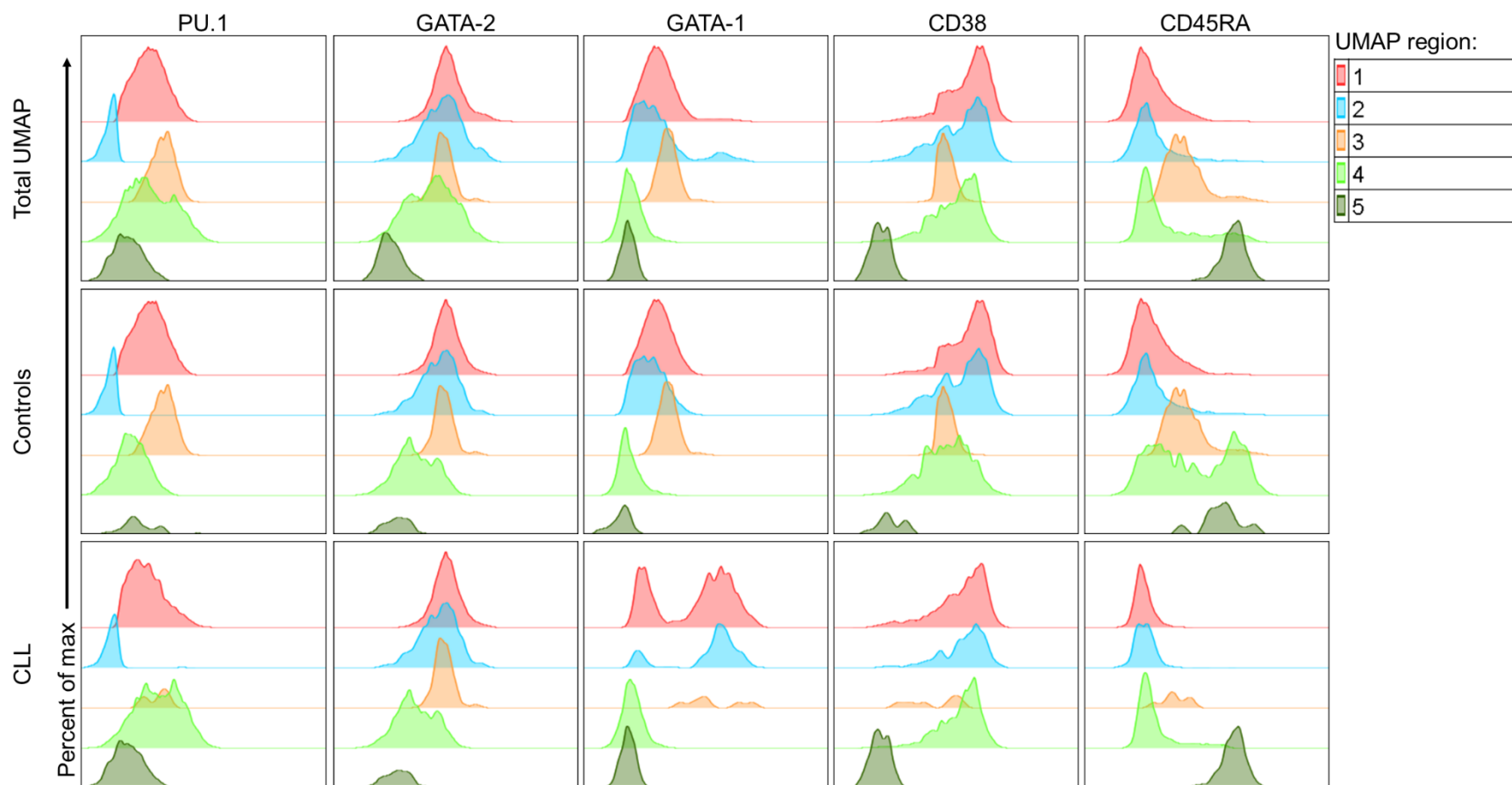
Figure S4. *ex vivo* UMAP marker expression patterns. Related to Figure 1. The analyzed *ex vivo* UMAP data from the cytofkit2 R package was imported back into FlowJo for further analysis. Each of the five individual regions were overlaid for the expression of the clustering markers. Each histogram represents the normalized expression data for each marker as calculated during the UMAP analysis. The histogram overlays are displayed as either the total UMAP (controls and CLL) or cells from the control or CLL cohorts only.
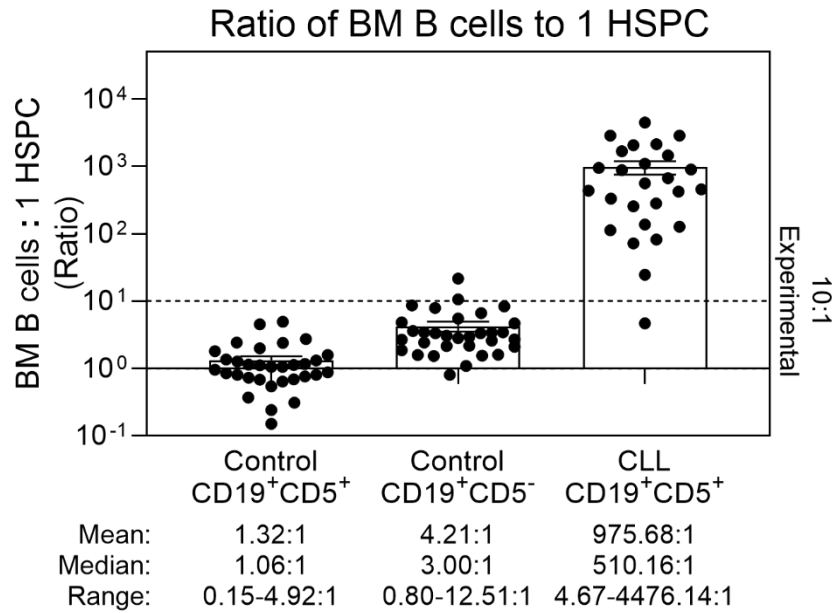
Figure S5. Determination of the *ex vivo* bone marrow B cell:HSPC BM ratio. Related to Figures 2-4. Freshly isolated control (n=32) or CLL (n=26) BM was analyzed by flow cytometry to determine the frequency of $CD19^+CD5^+$ and $CD19^+CD5^-$ B cells and Lin^-CD34^+ HSPCs among total BM cells. This data was generated from our previously published data set(Manso et al., 2019). The ratio for each individual sample (represented by a single point) was determined by dividing the frequency of either $CD19^+CD5^+$ or $CD19^+CD5^-$ B cells by the frequency Lin^-CD34^+ HSPCs. Data is presented as the mean +/- SEM.
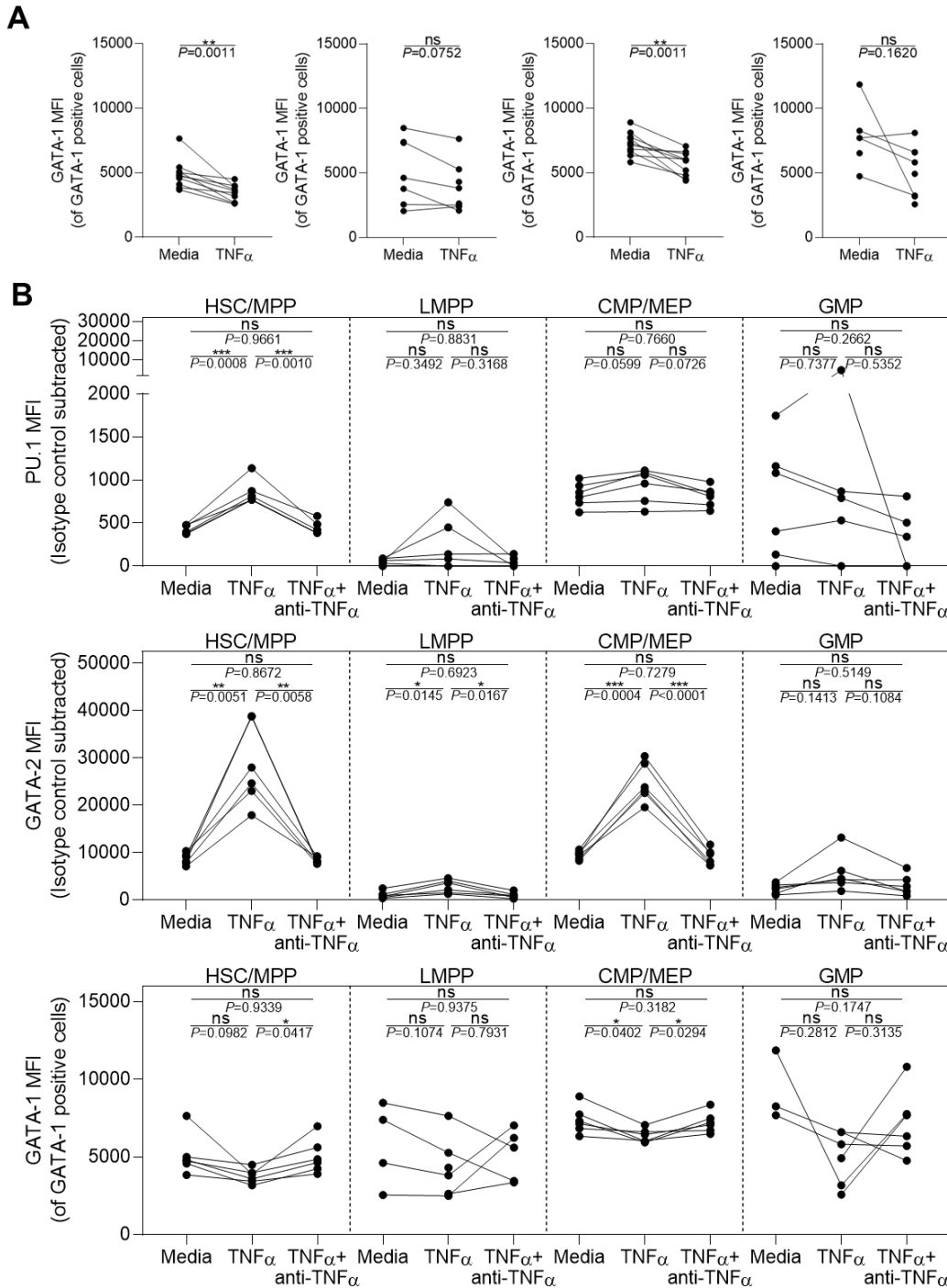
Figure S6. Effect of TNFα on transcription factor expression in HSPCs. Related to Figures 3 and 4.
(**A**) Freshly isolated control Lin⁻CD34⁺ HSPCs were exposed to TNFα as described in the Transparent Methods (n=10 or 6). Following 24 hours of culture, intranuclear flow cytometry was performed to determine the expression of GATA-1. **P<0.01 and ns by paired t-test. (**B**) Additional cultures were set up as in (A) and received a neutralizing antibody against TNFα (anti-TNFα) at the start of the experiment (n=6). Following 24 hours of culture, intranuclear flow cytometry was performed to determine the expression of PU.1, GATA-2, and GATA-1. Each point represents an individual donor with lines connecting the same donor across different experimental conditions. *P<0.05, **P<0.01, ***P<0.001, and ns by repeated measures one-way ANOVA adjusted for multiple comparisons.
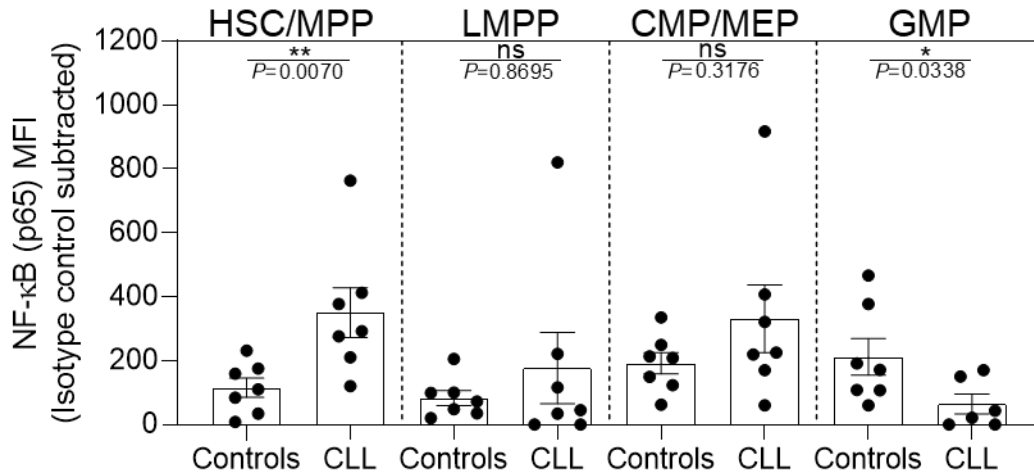
Figure S7. *ex vivo* analysis of NF-κB. Related to Figure 1. Freshly prepared BM from control and CLL patients were evaluated by intranuclear flow cytometry for expression of NF-κB p65 among HSC/MPPs, LMPP, CMP/MEPs, and GMPs. Each point represents individual donors and Data is presented as mean +/- SEM. Control n=7 and CLL n=7. *P<0.05, **P<0.01, and ns by Mann-Whitney *U* test.

Figure S8. Neutralization of TNFα normalizes transcription factor expression in CLL:HSPC co-cultures. Related to Figures 2-4. The HSC/MPP, LMPP, CMP/MEP, and GMP subsets were evaluated by flow cytometry for relative levels of PU.1, GATA-2, and GATA-1 protein levels (by MFI) between media controls and CLL+anti-TNFα cultures. All CLL co-cultures at a ratio of 10:1 (CLL cells:HSPCs). Evaluation of direct (**A**) and 1.0μm Transwell (**B**) co-cultures was performed. Each point and connecting line represents a unique pairing of a control HSPC donor and CLL patient (n=11 for direct co-culture and n=7 for TW co-cultures, 11 individual experiments). *P<0.05, **P<0.01, and ns by paired t-test.

Figure S9. IL-10 does not alter levels of PU.1, GATA-2, or GATA-1 in HSPCs or change colony forming unit (CFU) capacity. Related to Figures 2-4. (**A**) Freshly isolated control CD34+ HSPCs from BM were exposed *in vitro* to IL-10 for 24 hours. Following incubation, the HSPCs were analyzed by intranuclear flow cytometry for expression of PU.1, GATA-2, and GATA-1 as described in the Transparent Methods. Each point and connected line represents a unique donor (n=6). Some culture conditions did not contain detectable GATA-1 protein among GMPs and therefore are not represented in the graph. (**B**) Freshly isolated CD34+ HSPCs from control BM were plated in triplicate CFU assays. CFU assays were supplemented at assay initiation with 25 ng/mL IL-10 (n=5). Each point and connected line represents a unique donor. *P<0.05 and ns by paired t-test.

												Experimental use of samples:			
CLL ID	Age	Sex	Rai Stage	Cytogenetics	ZAP-70	CD38	CD49d	B2M	IGHV	Leukemic marrow involvement (%) – hematopathology	Sample tissue type	*ex vivo* TFs	*ex vivo* UMAP	*in vitro* co-culture	LTC-IC assay
CLL-1	66	M	0	Trisomy 12	+	-	+	+	UM	>90	BM	X			
CLL-2	68	F	I	13q-	-	-	-	+	M	10	BM	X			X
CLL-3	80	M	II	Trisomy 12	+	-	+	+	UM	90	BM	X			X
CLL-4	68	M	I	11q-	-	-	n.d.	+	UM	90	BM	X			X
CLL-5	58	M	I	13q-	+	-	-	+	UM	90	BM	X			X
CLL-6	58	F	III	Trisomy 12	+	-	n.d.	+	UM	80	BM	X			X
CLL-7	75	F	0	13q-	-	-	n.d.	+	UM	80	BM	X	X		
CLL-8	79	F	II	Trisomy 12	-	-	+	+	M	30	BM	X	X		
CLL-9	40	M	II	Trisomy 12	n.d.	+	n.d.	+	M	n.d.	BM	X	X		
CLL-10	63	F	III	13q-	+	-	+	+	UM	90	BM	X	X		
CLL-11	58	M	II	Trisomy 12	-	+	+	+	M	90	BM	X	X		
CLL-12	63	M	IV	13q-	+	+	+	+	UM	95	BM	X	X		X
CLL-13	53	M	0	13q-	+	-	-	+	UM	n.d.	BM	X	X		X
CLL-14	71	M	IV	Normal	-	-	+	+	M	<1	BM				X
CLL-15	56	M	0	17p-	+	+	-	+	UM	80	BM				X
CLL-16	41	M	I	Normal	+	-	+	+	M	20	BM	X	X		
CLL-17	74	M	0	13q-	-	-	-	-	M	n.d.	Blood			X	
CLL-18	67	M	0	Normal	-	-	-	-	M	n.d.	Blood			X	
CLL-19	69	F	III	13q-	-	-	-	+	M	90	Blood			X	
CLL-20	67	M	0	13q-	-	-		+	M	n.d.	Blood			X	
CLL-21	55	F	I	13q-	-	-	-	+	M	n.d.	Blood			X	
CLL-22	44	M	I	NA	-	-	-	+	UM	n.d.	Blood			X	
CLL-23	80	M	0	n.d.	-	-	-	+	M	n.d.	Blood			X	
CLL-24	71	M	0	Trisomy 12	+	-	+	+	UM	80	Blood			X	
CLL-25	92	F	0	13q-	-	-	-	+	M	n.d.	Blood			X	
CLL-26	80	M	0	Normal	-	-	-	+	M	n.d.	Blood			X	
CLL-27	82	M	0	Trisomy 12	-	+	-	+	UM	n.d.	Blood			X	

Table S1. CLL patient characteristics. Related to Figures 1-5. Demographics for each CLL patient are listed and the tissue source and list of experiments the sample was used for is indicated. B2M=beta 2 microglobulin, IGHV=immunoglobulin heavy chain variable region, M=mutated, UM=unmutated, +=positive, -=negative, BM = bone marrow, TF = transcription factor, and n.d.=no data.

Reagent or antibody specificity	Fluorochrome	Vendor	Catalogue Number	Clone	
Sample preparation reagents					
1X phosphate buffered saline (PBS) prepared from 10X stock	N/A	Gibco	14200-075	N/A	
Human Fc block	N/A	Miltenyi Biotec	130-059-901	N/A	
1% paraformaldehyde (PFA) prepared from 10% stock	N/A	Electron Microscopy Sciences	15712-S	N/A	
Cell culture reagents					
CellGenix GMP SCGM media	N/A	CellGenix	20802	N/A	
Recombinant human stem cell factor	N/A	PeproTech	300-07	N/A	
Recombinant human IL-6	N/A	PeproTech	200-06	N/A	
Recombinant human IL-3	N/A	PeproTech	200-03	N/A	
Recombinant human Erythropoietin	N/A	PeproTech	100-64	N/A	
Recombinant human TNFα	N/A	PeproTech	300-01A	N/A	
anti-human TNFα	N/A	R&D Systems	AF-210-NA	N/A	
Recombinant human IL-10	N/A	BioLegend	573202	N/A	
1.0 μm TransWell insert/plate	N/A	Corning	CLS3380	N/A	
MethoCult H4435 media	N/A	StemCell Technologies	04435	N/A	
MyeloCult H5100 media	N/A	StemCell Technologies	05150	N/A	
Hydrocortisone	N/A	StemCell Technologies	07904	N/A	
Cell Surface Markers					
Fixable Viability Stain 510	V510	BD Biosciences	564406	N/A	
CD3	PerCP	BD Biosciences	347344	Sk7	Lineage antibodies/ cocktail
CD5	PerCP	BioLegend	300618	UCHT2	
CD11b	PerCP	BioLegend	101230	M1/70	
CD14	PerCP	BD Biosciences	340660	MφP9	
CD19	PerCP	BD Biosciences	347544	4G7	
CD56	PerCP	BioLegend	318342	HCD56	
CD34	PE-Cy7	BD Biosciences	348791	8G12	
CD38	V450	BD Biosciences	646851	HB7	
CD45RA	APC/Fire 750	BioLegend	304152	HI100	
CD19	APC/Fire 750	BioLegend	363030	SJ25C1	
CD5	PerCP-Cy5.5	BD Biosciences	341089	L17F12	
Intranuclear staining with the True-Nuclear staining buffer kit					
True-Nuclear staining buffer kit	N/A	BioLegend	424401	N/A	
PU.1	Alexa Fluor 647	BioLegend	658004	7C6B05	
Mouse IgG1, κ isotype control (for PU.1)	Alexa Fluor 647	BioLegend	400136	MOPC-21	
GATA-2	FITC	R&D Systems	IC2046F	N/A	
Goat IgG isotype control (for GATA-2)	FITC	R&D Systems	IC108F	N/A	
GATA-1	PE	Cell Signaling Technologies	13353S	D52H6	
NF-kB p65	PE	Cell Signaling Technologies	9609S	D14E12	
Rabbit IgG isotype control (for GATA-1 and NF-κB p65)	PE	Cell Signaling Technologies	5742S	DA1E	

Table S2. List of reagents used. Related to Figures 1-5.

Transparent Methods

Experimental model and subject details
Human subjects
These studies are restricted to untreated CLL patients to eliminate confounding factors introduced by treatment. Fresh BM and/or blood samples were obtained from consenting CLL patients (Mayo Clinic IRB 1827-00, Table S1) and BM from age-matched controls either undergoing hip replacement surgery or recruited (Mayo Clinic IRBs 1062-00 and 16-006204) who signed informed consent per the Declaration of Helsinki and Mayo Clinic guidelines. BM samples were subjected to mature erythrocyte depletion by ACK lysis then used immediately. CLL blood samples underwent Ficoll separation immediately before use.

Cell lines
The murine stromal cell line M2-10B4 was obtained from ATCC and maintained in RPMI 1640 (Gibco) media supplemented with 10% fetal bovine serum (FBS, Mediatech), 1% penicillin/streptomycin (Gibco), and 1% L-glutamine (Gibco). The M2-10B4 cells were cultured in an incubator at 37°C and 5% CO_2.

Method details
Flow cytometry
Following isolation, cells were Fc blocked (Miltenyi Biotec, San Diego, CA, USA). Surface staining with monoclonal antibodies and fixable viability dye was performed on ice in 1X PBS for 30 minutes. Following washing, the cells were fixed and permeabilized for 60 minutes according to manufacturer's directions (BioLegend, San Diego, CA, USA) to allow for intranuclear staining. Cells were then stained for 30 minutes at room temperature with monoclonal antibodies against nuclear targets or appropriate isotype controls. Following staining, cells were washed and suspended in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for acquisition. When cell surface staining only was performed, cells were washed twice following staining and fixed with 1% paraformaldehyde for acquisition. Flow cytometry data was collected on a FACS-Canto (Becton Dickinson, Franklin Lakes, NJ, USA) that is standardized daily to allow for direct comparisons of mean fluorescent intensity (MFI) across separate experiments(Manso et al., 2019; Perfetto et al., 2006; Perfetto et al., 2012).

Flow cytometry analysis
For analysis of PU.1, GATA-2, and NF-κB p65 relative protein levels, the MFI of the stained sample was subtracted against the appropriate isotype control as expression is unimodal (Figure 1D and Figure S1). The isotype-subtracted MFI is the value used in the figures. GATA-1 staining is bi-modal (Figure 1D and Figure S1). Therefore, relative protein levels of GATA-1 was determined by first gating on GATA-1 positive cells (determined by the isotype control), then calculating the MFI of GATA-1 among only the cells that are GATA-1^{+}. This process results in no isotype control staining in the GATA-1 histogram analysis by virtue of the initial GATA-1^{+} determination. Flow cytometry analysis was performed with FlowJo 10.5.3 (Becton Dickinson).

***In vitro* cell cultures**
CD34^{+} cells from freshly processed control BM were isolated by positive magnetic bead selection (Miltenyi Biotec). CLL cells were plated at 1.0×10^6 cells per well with 1.0×10^5 CD34^{+} cells (10:1 ratio) in a 96 well round-bottom plate. The CLL:CD34^{+} co-cultures were incubated undisturbed for 24 hours at 37°C 5%CO_2 in serum-free media (CellGenix, Portsmouth, NH, USA) supplemented with human recombinant stem-cell factor (10ng/mL), IL-6 (10ng/mL), IL-3 (1ng/mL), and erythropoietin (1ng/mL) (all from PeproTech, Rocky Hill, NJ, USA). Transwell (Corning, Corning, NY, USA) assays utilized a 1.0μm insert. CLL cells were plated in the Transwell with CD34^{+} cells in the lower chamber. Where indicated, 0.8μg of anti-TNFα antibody (R&D Systems, Minneapolis, MN, USA) was added to the cultures (in the lower chamber for Transwell experiments) at assay initiation. Recombinant human TNFα (R&D Systems) or IL-10 (BioLegend), when utilized, was added at 25ng/mL(Asano et al., 1999; Grigorakaki et al., 2011; Xiao et al., 2002) . In all cases, cells were stained for flow cytometry as outlined above to allow for detection of surface and intranuclear proteins.

Uniform manifold approximation and projection (UMAP)
The R program (version 3.6.1)(R Core Team, 2019) and the cytofkit2 package (Jinmiao Chen's Lab, version 2.0.1)(Becher et al., 2014; Chen et al., 2016; Wong et al., 2015) were used to generate UMAP(Leland McInnes, 2018) data and graphics plots from *ex vivo* and *in vitro* flow cytometry data. The single/live/Lin$^-$ CD34^{hi}CD38$^-$ and CD34$^+$CD38$^+$ populations from each individual *ex vivo* sample was concatenated in FlowJo and exported whereas each *in vitro* flow cytometry data file was gated and exported on single/live/Lin$^-$/CD34$^+$ cells. The *ex vivo* data was imported into cytofkit2 with the parameters outlined in Figure S3A. Due to increased numbers of *in vitro* conditions/samples, additional data preprocessing was performed (Figure S3B). Some UMAP plots underwent additional graphical modifications with the ggplot2 package to allow for color selection and identical axis scaling across plots(Wickham, 2016).

Long-term culture initiating cell (LTC-IC) assay
Fresh BM CD34$^+$ cells from controls and CLL patients were isolated as above and plated across 12 or 36 replicates in LTC-IC medium (H5100 MyeloCult with 10^{-6}M hydrocortisone, StemCell Technologies, Vancouver, Canada). Cells were plated at limiting dilution (1000, 100, 50, 25, 10, 5, and 1 cell(s) per well) on irradiated feeder M2-10B4 stromal cells (12,500 cells per well) in 96 well flat-bottom plates. The cultures were maintained for five weeks at 37°C 5%CO$_2$ with weekly half-media exchange and scoring for the presence or absence of one or more colonies per well. In experiments containing TNFα, a limited cell dilution range was utilized (1000, 100, 50, and 10) with 12 replicates per dilution. Fresh media with the indicated concentration of TNFα was added weekly to the cultures to mimic *in vivo* chronic exposure.

Limiting dilution analysis (LDA)
Results from the LTC-IC assays containing control or CLL CD34$^+$ cells were analyzed by LDA (http://bioinf.wehi.edu.au/software/elda/)(Hu and Smyth, 2009). The confidence interval was set at 0.95, with options for testing inequality in frequency between multiple groups and adequacy of the single-hit model selected as analysis parameters.

Colony forming unit (CFU) assays
Following isolation of CD34$^+$ cells from freshly processed control BM, CFU assays were performed by plating triplicates of 500 CD34$^+$ cells in semisolid MethoCult H4435 media (StemCell Technologies). This media formulation allows for differentiation and enumeration of CFU-granulocyte, monocyte (CFU-GM), CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), and CFU-erythroid (CFU-E) colonies by morphology. When utilized, recombinant human TNFα was added at 25 ng/mL(Asano et al., 1999; Grigorakaki et al., 2011; Xiao et al., 2002). Neutralization of TNFα was achieved by addition of 0.8 μg of an anti-TNFα antibody (R&D Systems). The CFU cultures were incubated at 37°C 5% CO$_2$ for 11-12 days then manually scored, counted, and averaged across triplicates.

Quantification and statistical analysis
Statistical tests are indicated in figure legends and performed using GraphPad Prism version 8.1.2 (GraphPad Software, San Diego, CA, USA). For all tests, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and "ns" indicates non-statistical significance. Note that, as some *ex vi*vo and *in vitro* conditions had no detectable GATA-1, some paired t-tests are missing data for one or multiple conditions. When statistical analysis was performed, only values with data in each column were used for statistical comparison, as is standard practice. A power calculation was not performed owing to limited sample availability. Bar graphs of summary statistics indicate the mean ± standard error of the mean (SEM). The number of individual experiments and samples are outlined in the figure legends.

Supplemental references

Asano, Y., Shibata, S., Kobayashi, S., Okamura, S., and Niho, Y. (1999). Effect of interleukin 10 on the hematopoietic progenitor cells from patients with aplastic anemia. Stem Cells *17*, 147-151.

Becher, B., Schlitzer, A., Chen, J., Mair, F., Sumatoh, H.R., Teng, K.W., Low, D., Ruedl, C., Riccardi-Castagnoli, P., Poidinger, M.*, et al.* (2014). High-dimensional analysis of the murine myeloid cell system. Nat Immunol *15*, 1181-1189.

Chen, H., Lau, M.C., Wong, M.T., Newell, E.W., Poidinger, M., and Chen, J. (2016). Cytofkit: A Bioconductor Package for an Integrated Mass Cytometry Data Analysis Pipeline. PLoS Comput Biol *12*, e1005112.

Grigorakaki, C., Morceau, F., Chateauvieux, S., Dicato, M., and Diederich, M. (2011). Tumor necrosis factor alpha-mediated inhibition of erythropoiesis involves GATA-1/GATA-2 balance impairment and PU.1 over-expression. Biochem Pharmacol *82*, 156-166.

Hu, Y., and Smyth, G.K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods *347*, 70-78.

Leland McInnes, J.H. (2018). UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. Preprint at https://arxivorg/abs/180203426.

Manso, B.A., Zhang, H., Mikkelson, M.G., Gwin, K.A., Secreto, C.R., Ding, W., Parikh, S.A., Kay, N.E., and Medina, K.L. (2019). Bone marrow hematopoietic dysfunction in untreated chronic lymphocytic leukemia patients. Leukemia *33*, 638-652.

Perfetto, S.P., Ambrozak, D., Nguyen, R., Chattopadhyay, P., and Roederer, M. (2006). Quality assurance for polychromatic flow cytometry. Nat Protoc *1*, 1522-1530.

Perfetto, S.P., Ambrozak, D., Nguyen, R., Chattopadhyay, P.K., and Roederer, M. (2012). Quality assurance for polychromatic flow cytometry using a suite of calibration beads. Nat Protoc *7*, 2067-2079.

R Core Team (2019). R: A language and environment for statistical computing (Vienna, Austria: R Foundation for Statistical Computing).

Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wong, M.T., Chen, J., Narayanan, S., Lin, W., Anicete, R., Kiaang, H.T., De Lafaille, M.A., Poidinger, M., and Newell, E.W. (2015). Mapping the Diversity of Follicular Helper T Cells in Human Blood and Tonsils Using High-Dimensional Mass Cytometry Analysis. Cell Rep *11*, 1822-1833.

Xiao, W., Koizumi, K., Nishio, M., Endo, T., Osawa, M., Fujimoto, K., Sato, I., Sakai, T., Koike, T., and Sawada, K. (2002). Tumor necrosis factor-alpha inhibits generation of glycophorin A+ cells by CD34+ cells. Exp Hematol *30*, 1238-1247.