Supplement

Supplementary Figure Legends

Figure S1.

A. L1236 SUP was collected and chromatographic fractionation was performed using a Q and a Heparin Sepharose column. Red represents the absorption at 260 nm, blue the absorption at 280 nm and green shows the salt concentration used for elution. **B.** Fractions were TCA precipitated and run on an SDS-PAGE gel. The gel was stained with Imperial protein stain. FQ was the flow-through of the Q Sepharose, FH was a wash fraction of the heparin column. Fractions 1-5 resulted from the gradient salt elution and fraction 6 was the final 2 M NaCl elution. Fractions 1 and 2 contained the NF-κB activating factor, whereas Fraction 6 was used as a control fraction. **C**. $5x10^5$ cells/ml of the indicated cell lines (blue, cHL; black, Burkitt lymphoma, pre-B-cell leukemia and DLBCL) were seeded in 24-well plates, culture medium was collected after 24 h and LTA amounts were determined by ELISA.

Figure S2.

A. Cytoplasmic (C) or nuclear (N) extracts of cHL cell lines were analyzed by Western blotting for cellular localization of NF-κB subunits, as indicated. LDH-A and PARP1 served as fractionation and loading controls. **B**. $3.5x10^5$ L1236 cells/ml were cultured for 24 h. Medium was then removed, filtered (SUP) and used to treat HeLa cells for indicated time points. Protein localizations in C and N extracts were determined by Western blotting and global NF-κB DNA binding activity was determined by EMSA. Bottom panel, DNA binding activity of NF-κB subunits was determined at two different time points by supershift assay, as indicated. IgG, isotype control. **C.** HeLa cells were treated with 10 ng/ml LTA for 15 min or 24 h. Cytoplasmic (C) and nuclear (N) extracts were analyzed for subcellular localization of NF-κB subunits by Western blotting. Bottom panel, supershift of NF-κB subunits was performed.

Figure S3.

A. Cell surface expression of TNFRSF1A, **B.** TNFRSF1B and **C.** TNFRSF14 on the cHL cell line panel as well as control cell lines was assessed by flow cytometry (FC). Filled curves represent the isotype control; black curves the receptors. Representative histograms of three experiments are shown.

Figure S4.

A. Semi-quantitative PCR was performed with cDNA of indicated cell lines. Expression of mRNA of *LTB*, *LTBR* and *BTLA* was determined. *RPL13A* served as control. Indicated cell lines were analyzed by FC for cell surface expression of **B.** BTLA, **C.** CD160 or **D.** LTBR, as indicated. Filled curves represent the isotype control; black curves the receptors. Representative histograms of three experiments are shown.

Figure S5.

A. left, Knockdown of *NFKB1*/*RELA,* or right, *NFKB2*/*RELB* was performed in L1236 cells. Total RNA was isolated and the cDNA was analyzed by RT-qPCR for expression of the respective NF-κB subunits, *LTA*, *TNFRSF14*, *TNFRSF1A* and *TNFRSF1B*. Error bars represent the standard error of the mean (SEM). **B.** Expression levels of negative regulators of the NF-κB system in cHL cell lines were determined by Western blot analysis as indicated. LDH-A was used as loading control. **C.** L1236 cells were transduced with either pWPI control vector or A20. LTA mRNA expression was determined using RT-qPCR. Error bars represent SEM.

Figure S6.

A. The CRISPR/Cas9 system was used to create stable L1236 LTA-KO cell lines. Two guideRNAs specific for LTA were designed, targeting either the second or third exon. Thin lines, introns; medium lines, non-coding exons; thick lines, coding exons; white triangles, transcription direction. **B.** GSEA was performed comparing the LTA regulated gene list with

the Molecular Signature Database v6.1. Enrichment plots for hallmarks 'TNF_signaling_via_NF-κB', 'Inflammatory_response' and 'IL6_JAK_STAT3_Signaling' are shown. NES, Normalized Enrichment Score; FDR, False Discovery Rate.

Figure S7.

A. Following 72 h of cultivation without conditioned medium, RNA was isolated from L1236 wildtype, control (v2) and *LTA* KO clonal cells to determine mRNA expression of NF-κB subunits. Error bars represent SEM. **B.** DNA binding activity of STAT6 in L1236 WT and *LTA* KO cells was analyzed by EMSA using the *CCL2* promoter site. Supershift analysis was performed using anti p-STAT6 antibody and IgG isotype control in L1236 wildtype cells.

Figure S8

Graph showing percentage of dead cells as quantitated (blind count from two independent experiments) with Acridine orange/Ethidium bromide staining. Graph shows cells staining orange/red corresponding to dead cells as a percentage of total cells, staining blue/green. UT – untreated cells. IR – cells irradiated with 20 Gy and harvested 48 hours later. P value determined by two tailed T test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure S9.

A. The indicated cells were cultured for 72 h without conditioned medium. RNA was then extracted and subjected to RT-qPCR to determine the expression levels of the shown genes. Error bars represent SEM. **B.** L1236, control, g2_1 and g3_4 cells were cultivated at a cell density of $5x10^5$ cells/ml in RPMI without FCS. The medium was collected after 24 h and cytokines secretion was analyzed using the Cytokine Array C3. RMPI was used as a medium control.

Figure S10.

High-resolution figure of the co-expression analysis of LTA target genes in healthy PBMCs (1). For further details see Figure legend 2E. In addition, this figure contains gene names and a heatmap showing the percentage of cells in the cell population which express the corresponding gene. Figure can be zoomed in.

Supplementary Table Legends

Supplementary Table 1

Extracellular proteins enriched in Fraction 1 and 2 identified by mass spectrometry analysis

Supplementary Table 2

LTA regulated gene expression

Supplementary Table 3

ENRICHR significant Cancer Cell Line Encyclopedia dataset

Supplementary Materials and Methods

Cell lines, culture conditions and treatment

L1236, L428, KM-H2, Reh, Namalwa and HBL1 cells were cultured in RPMI (Gibco) with 10% heat-inactivated (h-i) fetal calf serum (FCS; Gibco). HDLM-2, L540 and Ramos were cultured with RPMI supplemented with 20% h-i FCS. SUP-HD1 cells were grown in McCoy5a medium (Gibco) with 20% h-i FCS and U-HO1 in IMDM/RPMI (4:1) medium (Gibco) supplemented with 20% h-i FCS and 2 mM L-glutamine. HeLa and HEK283T cells were cultured in DMEM (Gibco) supplemented with 10% FCS.

Cells were treated with 10 ng/ml LTA (Peprotech) for indicated time points. To analyze the effect of cell culture supernatant, $3.5x10^5$ cHL cells/ml were cultured in normal culture medium for 24 h. Cells were then removed by centrifugation and the supernatant was filtrated through a 0.2 µm Minisart syringe filter (Sartorius Stedim). Filtered supernatant (SUP) was then used to treat HeLa cells for indicated time points. To analyze to impact of LTA on NF-κB activation, L1236 cells were treated with 80 µg/ml LTA neutralizing antibody (359-81-11, sc-32767 L) or IgG_1 control (554721, BD Pharmingen) for 24 h. SUP was then used to treat HeLa cells. 100 µg/ml of SUP Enbrel (Pfizer) was used to treat HeLa cells, when SUP was added. Human Fc (110-HG-100, R&D Systems) was used as a control.

Acridine orange/Ethidium bromide staining of cells for cell death analyses were performed according to the protocol of Kasibhatla, S. et al. (2006) *Cold Spring Harb Protoc* http://cshprotocols.cshlp.org/content/2006/3/pdb.prot4493.citation

Cell extracts and Western blots

For preparation of cytoplasmic (C) and nuclear (N) extracts, cells were washed with PBS and incubated for 15 min on ice with Buffer A (10mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.4 mM Pefabloc, 20 mM β-glycerophosphate, 10 mM NaF and complete

protease inhibitor cocktail tablets (Roche)). Samples were then centrifuged and the pellet was resuspended in Buffer A supplemented with 0.3% Nonidet P-40 to obtain the cytoplasmic extract. Samples were washed in Buffer A and resuspended in Buffer C (20 mM Tris-HCl (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.4 mM Pefabloc, 20 mM β-glycerophosphate, 10 mM NaF, complete protease inhibitor cocktail tablets (Roche) and 0.5% Nonidet P-40) and incubated for 30 min at 4°C. To remove cell debris and obtain nuclear extracts, lysates were centrifuged for 10 min at 14000 rpm.

20 µg of whole-cell extract, cytoplasmic or nuclear extract were used for Western blot as previously described (2). The following antibodies were used: PARP1 (F2, sc-8007), p65 (F6, sc-8008), p50 (H119, sc-7178), RelB (C1E4, 4922, Cell Signaling), IκBα (C-21, sc-371), p52 (05-301, Millipore), LTA (E6, sc-28345), pIKK (2681, Cell Signaling), LDH-A (N-14, sc-27230) and pSMAD2/3 (3101, Cell Signaling). Blots were analyzed by chemiluminescence using the Fusion Solo (Vilber). ECL was used following manufacturer's protocol (Millipore).

Electrophoretic Mobility Shift Assay (EMSA) and supershift analysis

For EMSA, the H2K (5'- gatcCAGGGCTGGGGATTCCCCATCTCCACAGG -3') oligonucleotide was labeled to detect NF-κB DNA binding activity and the CCL2 promoter oligonucleotide (5'- gatcCCTTTCCTACTTCCTGGAAATCCACAGGA -3') was labeled to detect STAT6 DNA binding activity. A total of 3 µg protein was incubated with 2 µg poly(dI-dC), 1 µg BSA, 5 mM DTT, 20 mM HEPES (pH 8.4), 60 mM KCl and 8 % Ficoll for 30 min at RT. The following antibodies were used for supershift: p65 (C20, sc-372), p50 (H119, sc-7178), p52 (05-301, Millipore), RelB (C19, sc-266, Santa Cruz) and p-STAT6 (pY641.18, sc-13601) or the isotype controls: Rabbit IgG (Sigma), Goat IgG (sc-2028) and Mouse IgG (BD).

Chromatin immunoprecipitation (ChIP) assays

For ChIP, cells were fixed using 1 % formaldehyde for 5 min at RT and lysed with 50 mM Tris-HCl, pH 8.0 / 5mM EDTA / 1 % SDS. Samples were then sonicated with the Bioruptor Plus (Diagenode; 13 cycles, intensity High, sonication 30 s/break 30 s per cycle). Chromatin

was pre-cleared with BSA-saturated Protein A-sepharose and incubated overnight at 4 °C with the p52- or RelB-specific antibody (concentration as indicated by the manufacturer). Immuno-complexes were collected with BSA-saturated Protein A-sepharose for 1 h at 4 °C. The protein-DNA complexes were washed and then eluted using 1 % SDS / 0.1 M $NaHCO_3$. Reversion of the cross-linking, RNAse treatment, proteinase K digestion, and DNA purification by phenol-chloroform extraction were performed according to standard protocols.

Enrichment of genomic regions was determined by quantitative PCR (qPCR) using the CFX96 Real Time System (Bio-Rad) and GoTaq® qPCR Master Mix (Promega). ChIP-DNA was used corresponding to 3 x 10^5 cell equivalents with 4 ng of input DNA. All samples were analyzed in triplicates and values were normalized to non-recruiting intergenic regions on chromosome 4 and 12. The normalized enrichment (ΔΔCq) was calculated by the CFX manager software (Bio-Rad). Primer sequences are shown below:

Gene	Acc.Nr.	sequence (5' → 3')	Primer Position (TSS=1)	Efficiency (%)
Reference	GRCh37:4:58100351-58100438	TGCAGGGGCAAGCATATTCA GGAGATAAAGCTGGGCGACA	na	94,4
Reference	GRCh37:12:38676107-38676214	TGCCTTAGTTTTGTTGGTCCT GTTGGGAGAAAAATGCACGGTTA	na	101,4
LTA	GRCh37:6:31539831:31542101:1	CACGATTCCCCTGACCCGAC AGGAGAGAACCACAGGCTGG	-8 -78	95,5
TNFRSF14	GRCh37:1:2487078:2496821:1	AGGGCCCCTTTATTCGGCAA CCCCACCTCACTCACACACT	-99 -179	90,7

RNA isolation, cDNA synthesis and quantitative RT-PCR (RT-qPCR)

RNA extraction was performed according to manufacturer's protocol using RNeasy kit (Qiagen). cDNA was produced using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's protocol.

RT-qPCR was run using the CFX96 Real Time System (Bio-Rad) and GoTaq® qPCR Master Mix (Promega). The normalized expression (ΔΔCq) and primer efficiencies were calculated by the CFX manager software (Bio-Rad). RT-qPCR results are shown as mean, with the error bar representing the SEM. Primer sequences are shown below:

Gene name	Accession no.	Sequence (5'->3')	position (TSS=1)	Efficiency (%)
HPRT1	NM_000194.2	TGTAATGACCAGTCAACAGGG	483	
		GGATTATACTGCCTGACCAAGG	631	101,5
RPL13A	NM_012423.2	AAAGCCAAGATCCACTACCG	509	
		GGAATTAACAGTCTTTATTGGGCTC	657	105,7
RELA	NM_021975.3	CCTGTCCTTTCTCATCCCATC	668	
		ACCTCAATGTCCTCTTTCTGC	816	87,3
RELB	NM_006509.3	CTACAACGCTGGGTCCCTGA	878	
		TAGACGGGCTCGGAAAGCAC	1009	103,9
REL	NM_002908.2	ATGGCCTCCGGTGCGTATAA	225	
		GGAATGCTGCCTGCTGATCG	331	106,8
NFKB1	NM_003998.3	CATCCACCTTCATTCTCAACTTG	2123	
		ACCACATCTTCCTGCTTAGTG	2263	100,0
NFKB2	NM_002502.3	CCGGATCTCGCTCTCCACCGGA	65	
		GGGCTAGGCCCGGCTCTGTCTA	188	86,2
NFKBIA	NM_020529.2	GAGGACGAGCTGCCCTATGA	1008	
		AGCCCCTTTGCGCTCATAAC	1077	98,9
LTA	NM_000595.3	CATGACACCACCTGAACGTC	162	
	NM_001159740.2	TGCTCAAGGAGAAACCATCC	448	87,1
TNFRSF14	NM_003820.2	AGGACGAGTACCCAGTGG	430	
		ACTTGCTTAGGCCATTGAGG	576	91,5
TNFRSF1A	NM_001065.3	GCCCCTGGTCATTTTCTTTG	945	
		GTAGTTCCTTCAAGCTCCCC	1091	100,8
TNFRSF1B	NM_001066.2	CTGGCGACTTCGCTCTTCCA	853	

		AAGTGAGGCACCTTGGCTTCT	997	100,0
BCL2L1	NM_138578.1	GCGGGGCACTGTGCGTGGAA	806	
		AAGTATCCCAGCCGCCGTTCTCCT	937	103,5
TXNIP	NM_006472.3	ACCCCAGAAGCTCCTCCCTGCT	1320	
		GCAGAGGAGTGGTTGGGCTCTCCA	1395	98,6
TNF	NM_000594.3	CTCTAATCAGCCCTCTGGCCC	378	
		CAGCTTGAGGGTTTGCTACAACA	470	98,9
CSF2	NM_000758.3	GGCGTCTCCTGAACCTGAGT	151	
		GTCGGCTCCTGGAGGTCAAA	214	105,2
CCL22	NM_002990.4	GAAACACTTCTACTGGACCTC	184	
		GCACAGATCTCCTTATCCCT	264	84,5
CCL5_1	NM_002985.2	ACACCCTGCTGCTTTGCCTA	159	
		CGGGTGACAAAGACGACTGC	268	88,4
CCL5_2	NM_001278736.1	AGCAGTCGTCCACAGGTCAA	248	
		CTTGGCGGTTCTTTCGGGTG	364	101,6
CXCL10	NM_001565.3	CCAGAATCGAAGGCCATCAAGA	334	
		TCGATTTTGCTCCCCTCTGGT	424	106,8
CD40	NM_001250.4 NM_152854.2	GAGAGCCCTGGTGGTGATCC	653	
		GGCTTCTTGGCCACCTTTTTGAT	742	96,8
CD80	NM_005191.3	CTGGGAAGTGCCCTGGTCTT	325	
		TCACGTGGATAACACCTGAACAGA	510	94,8
CD44	NM_001001390.1 NM_001001391.1 NM_001202555.1 NM_001202556.1	TACATCCTCACATCCAACACC	747	
		GTGCCATCACGGTTAACAATAG	892	97,9
CCR4	NM_005508.4	CTTCCTGAGCAAGCCTGG	55	
		GTGTCTGCTATATCCGTGGG	194	113,3
ICAM1	NM_000201.2	CCATCTACAGCTTTCCGGCG	1236	
		GGTGGGCCTCACACTTCACT	1325	106,3
VCAM1	NM_001078.3 NM_080682.2 NM_001199834.1	TGGAAATGACCTTCATCCCTACCA	1618	
		TCTCTGGGGGCAACATTGACA	1759	84,6

Semi-quantitative PCR

Semi-quantitated PCR was performed using TrueStart™ Hot Start *Taq* DNA Polymerase according to manufacturer's instructions (Thermo Fisher Scientitic). The samples were then run on an agarose gel and visualized using the Herolab Gel Documentation System. The following primers were used:

Gene name	Accession no.	Sequence (5'->3')	position (TSS=1)	Efficiency (%)
RPL13A	NM_012423.2	AAAGCCAAGATCCACTACCG	509	
		GGAATTAACAGTCTTTATTGGGCTC	657	105,7
LTB	NM_002341.1	GTCAACATCAGTCACCCCG	669	
		CAATATTCACGCACTCGCAC	781	97,5
LTBR	NM_002342.2	GGCACCTATGTCTCAGCTAAAT	519	94,7
		GTAGTTCCAGTGCTCGTTGTAG	608	
BTLA	NM_001085357.1 NM_181780.3	TGCCTGCCATGCTTGGAACT	214	
		TCACATGATTCTTTCCCATGGATGT	307	95,2

Chromatography

L1236 cells were cultured in RPMI medium without FCS for 48 h. Filtered (2 µm filter) cell culture SUP was used for further analysis. Chromatographic fractionation was performed using a Q Sepharose (GE Healthcare) and Heparin Sepharose column (GE Healthcare) and the ÄKTA protein purification system (GE Healthcare). Both columns were previously equilibrated with wash buffer containing 137 mM NaCl and 20 mM HEPES (pH 7.4). Culture supernatant was loaded directly onto the Q Sepharose. The flow-through of the Q column was then loaded onto the heparin column. The heparin column was washed with wash buffer. The column was eluted using step gradients of 0.2, 0.3, 0.4 and 2 M NaCl in 20 mM HEPES buffer (pH 7.4) and 5 ml fractions were collected. Fractions were pooled and checked for the ability to activate NF-κB in HeLa cells. Activation of NF-κB was determined after 15 min or 24 h by analyzing nuclear translocation of NF-κB subunits in WB.

TCA precipitation

TCA precipitation was performed according to standard protocols and samples were run on SDS-PAGE gel, which was then stained with Imperial Protein Stain (Thermo Scientific) to visualize protein content.

Mass spectrometry

The chromatographic fractions 1 and 2 containing the NF-κB activating factor were analyzed by mass spectrometry and compared to the control fraction 6, which did not induce NF-κB in order to identify the factor.

Samples for mass spectrometric analysis were produced as previously described (3). In short, proteins were precipitated by Wessel-Flügge precipitation (4). The retrieved protein pellets were air-dried before being resuspended in 6 M Urea, 2 M Thiourea in 10 mM HEPES (pH 8). Proteins were denatured by adding 10 mM DTT in 50 mM ammoniumbicarbonate (ABC buffer) and then alkylated by the addition of 55 mM iodoacetamide. Alkylated proteins were sequentially digested by LysC followed by trypsin. The resulting peptide solution was acidified by trifluoroacetic acid (TFA, Sigma-Aldrich) before peptides were desalted and stored on StageTips (5). Peptides were eluted from the StageTips using Buffer B (80% Acetonitrile and 0.1% formic acids) and dried using a speedvac (Eppendorf). The peptides were resuspended in Buffer A (5% acetonitrile and 0.1% formic acid) before being separated on a 15 cm column (inner diameter of 75 μm) packed with C18 beads (Dr.Maisch, GmbH) coupled to a high-performance liquid chromatography (HPLC) system deploying 2 hr gradients of increasing Buffer B concentration (250 nl/min flow rate). Peptides were ionized using an electrospray ionization (ESI) source (ThermoScientific) and analyzed on a Q Exactive mass spectrometer (ThermoScientific). The MS scans were run at a 70,000 resolution, with a target value of 3,000,000 ions and a maximum injection time of 20 ms. The top 10 most intense ions in the MS full scans were selected for higher energy collision induced dissociation. The MS/MS scans were collected at a 17,500 resolution, with a target value of 1,000,000 ions and a maximum injection time of 60 ms.

The resulting raw files were analyzed using MaxQuant software version 1.6.1.0 with default settings (6). The mass spectra were matched to an *in silico* digested human Uniprot database (UP000005640) and a database containing common contaminants. The resulting text files were filtered to exclude reverse database hits, potential contaminants, and proteins only identified by site.

Targets were filtered for enrichment in fraction 1 and 2, lacking in faction 6. Gene Ontology terms were used to filter for extracellular presence of the identified proteins.

Mass spectrometry data are deposited on the PRIDE server (https://www.ebi.ac.uk/pride/archive/) under the Accession-ID PXD010399.

ELISA

$5x10^5$ cells/ml were seeded into a 24-well plate (TPP) and cultured for 24 h. Cell culture medium was collected and sterile filtered. 100 µl was used to determine LTA amounts in the medium using a Human TNF-beta ELISA kit (ELH-TNFb-1, RayBiotech) according to manufacturer's protocol. Samples were analyzed in duplicates and the mean concentration of LTA was calculated.

Cytokine Array

To determine cytokine secretion, cells were washed and seeded into a 6-well plate (TPP) at a density of $5x10^5$ cells/ml. The cells were cultured in RPMI (Gibco) without FCS for 24 h. The conditioned medium was then collected, centrifuged and filtered. The cytokine array C3 (AAH-CYT-3-2, RayBiotech) was used according to manufacturer's protocol and incubated overnight with 1 ml of conditioned medium. RPMI without FCS was analyzed in parallel as a control.

Flow Cytometry

$1x10^6$ cells/sample were washed in FC Buffer (1x PBS containing 2% h-i FCS and 10 mM EDTA) and blocked with CD16/32 (101302, biolegend) in FC Buffer for 20 min. Cells were then washed with FC Buffer and incubated for 20 min at RT with the following anti-human antibodies:

Antibody	Type	Company	Order no.	Conc.
PE-CD272 (BTLA)	Mouse IgG2a, κ	biolegend	344506	1:40
PE- Mouse IgG2a, κ	Mouse IgG2a, κ	biolegend	400212	1:40
PE-CD160	Mouse IgM, κ	biolegend	341206	1:20
PE- Mouse IgM, κ	Mouse IgM, κ	biolegend	401611	1:20
PE-CD120b (TNFRSF1B)	Rat IgG2a, κ	biolegend	358404	1:20
PE-Rat IgG2a, κ	Rat IgG2a, κ	biolegend	400508	1:40
PE-LTBR	Mouse IgG2b, κ	biolegend	322008	1:40
PE-Mouse IgG2b, κ	Mouse IgG2b, κ	biolegend	400314	1:40
BV421-CD120a (TNFRSF1A)	Mouse IgG2a, κ	BD	743503	1:100
BV-421-Mouse IgG2a, κ	Mouse IgG2a, κ	BD	562439	1:100
PE-CD270 (TNFRSF14)	Mouse IgG1, κ	biolegend	318806	1:40
PE-Mouse IgG1, κ	Mouse IgG1, κ	biolegend	400114	1:40

Cells were gated based on SSC/FSC scatter plot and fluorescent signal. Data was acquired on a LSR II flow cytometer (BD) and analyzed with FlowJo software (TreeStar).

CRISPR/Cas9 mediated KO

The lentiCRIPSPR v2 vector was a gift from the Feng Zhang (Addgene plasmid #52961). The gRNAs were designed using CrispRGold (7). Two gRNAs were used for further experiments which target the second exon (g2) (LTA_g2.for: 5'-

CACCGCTACTTCGTCTACTCCCAGG-3', LTA_g2.rev: 5'-AAACCCTGGGAGTAGACGAAGTAGC-3') and the fourth exon (g3) (LTA_g3.for: 5'-CACCGTGGTGCCACACACCCTTGGG-3', LTA_g3.rev: 5'-AAACCCCAAGGGTGTGTGGCACCAC-3') of *LTA*.

The cloning was performed according to the standard protocol of the Zhang lab (8). In brief, the vector was digested with BsmBI (Thermo Fisher Scientific) and dephosphorylated using rSAP (NEB) Digested vector was run on a 1% agarose gel and purified using the QIAquick gel extraction kit (Qiagen). LTA gRNA oligonucleotides were annealed and phosphorylated. Oligonucleotides and vector were then ligated overnight at 16°C using the T4 Ligase (NEB). The plasmids were transformed into One Shot Stbl3 chemically competent bacteria (Thermo Fisher Scientific). Single colonies were selected for plasmid production and gRNA incorporation was validated by sequencing.

Lentiviral packaging and transfection

HEK-293T17 cells were seeded into 6-well plates at a cell density of 8 x10^5 cells/well 24 h prior to transfection. For lentiviral packaging cells were transfected with 1.5 µg lentiCRISPRv2 plasmids containing the gRNAs, 1 µg pCMV-VSG-G (a gift from Bob Weinberg (Addgene plasmid # 8454)) and 1 µg psPAX2 (a gift from Didier Trono (Addgene plasmid # 12260)). Plasmids were diluted in 1 ml OptiMEM. 7 µl Lipofectamin 2000 was added to 1 ml OptiMEM. Both mixtures were incubated for 5 min at RT. Mixtures were then combined and incubated for additional 20 min. Medium of HEK293/T17 cells was exchanged to OptiMEM and reaction mixture was added drop wise while gently rocking the plates. After 6 h of transfection medium was replaced to normal culture medium. For lentiviral production cells were incubated for 48 h at 37°C and 5% CO_2. Medium was then harvested and filtered through 0.45 µm filter. Viral supernatant was either stored at -80°C or directly used for transduction.

Transduction of cHL cell lines and single cell selection

For lentiviral transduction $1x10^6$ L1236 cells were seeded into 6-wells. 2 ml of viral supernatant was added and the plate was centrifuged at 2000 rpm for 90 min at 37°C. Cells were then incubated for 4 days. Medium was then replaced with normal culture medium. For selection of transduced cells, 2 µg/ml puromycin was added for 5 days. Normal culture conditions were then re-established. Single cell clone selection was performed by serial dilutions. Cells were cultured in 96-well with conical bottom and with conditioned medium derived from L1236. Medium was regularly replaced and growing colonies were analyzed for mRNA and protein levels of LTA to determine ideal clones for further analysis.

Gene-wide expression analysis

Analysis of the transcriptome-wide gene expression levels was performed using the human Clariom™ S Assay (Thermo Fisher Scientific) according to manufacturer's instructions. To this end, L1236 control cells (v2) and the two LTA KO clones g2_1 and g3_4 were cultured in normal culture medium for 72 h. The RNA was then extracted using the RNeasy kit (Qiagen) according to manufacturer's instructions. The RNA concentration and quality was determined using the NanoDrop. Furthermore, RNA quality was analyzed using the Agilent Bioanalyzer 6000 Nano kit according to the manufacturer's protocol. Only RNA samples with a RIN value of 9-10 were used for further analysis. In total, three replicates were analyzed.

Data analysis was performed using the Transcriptome Analysis Console 4.0 (Thermo Fisher Scientific). All samples passed the following quality control thresholds: labeling controls, hybridization controls, positive vs. negative area under the curve.

The *LTA* KO clones were regarded as one group and compared to the L1236 v2 control cells. The transcripts were annotated according to the genome version hg38 (Homo sapiens). An FDR of <0.05 and the gene expression level fold change of ≤-2 and ≥2 were used as cut-offs to determine activated and repressed genes, respectively.

Microarray data was deposited in the ArrayExpress database (9) at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6896.

http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6896

GSEA

GSEA was performed as described (10) using the Molecular Signature Database v6.1.

To create gene sets from our previously reported canonical or non-canonical activated or repressed NF-κB targets (11), we filtered the corresponding gene lists by log2fc <-0.415 or >0.451.

Comparison of LTA expression in human peripheral blood cells and lymphomas

Data from Brune *et al.* (12) was downloaded from the GEO database (GEO ID: GSE12453). Samples were processed using the RMA normalization method (10.1093/biostatistics/4.2.249). Sample with the ID GSM312946 was removed prior to the normalization due to low quality. Differential expression analysis was done in a pairwise manner between all combinations of samples, using limma (10.1093/nar/gkv007), Genes with absolute fold change greater than 1, and the adjusted p value lower than 0.05 were marked as differentially expressed.

Annotations for the Affy hg u133p2 platform were downloaded from Biomart. Probesets which were annotated to multiple genes were filtered out of the subsequent analysis steps.

Integration of target genes with gene expression in human peripheral blood cells

Peripheral blood mononuclear cell (PBMC) datasets were downloaded from the 10x Genomics web resource (https://support.10xgenomics.com/single-cell-gene-expression/datasets/2.1.0/pbmc8k). The data was processed using Seurat (https://doi.org/10.1038/nbt.4096) in the following manner: cells with less than 200 and more

than 2500 detected genes were filtered out of the analysis. Subsequently cells containing more than 5% of mitochondrial transcripts were filtered out. The data was log normalized with the scaling factor equal to 10000. Variable genes were detected using the default Seurat parameters. Cell clusters were identified using the graph clustering method implemented in the FindClusters function, with the resolution parameter set to 0.6. First 10 principal components were used for the clustering. The resulting clusters were annotated with corresponding blood cell types based on known marker genes. Cell-specific gene expression profile was obtained by averaging, per gene, the expression of all cells corresponding to each cell type.

Integration of the single cell expression data with the LTA signature

Cell type specificity of expression LTA controlled genes was explored by integrating genes downregulated upon *LTA* KO (log2 FC LTA KO / WT < -1 and p value < 0.0005) with their expression in PBMCs. Gene sets were merged based on HGNC symbols. The LTA signature co-expression modules were constructed in the following way; the expression levels of all LTA signature genes were compared in previously defined blood cell types using the Pearson correlation coefficient. The resulting values were clustered using hierarchical clustering with Euclidean distance and complete linkage. The co-expression matrix was split into 13 modules.

Heatmap visualizations were constructed using the ComplexHeatmap Bioconductor package (10.1093/bioinformatics/btw313).

Supplementary References

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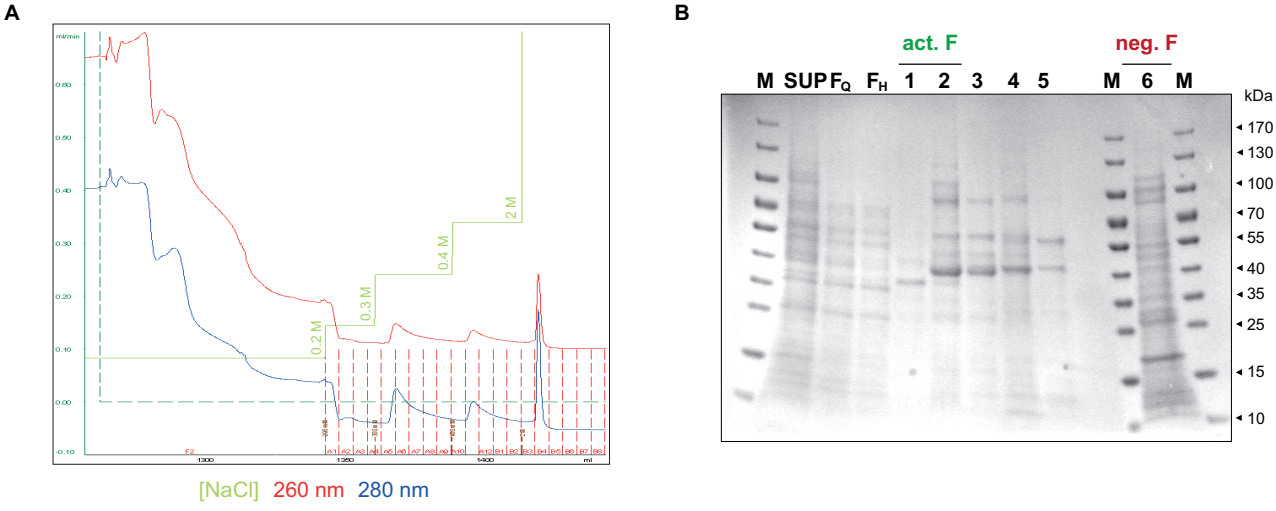
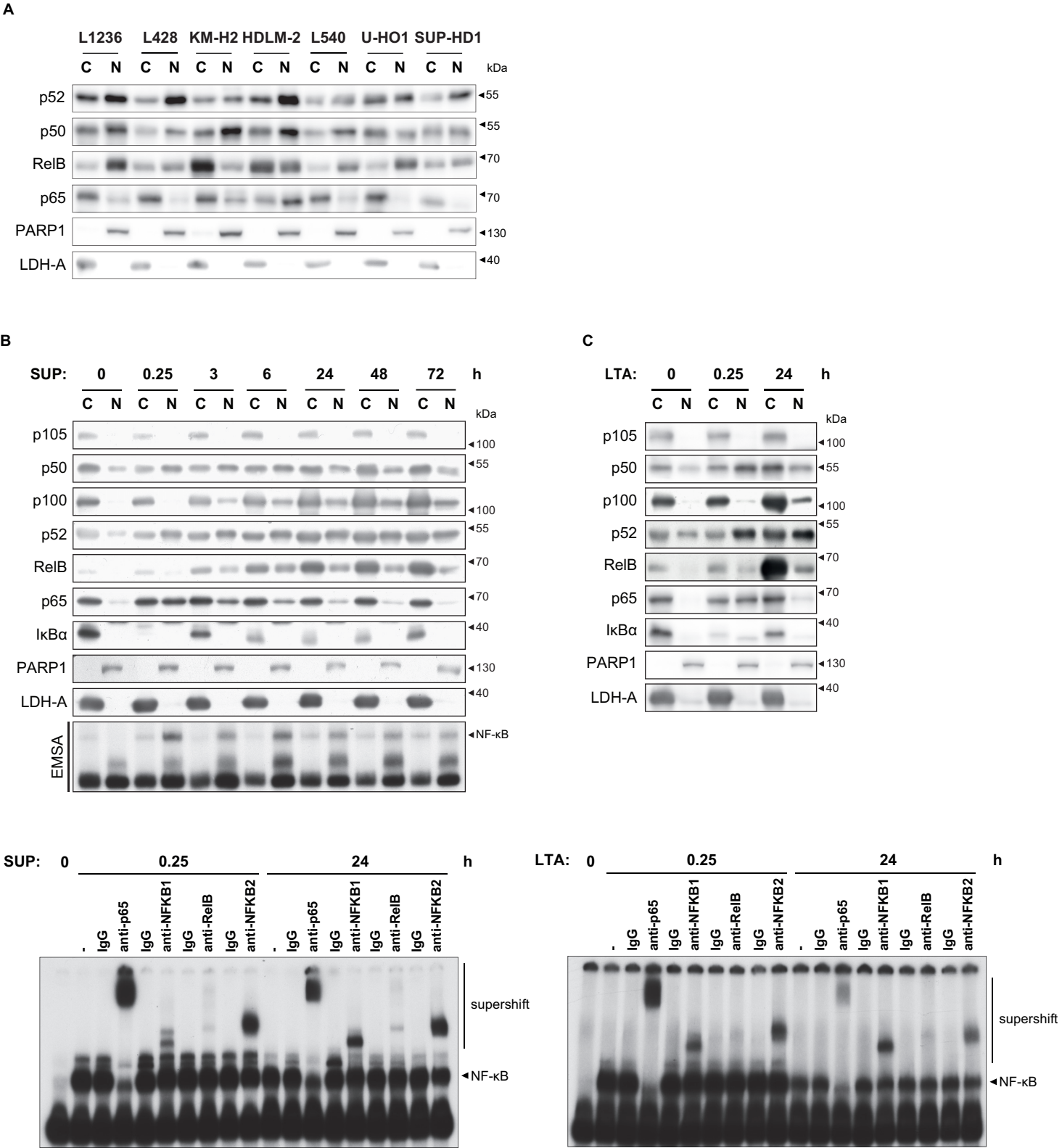
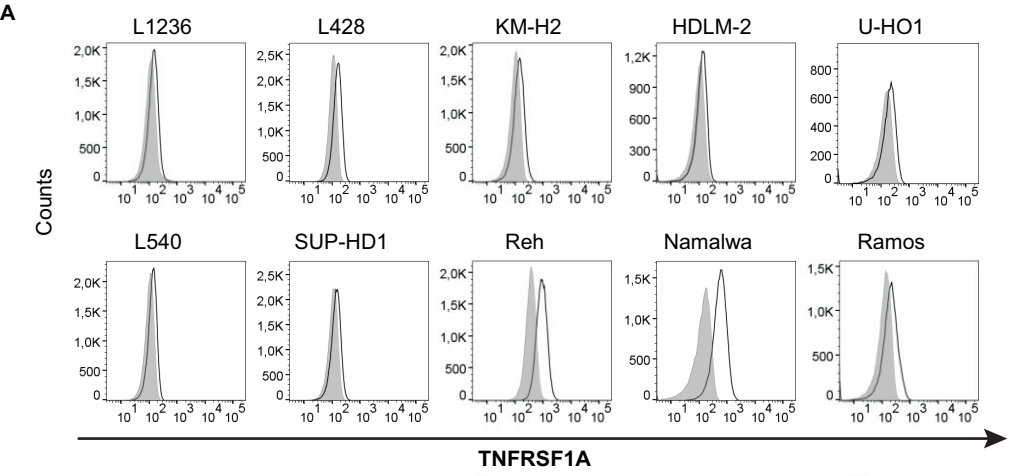
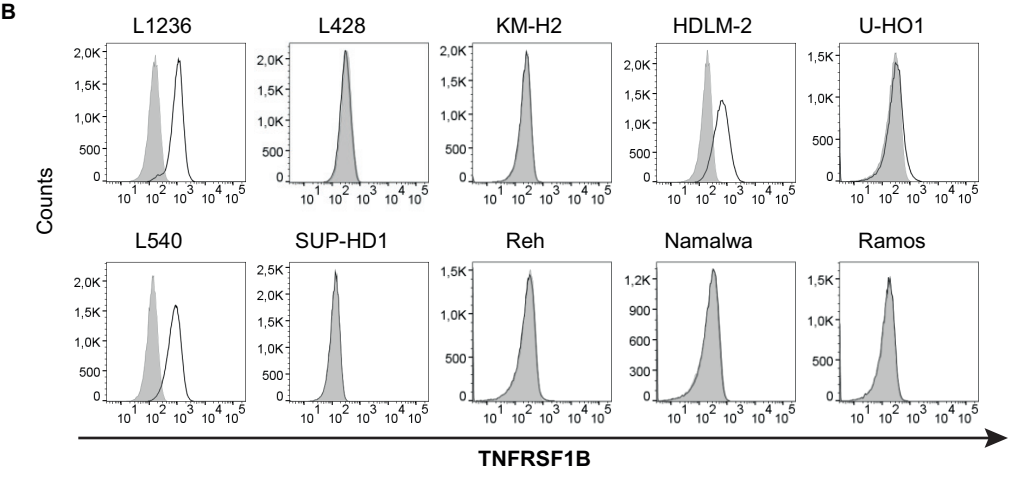
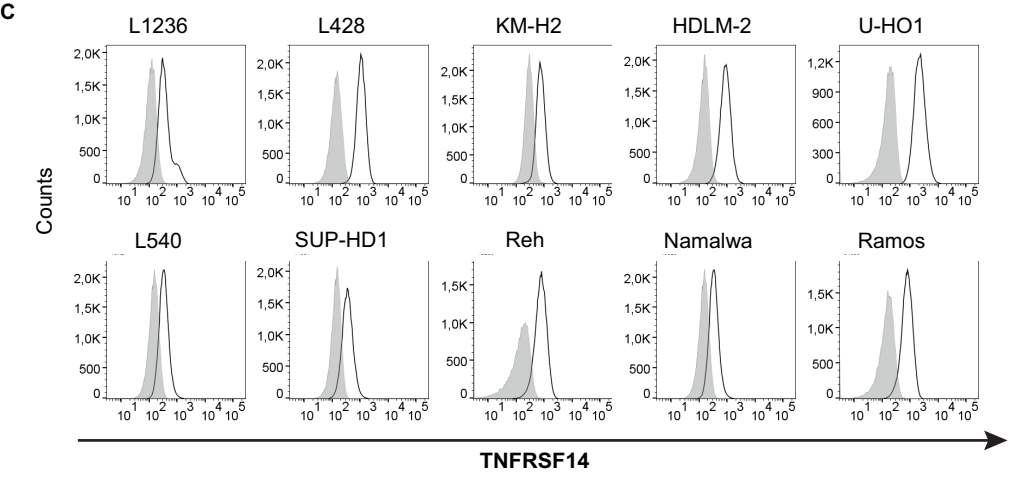
SUPPLEMENTAL FIGURE 1

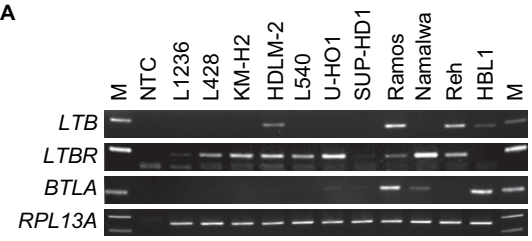


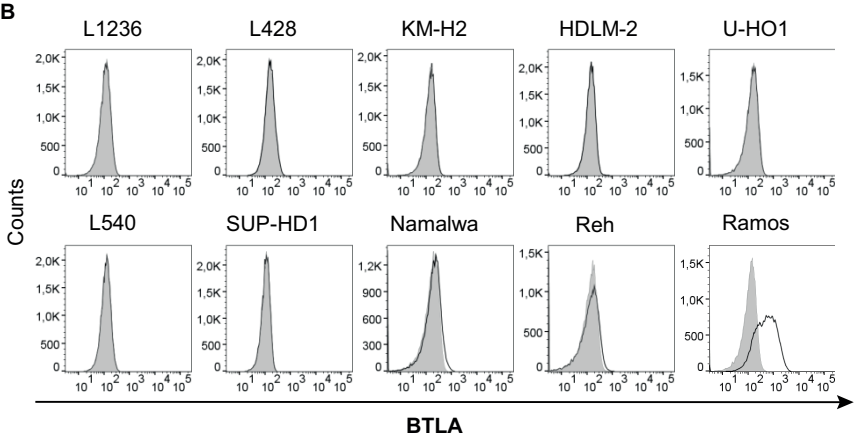

A
L1236
L428
KM-H2
HDLM-2
L540
U-HO1
SUP-HD1
C N C N C N C N C N C N C N
kDa
p52
p50
RelB
p65
PARP1
LDH-A
55
55
70
70
130
40
B
SUP: 0 0.25 3 6 24 48 72 h
C N C N C N C N C N C N C N
kDa
p105
p50
p100
p52
RelB
p65
IκBα
PARP1
LDH-A
EMSA
100
55
100
55
70
70
40
130
40
NF-κB
C
LTA: 0 0.25 24 h
C N C N C N
kDa
p105
p50
p100
p52
RelB
p65
IκBα
PARP1
LDH-A
100
55
100
55
70
70
40
130
40
SUP: 0 0.25 24 h
-
IgG
anti-p65
IgG
anti-NFKB1
IgG
anti-RelB
IgG
anti-NFKB2
supershift
NF-κB
LTA: 0 0.25 24 h
-
IgG
anti-p65
IgG
anti-NFKB1
IgG
anti-RelB
IgG
anti-NFKB2
supershift
NF-κB

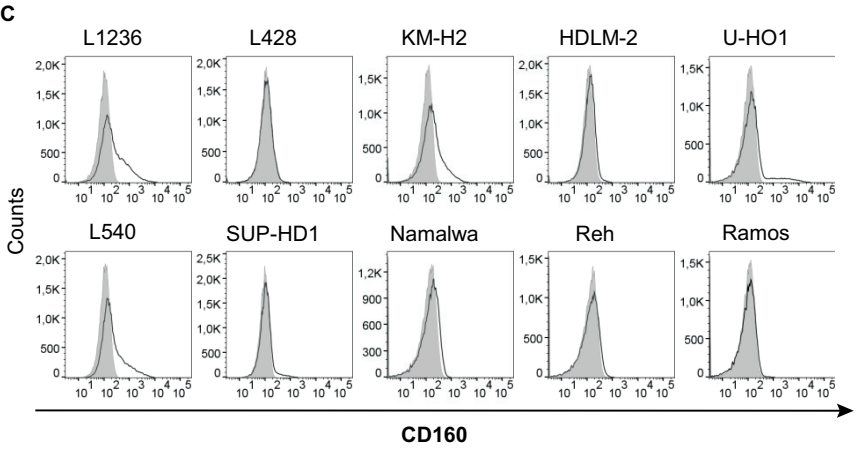

A
L1236
L428
KM-H2
HDLM-2
U-HO1
L540
SUP-HD1
Reh
Namalwa
Ramos
Counts
TNFRSF1A

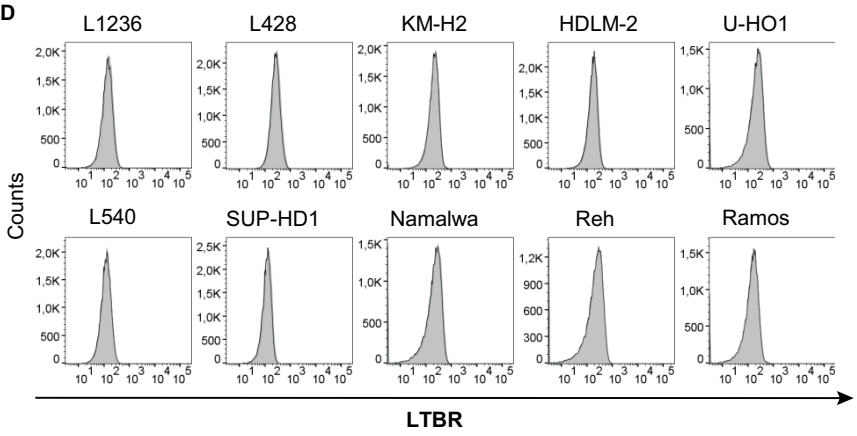

B
L1236
L428
KM-H2
HDLM-2
U-HO1
L540
SUP-HD1
Reh
Namalwa
Ramos
Counts
TNFRSF1B


C
L1236
L428
KM-H2
HDLM-2
U-HO1
L540
SUP-HD1
Reh
Namalwa
Ramos
Counts
TNFRSF14

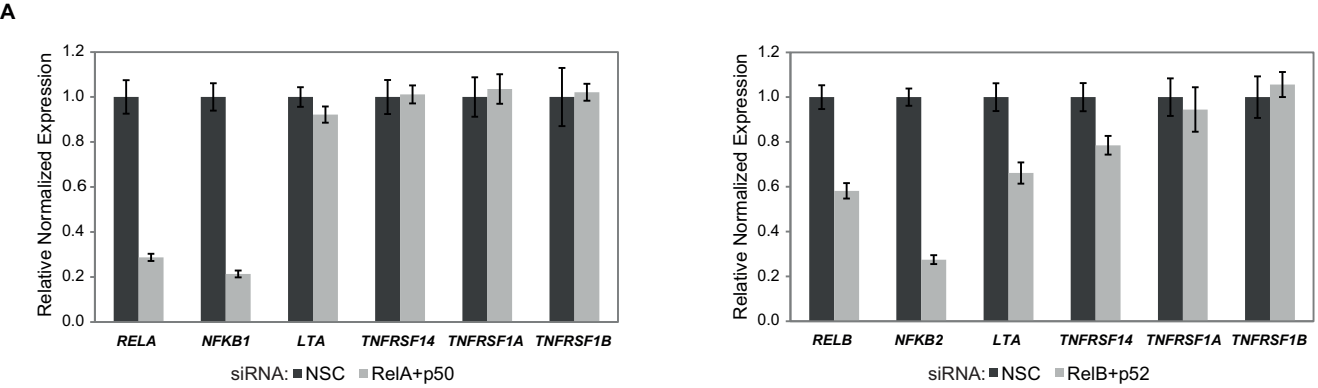

A
M
NTC
L1236
L428
KM-H2
HDLM-2
L540
U-HO1
SUP-HD1
Ramos
Namalwa
Reh
HBL1
M
LTB
LTBR
BTLA
RPL13A

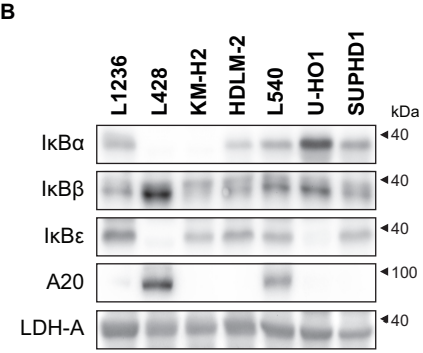

B
L1236
L428
KM-H2
HDLM-2
U-HO1
L540
SUP-HD1
Namalwa
Reh
Ramos
Counts
BTLA

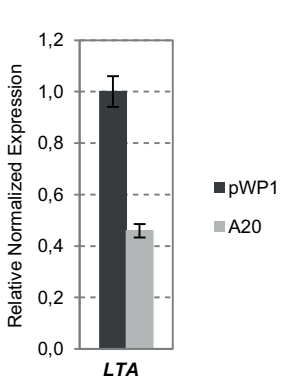

C
L1236
L428
KM-H2
HDLM-2
U-HO1
L540
SUP-HD1
Namalwa
Reh
Ramos
Counts
CD160


D
L1236
L428
KM-H2
HDLM-2
U-HO1
L540
SUP-HD1
Namalwa
Reh
Ramos
Counts
LTBR

SUPPLEMENTAL FIGURE 5



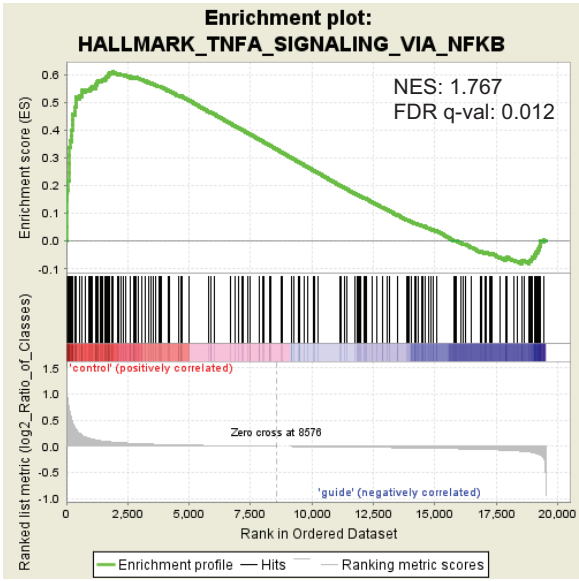


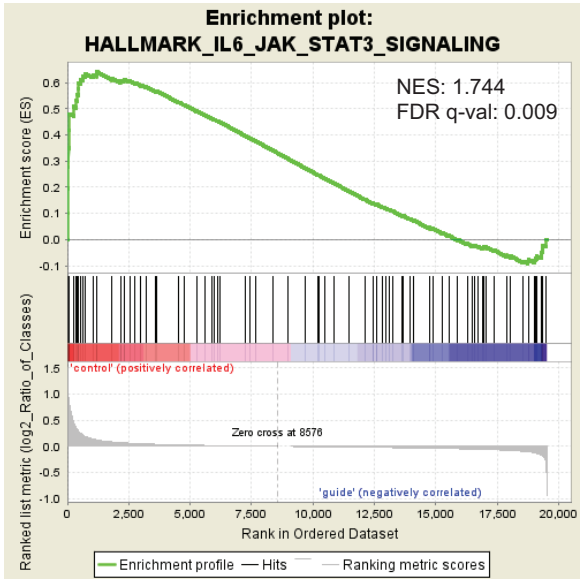


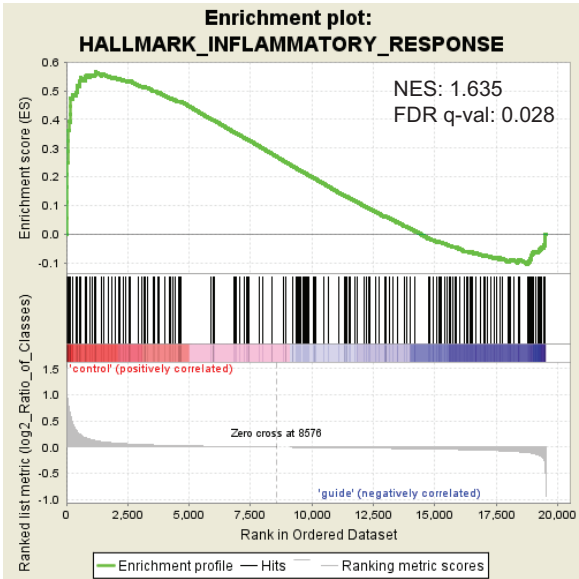
A

LTA: 6p21.33
gRNA #2
gRNA #3

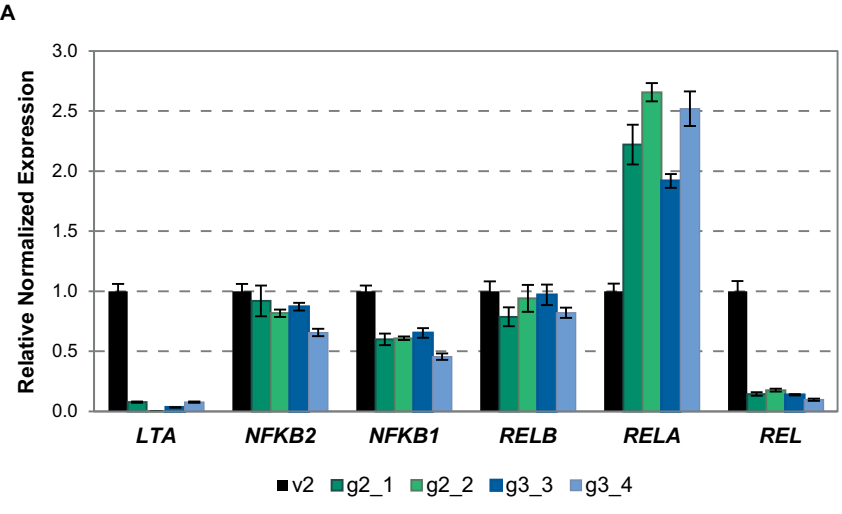
B

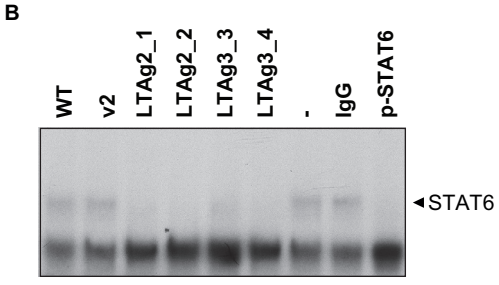

Enrichment plot:
HALLMARK_TNFA_SIGNALING_VIA_NFKB
NES: 1.767
FDR q-val: 0.012
Enrichment score (ES)
'control' (positively correlated)
Zero cross at 8576
'guide' (negatively correlated)
Ranked list metric (log2_Ratio_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


Enrichment plot:
HALLMARK_IL6_JAK_STAT3_SIGNALING
NES: 1.744
FDR q-val: 0.009
Enrichment score (ES)
'control' (positively correlated)
Zero cross at 8576
'guide' (negatively correlated)
Ranked list metric (log2_Ratio_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

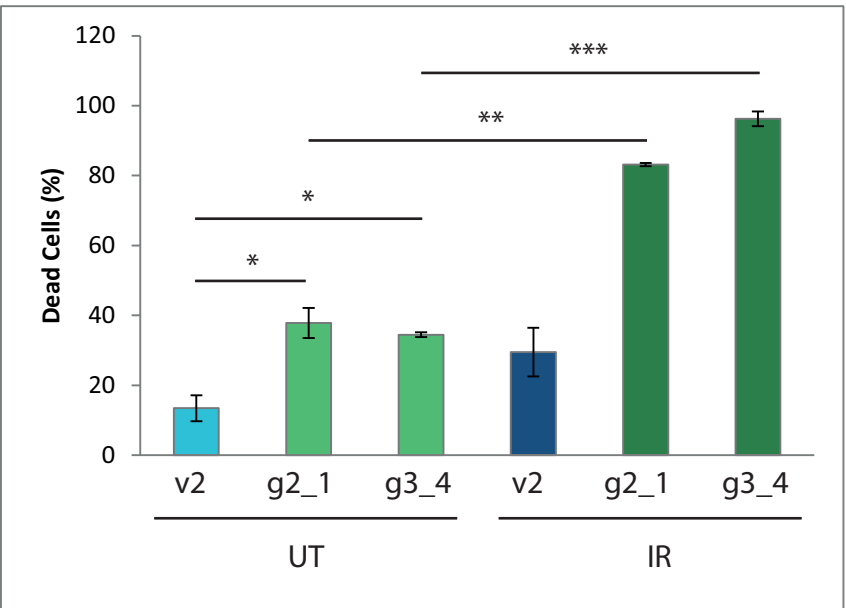

Enrichment plot:
HALLMARK_INFLAMMATORY_RESPONSE
NES: 1.635
FDR q-val: 0.028
Enrichment score (ES)
'control' (positively correlated)
Zero cross at 8576
'guide' (negatively correlated)
Ranked list metric (log2_Ratio_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

SUPPLEMENTAL FIGURE 7

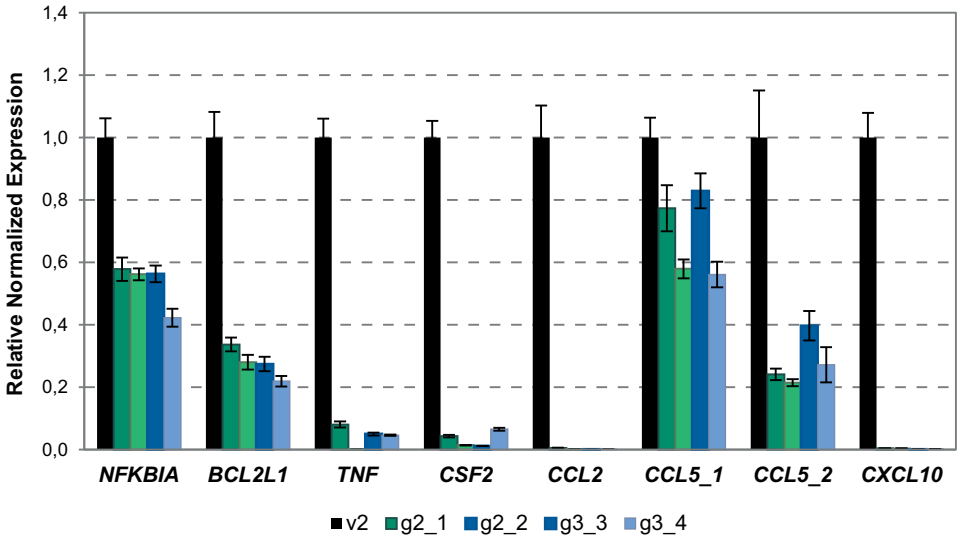




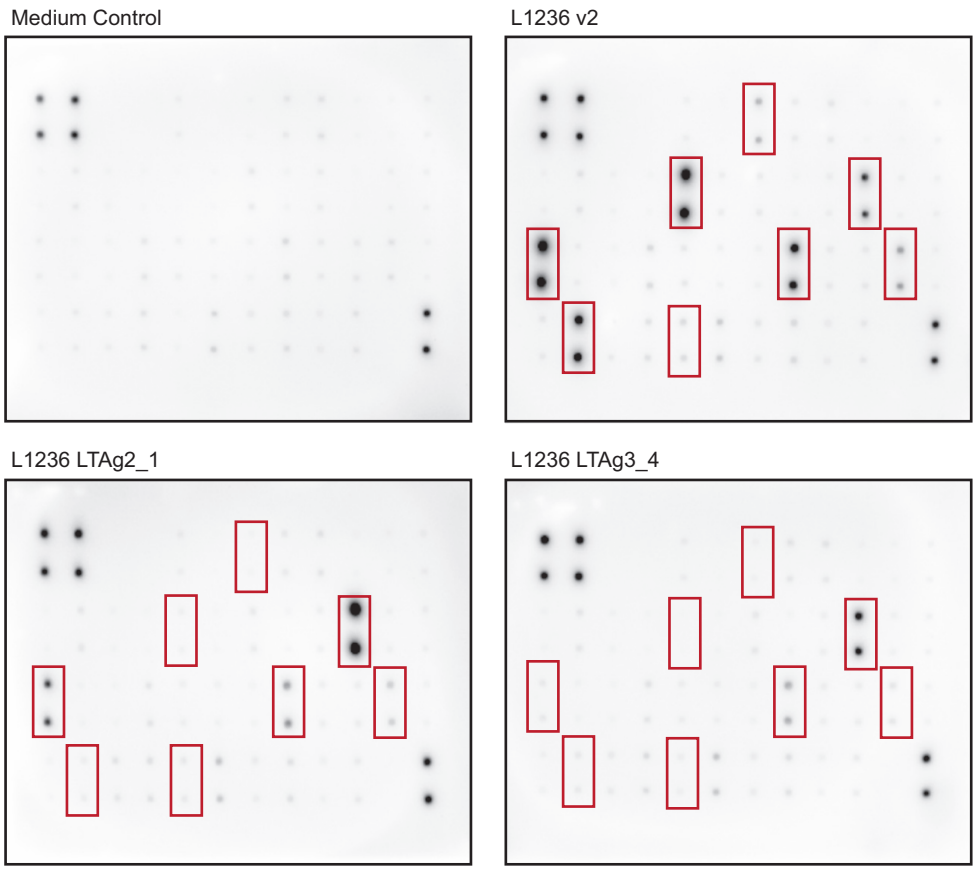
SUPPLEMENTAL FIGURE 8



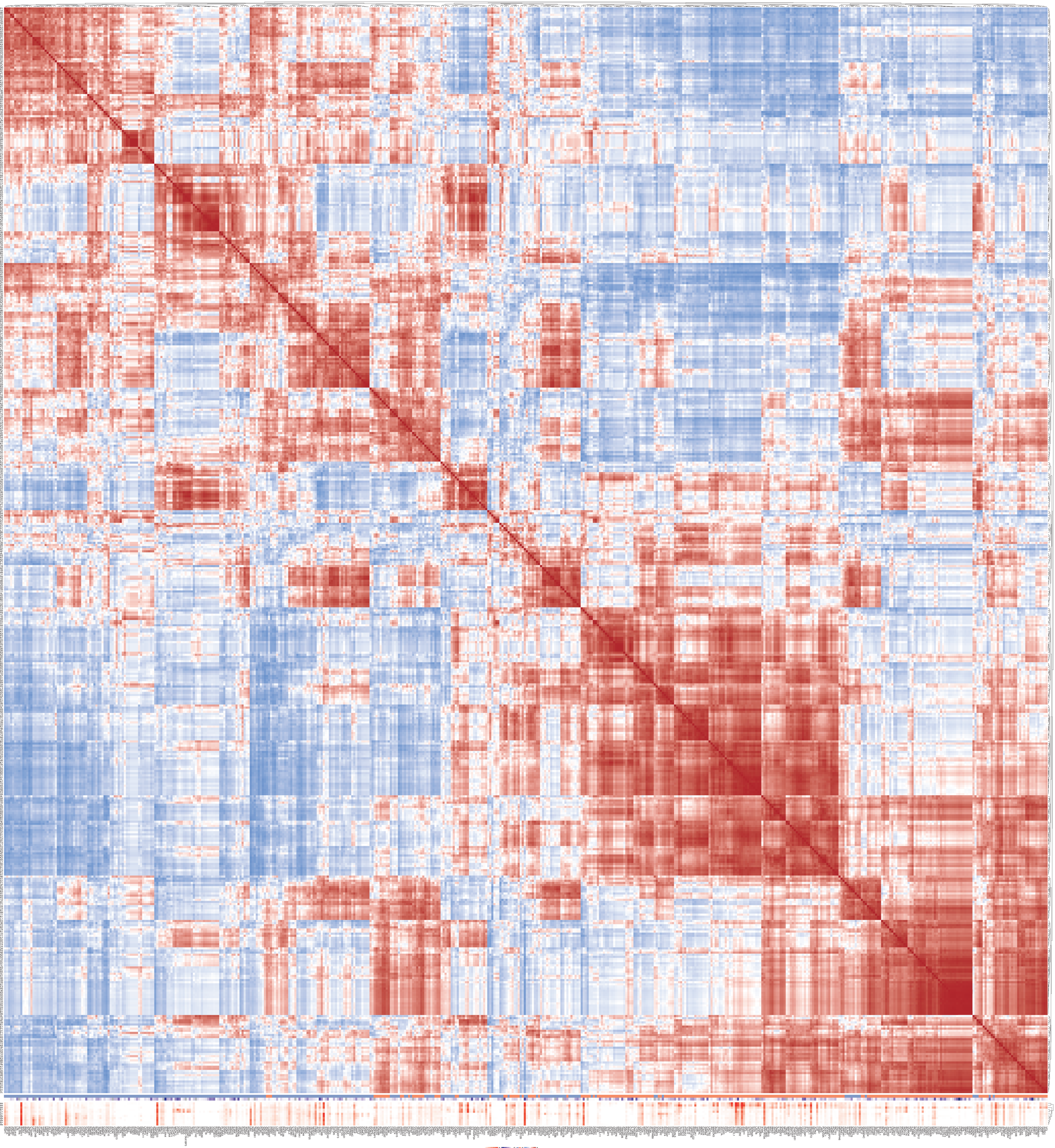
A



B



	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	CXCL5	GCSF	GM-CSF	GRO a/b/g	CXCL1	CCL1	IL-1 alpha	IL-1 beta
2												
3	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	Il-8	IL-10	Il-12 p40/p70	Il-13	Il-15	IFN-gamma
4												
5	CCL2	CCL8	CCL7	M-CSF	CCL22	CXCL9	MIP-1 delta	RANTES (CCL5)	SCF	SDF-1	CCL17	TGF beta 1
6												
7	TNF alpha	LTA	EGF	IGF-1	Angio-genin	OSM	TPO	VEGF-A	PDGF-BB	Leptin	NEG	POS
8												


CD14+ Monocytes
FCGR3A+ Monocytes
Megakaryocytes
CD79A+ MS4A1+ Bcells
MS4A1+ CD8A+ TGC+
GNLY+ CCL4+ PRF1+ tdTGFBP2+
NK cells
CD8 T cells
Naive CD4 T cells
B cells
Log2FC
-log10(p-adj)