An intronic deletion in megakaryoblastic leukemia 1 is associated with hyperproliferation of B cells in triplets with Hodgkin lymphoma

Julien Record,[1*] Anton Sendel,[1*] Joanna S. Kritikou,[1] Nikolai V. Kuznetsov,[1] Hanna Brauner,[1] Minghui He,[1] Noemi Nagy,[1] Mariana M.S. Oliveira,[1] Elena Griseti,[1] Christoph B. Haase,[1] Jenny Dahlström,[2] Sanjaykumar Boddul,[2] Fredrik Wermeling,[2] Adrian J. Thrasher,[3] Chaohong Liu,[4] John Andersson,[1,5] Hans-Erik Claesson,[2] Ola Winqvist,[2] Siobhan O. Burns,[6,7] Magnus Björkholm[2] and Lisa S. Westerberg[1]

[1]Department of Microbiology, Tumor and Cell Biology, Biomedicum, Karolinska Institutet, Stockholm, Sweden; [2]Department of Medicine Solna, Karolinska University Hospital, Stockholm, Sweden; [3]Institute of Child Health, University College London, London, UK; [4]Department of Pathogen Biology, School of Basic Medicine, Huazhong University of Science and Technology, Wuhan, China; [5]Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden; [6]Institute of Immunity and Transplantation, University College London, London, UK and [7]Department of Immunology, Royal Free London NHS Foundation Trust, London, UK

** JR and AS contributed equally to this work*

 doi:10.3324/haematol.2019.216317

Received: March 7, 2019.

Accepted: September 26, 2019.

Pre-published: October 3, 2019.

Correspondence: *LISA WESTERBERG* - lisa.westerberg@ki.se

Extended Methods

Human blood samples

Whole blood samples were obtained from the triplets and age-matched controls after informed consent was given. This study was performed according to the principles expressed in the Helsinki Declaration and with approval from the local ethics committee (Dnr 2015/416-31). For analysis of primary cells, the first experiment included samples from HL0, HL1, and ctrl (not used for EBV-transformation) collected in February 2015 and the second experiment HL2 and C1, collected in May 2015. To establish EBV-transformed LCLs, PBMCs from HL0, HL1, and HL2, and two age- and sex-matched controls (C1 and C2), all collected in November 2015, were cultured with supernatant of the virus-producing B95-8 line (1).

Clinical status of patients

HL0 has remained free from HL including the time of blood sampling. He suffered one stroke in 2009 and another in 2010. However, he still (2019) remains free from HL. The triplet HL1 was diagnosed in 1985 at age 40 with stage IIIA HL of Epstein-Barr virus (EBV)–positive mixed cellularity subtype. He has been treated with mustargen, oncovin, procarbazine, prednisone/adriamycin, bleomycin, vinblastine, and dacarbazine chemotherapy. The triplet HL1 had enjoyed a 31-year continuous first complete remission at the time of blood sampling. To date he remains free from HL but underwent surgery for a prostate cancer in 2011. He suffers from cardiovascular disease. The triplet HL2 developed stage IIIB HL in 2008. His tumor was EBV-positive and of nodular sclerosis (NS) subtype. A complete and lasting remission was achieved following adriamycin, bleomycin, vinblastine and dacarbazine chemotherapy. The triplet HL2 died from cardiac insufficiency in January 2017. There were no clinical or laboratory signs of HL at the time of blood sampling or death.

Mice

NOD/SCID-IL2rγ^{null} (NSG) mice (2) were bred and maintained at the animal facility of the Department of Microbiology, Tumor and Cell Biology at Karolinska Institutet under specific pathogen-free conditions. Female mice were used 6-12 weeks of age and all animal experiments were performed after approval from the local ethics committee (the Stockholm District Court, permit N77/13 and N272/14).

Flow-cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA)

Whole blood samples were subjected to Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole blood (FASCIA) (3) to examine the capacity to form blast cells upon stimulation with different antigens. Heparinized whole blood was diluted 1:10 in RPMI-1640 supplemented with 10-20 % fetal calf serum (FCS), L-glutamine, penicillin-streptomycin, and Na-pyruvate (complete RPMI). The blood-medium mixture was stimulated with medium alone or with medium containing Phytohemagglutinin, Pokeweed mitogen, Concanavalin A, Prevenar vaccine, Tetanus toxin, Influenza A vaccine, PPD, Candida, Staphylococcal enterotoxin A/B, or Varicella zoster virus and cultured for 7 days at 37 °C with 5 % CO_2. At day 7, cells were stained with CD3-FITC/CD4-PE Simultest mix (BD Biosciences, San Jose, CA, USA) and in samples with Pokeweed mitogen with CD19-PC7 (Beckman Coulter, Brea, CA, USA). Blast numbers were acquired during 80 seconds using a FC500 or Navios flow cytometer (Beckman Coulter). To obtain an absolute number of proliferating (blast) cells per µL whole blood, Trucount (BD Biosciences) tubes were used (3). Table 1 shows blast cell numbers at day 7.

Primary B cell isolation and culture

Ten mL of whole blood was diluted 1:4 with 0.9 % NaCl and underlaid with 10 mL of Ficoll-Paque PLUS (GE Healthcare Life Sciences, Marlborough, MA, USA); after centrifuging at 400 *g* for 40 minutes at room temperature (rt), the peripheral blood mononuclear cell (PBMC) layer was aspirated and washed twice with Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (ThermoFisher Scientific, Waltham, MA, USA). B cells were isolated using the CD19 MicroBeads kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the company protocol. MACS buffer was prepared with phosphate-buffered saline (PBS), pH 7.2, 0.5 % bovine serum albumin (BSA), and 2 mM EDTA. Cells were suspended in 80 µL of buffer and 20 µL of CD19 MicroBeads per 10^7 cells, incubated for 15 minutes at 4 °C, and subsequently washed with buffer. Labeled cells were separated by passing cell suspension through MACS® MS columns (Miltenyi Biotec). For primary cell culture, B cells were suspended at $0.5x10^6$ cells/ml in complete RPMI with 30 ng/mL human IL-4, (Peprotech, London, UK), and 3 µg/mL anti-human CD40 (S2C6; MabTech, Cincinnati, OH, USA).

EBV transformation

For the establishment of EBV-transformed LCLs, PBMCs from HL0, HL1, and HL2, and two age- and sex-matched controls (C1 and C2) were isolated from blood by Ficoll-Paque gradient separation. B cells were infected with EBV by incubating the PBMCs with supernatant of the virus-producing B95-8 line for 90 minutes at 37 °C (1). Thereafter the cells were washed, re-suspended in complete RPMI-1640 medium and cultured until stable LCLs were obtained. Cyclosporin A was added to the medium (0.5 µg/mL) for the first 4 weeks in order to inhibit EBV-specific T cells. LCLs were cultured in complete RPMI at $0.3–1.5x10^6$ cells/mL.

Reverse transcriptase-quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA from LCLs was isolated with the RNeasy kit (QIAGEN GmbH, Hilden, Germany). Each sample was treated with RNAse-free DNAse (QIAGEN) and RNA concentration was measured with the NanoDrop 2000 (ThermoFisher Scientific). The visual quality control for RNA integrity and purity was performed by 1 % UltraPure Agarose (ThermoFisher Scientific), 1x TAE gel electrophoresis analysis. Gel images were created with the ImageQuant LAS4000 Image System using the ImageQuant LAS4000 Control Software (GE Healthcare). Two µg of total RNA per sample was used for each cDNA synthesis reaction together with SuperScript II Reverse Transcriptase (ThermoFisher Scientific). The following primer pairs was used; MKL1-forward 5'-ACTAGCCGATGACCTCAATGA-3' and MKL1-reverse 5'-TTCACCTGGCCCACAATGATG-3'; SRF-forward 5'-CGAGATGGAGATCGGTATGGT-3' and SRF-reverse 5'-GGGTCTTCTTACCCGGCTTG-3'; ACTB-forward 5'-CATGTACGTTGCTATCCAGGC-3' and ACTB-reverse 5'-CTCCTTAATGTCACGCACGAT-3'; ITGAL-forward 5'-TGCTTATCATCATCACGGATGG-3' and ITGAL-reverse 5'-CTCTCCTTGGTCTGAAAATGCT-3'. RT-qPCR reactions were performed using SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad, Hercules, CA USA) in the C1000/CFX96 RT-qPCR System (Bio-Rad). Gene expression data were analyzed using Excel (Microsoft, Redmond, WA, USA). The arithmetic mean Ct of the triplicate, ΔCt, and RQ values were calculated, and then RQ values were normalized by average RQ of healthy controls (C1 and C2). The normalized RQ mean of biological replicates was determined and shown as "Expression fold change" with error bars representing the average deviation.

Western blotting

Cell lysates from LCLs were obtained by incubating $2x10^6$ cells for 30 minutes at 4 °C in 100 µL of NP-40 based buffer (NaCl: 150 mM, NP40: 0.5 %, tris(hydroxymethyl)aminomethane

(Tris)-Cl, pH 8.0: 50 mM) or radioimmunoprecipitation assay buffer (G-Biosciences, St. Louis, MO, USA) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). During the lysis, the samples were vortexed every 10 minutes. The samples were then centrifuged at 13,000 rpm at 4 °C for 10 minutes before the supernatant was collected and mixed with Laemmli sample buffer in a 1:1 ratio and boiled at 95 °C for 10 minutes. Cell lysates (20 µL per well) were separated on Bolt 4–12 % Bis-Tris Plus polyacrylamide gels (ThermoFisher Scientific) in 1x MOPS (3-[N-morpholino]propanesulfonic acid) SDS Running Buffer (ThermoFisher Scientific). SeeBlue® Plus2 Pre-stained Protein Standard (ThermoFisher Scientific) was included as a size marker. The membrane was then blocked with 5 % BSA/PBS-Tween 0.1 % (PBST) solution before being incubated overnight at 4 °C in 5 % BSA/PBST with the anti-MKL1 antibodies sc-21558 (Santa Cruz Biotechnology, Dallas, TX, USA) or HPA030782 (Sigma-Aldrich) at 1:500 and 1:250, respectively. The membrane was then incubated for 1 hour at rt with HRP-conjugated secondary antibodies mouse anti-rabbit (sc-2357; Santa Cruz Biotechnology) or mouse anti-goat (sc-2354; Santa Cruz Biotechnology) in 5 % BSA/PBST at 1:5000 and 1:10,000 respectively. The blot membrane was then developed with PIERCE ECL Western Blotting substrate and blot images were acquired using the ImageQuant LAS4000 Image system and ImageQuant LAS4000 Control Software (GE Healthcare). An anti-GAPDH antibody (sc-25778; Santa Cruz Biotechnology) was used as loading control.

Flow cytometry

Flow cytometry was performed on PBMCs, LCLs, and cultured primary B cells using the LSRFortessa X-20 (BD Biosciences) and results were processed using FlowJo v10 software (TreeStar Inc., St. Ashland, Oregon, USA). Whole blood was diluted 1:20 in FACS Lysing solution (BD Biosciences) to lyse erythrocytes, washed twice with PBS after 5 minutes of

incubation. Cultured primary B cells and LCLs were washed with PBS and analyzed in single-cell suspensions.

Analysis of primary lymphocytes was performed by first incubating the cells for 10 minutes in PBS with FcBlock (1:200; BD Biosciences) for blocking together with the Aqua Dead Cell Stain (1:300; ThermoFisher Scientific) diluted at 1:300 to determine living cells. The cells were then fixed and permeabilized with the Foxp3/Transcription Factor Fixation/Permeabilization kit (ThermoFisher Scientific). Proliferation was assessed using an anti-human Ki-67-Alexa647 antibody (Biolegend) at 1:100 in 5 % BSA/PBS for 30 minutes at rt.

For MKL1 expression in primary B cells, cells were fixed with Fix/Perm solution (BD Bioscience) for 30 minutes before being stained with rabbit anti-human MKL1 at 1:50 (Sigma-Aldrich) for 30 minutes at rt. The cells were then incubated with a goat Alexa568-conjugated anti-rabbit antibody at 1:1000 (ThermoFisher Scientific) for 30 minutes. To determine the F-actin content, the cells were, after fixation and permeabilization, incubated with a solution of biotinylated phalloidin (ThermoFisher Scientific) at 1:1000 for 30 minutes at rt. The cells were then incubated with streptavidin-APC/Cy7 (BioLegend, San Diego, CA, USA) at 1:1000 for 30 minutes at rt. All steps above were followed by three washes with PBS.

Cell cycle analysis of LCLs was performed by collecting cells directly from culture and labelling them with 5 µg/mL Hoechst-33342 (ThermoFisher Scientific) in PBS for 30 minutes at 37 °C, and then washed twice in PBS before analysis.

To determine integrin expression at the cell surface, LCLs were collected and washed twice with ice-cold PBS before being resuspended in 100 µL of 2 % BSA/FcBlock (BD Bioscience)

in PBS and incubated for 20 minutes on ice. The cells were then stained for 30 minutes on ice with an anti-human CD11a antibody (TS2/4; Biolegend) for total CD11a expression, or an anti-human CD11a antibody (Hl111; Biolegend) for inactive/closed conformation-CD11a expression, or the anti-human CD54 antibody (Biolegend). The cells were incubated with an anti-mouse-Alexa647 antibody (ThermoFisher Scientific) for 30 minutes on ice before being analyzed. All staining steps were followed by two washes with ice-cold PBS and one wash with ice-cold 2 % BSA/PBS.

To determine F- and G-actin content in two LCL samples side by side, one sample was incubated with an anti-human CD54 antibody (Biolegend) for 30 minutes on ice. The cells were washed three times with ice-cold PBS before being incubated with an anti-mouse-Alexa647 antibody (ThermoFisher Scientific) for 30 minutes on ice. The labeled sample were mixed with an unlabeled sample in the same tube and then fixed using 4 % formaldehyde (FA) for 20 minutes and permeabilized using 0.1 % Triton X-100 (Sigma-Aldrich) for 10 minutes. PBS containing 2 % BSA was used as blocking agent for 30 minutes before the cells were stained for 30 minutes with a 2 % BSA/PBS solution with DNaseI-Alexa488 (ThermoFisher Scientific) and phalloidin-Alexa568 (ThermoFisher Scientific) at 1:1000 and 1:400, respectively. The cells were washed three times for 5 minutes with PBS before being analyzed.

Fluorescence-activated cell sorting

HL1 cells were sorted according to their CD11a expression. $10x10^6$ cells or more were collected and washed twice with ice-cold PBS before being resuspended in 2 % BSA/FcBlock (1:50, BD Bioscience) in PBS and incubated for 20 minutes on ice. Then, cells were stained for CD11a for 30 minutes on ice with an anti-human CD11a antibody (TS2/4; Biolegend). Subsequently, cells were incubated with Alexa647-conjugated anti-mouse antibody

(ThermoFisher Scientific) for 30 minutes on ice before being resuspended in 1 % FBS/Gey's balanced salt solution (GBSS). The cells were sorted using a FACSAria (BD Biosciences) and collected in 50 % FBS/GBSS.

Immunocytochemistry

Glass slides were coated with a fibronectin solution (50 µg/mL in PBS; Invitrogen) for 1 hour at 37 °C (ThermoFisher Scientific) and then with anti-CD19 antibody (BD Biosciences) (20 µg/mL in PBS) for 1 hour at 37 °C before being washed twice with PBS. Cells in complete RPMI were seeded at 10^6 cells/mL and were let to adhere for 1 hour and 40 minutes at 37 °C. Cells were then fixed with 3.7 % FA for 15 minutes and permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich) for 15 minutes. 10 % FCS/PBS was used for blocking for 1 hour before F-actin was stained with phalloidin-Alexa488 (ThermoFisher Scientific) at 1:500 in 10 % FCS/PBS. Each step listed above was followed by three 5-minute PBS washes. Slides were mounted with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The cells were imaged using a Leica DMRE epi-fluorescence microscope equipped with 25x/0.75 PL-FL and 40x/1.00-0.50 PL-FL objectives. Images were acquired at rt using the Hamamatsu C4880 camera and the HiPic 32 software (Hamamatsu, Shizuoka, Japan). Original magnification x400. Brightness and contrast were adjusted using ImageJ/FIJI(4). Spread cells were defined as having lamellipodia-like structures and/or long protrusions and manually counted. For nuclei counting, cell borders were determined by phalloidin staining and nuclei identified by DAPI (4',6-diamidino-2-phenylindole) staining.

Interference reflection microscopy

To determine cell adhesion area, $0.25x10^6$ LCLs were transferred to glass cover slips coated with 50 µg/mL fibronectin (Invitrogen) and 5 µg/mL purified anti-CD19 antibody (BD

Biosciences) and thereafter incubated for 1 hour and 40 minutes at 37 °C. Afterwards, cells were fixed with 1.3 % FA and mounted with Aqua Poly/Mount (Polysciences Inc., Taipei, Taiwan). Cells were assessed with interference reflection microscopy using a Zeiss Axio Observer Z1 microscope equipped with a Zeiss analyzer slider D/A, 360° rotatable position, polarizer and a Hg/Mercury-lamp. Pictures were acquired using a 63x oil-immersion lens and the ZEN software (Zeiss, Oberkochen, Germany). ImageJ was used for data analysis of adhesion area, following normalization of brightness and contrast by setting the range equal to all pictures within the experiment, subtraction of the background using a 50 pixel rolling ball radius and normalization of threshold.

Time-lapse imaging of cell aggregation

LCLs were washed twice with PBS and resuspended at $1.6x10^6$ cells/mL in RPMI-1640 containing 0.5 % BSA and 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). 100 µL of cell suspension per sample was seeded in a flat bottom 96-well plate. The plate was placed in the microscope environmental chamber at 37 °C, incubated for 10 minutes, whereby 100 µL of warm medium was added to the wells and incubated for 10 minutes. Aggregation was imaged every 2 minutes at 37 °C with 5 % CO_2 using a Zeiss CellObserver Z1 equipped with a EC Plan-Neofluar 10x/0.30 Ph1 objective and a Zeiss Axiocan MR R3 camera. Images were processed using ImageJ/FIJI. To detect aggregates, the “Find edges” function and a Gaussian filter (Sigma: 4) were applied resulting in the aggregates displayed as bright objects over a dark background. A signal threshold was then applied to generate a binary image and to locate aggregates. To discriminate aggregates from single cells that were not filtered out by the threshold, aggregates of 5 cells or more were detected with the “Analyze particles” function (size filter: 500 μm^2–∞). Results were analyzed in Microsoft Excel (Microsoft, Redmond, Washington, USA).

DNA synthesis assessment

LCLs were seeded at $3x10^5$ cells/mL and cultured for 48 hours with a 2.5 μCi/mL ^{3}H-thymidine (1 Ci = 37 GBq) pulse the last 20 hours. Cells were harvested onto a membrane, scintillation liquid was added, and incorporated radioactivity was measured using a Wallac microplate scintillation counter (Wallac Oy, Turku, Finland).

Metaphase preparation and telomere-FISH

Telomere-fluorescent in situ hybridization was performed as previously described (5). LCLs in culture were seeded at 10^6 cells/mL and arrested in metaphase with the addition of 0.1 μg/mL colcemide (ThermoFisher Scientific) for 5 hours at 37 °C. After PBS washing, 10 mL of 0.075 M KCl was added slowly to the cells to allow them to swell, while vortexing them gently to avoid aggregation. Cells were incubated at 37 °C for 15 minutes whereafter two drops of fixative consisting of 3:1 of methanol and glacial acetic acid (Sigma-Aldrich) were added, before pelleting the cells at 700 rpm for 7 minutes and discarding supernatant. Cells were resuspended slowly in 10 mL of fixative while vortexing gently. The suspension was centrifuged at 700 rpm for 7 minutes and the fixation procedure was repeated twice before cells were dropped onto Superfrost Poly+ slides (ThermoFisher Scientific) to let cells burst and chromosomes adhere to the surface. Slides were held with surfaces facing down towards water vapor for 5 seconds and then dried on a heating block, after which they were incubated with 1 mg/mL of pepsin in H_2O at 37 °C for 10 minutes. TelG-Cy3 PNA probe (Panagene, Daejeon, South Korea) was added 1:20 in hybridization buffer (consisting of 10 mM $NaHPO_4$ pH 6.4, 10 mM NaCl, 20 mM Tris pH 7.5, 70 % FA, Denhardt's solution, and 10 μL/mL ssDNA) together with 100 μg/mL cot1 DNA, hybridized at 80 °C for 3 minutes and subsequently incubated at rt for 2 hours. Slides were washed 3 times in 0.1 % Tween/PBS preheated to 55

°C and mounted in Vectashield Antifade Mounting Medium with DAPI. Cells were imaged at rt using a Leica DMRE microscope equipped with a 100x/1.25 N PLAN objective. Images were taken using the Hamamatsu C4880 camera and HiPic 32 software (Hamamatsu). Image brightness and contrast were adjusted using ImageJ.

Inhibition of MKL1 using CCG-1423

HL0 cells were incubated with indicated concentration of CCG-1423 for 24 or 48 hours at 37 °C and 5 % CO_2. The DMSO control used was equivalent to the highest amount of DMSO of the experiment, i.e. 10 or 20 µM condition. For *in vivo* administration, NSG mice were injected with HL0 cells by subcutaneous injection. After 6 days, 50 µl of 10 µM CCG-1423 or DMSO was injected intratumorally for 6 consecutive days. The volume of the tumor was calculated at the endpoint using a caliper.

Statistical analysis

Data are expressed as means +/- SD where indicated. Statistical analyses were performed using the GraphPad Prism 7 software (GraphPad Software, La Jolla, California, USA). Statistical significance between groups was assessed by 2-tailed Student *t*-test or one-way ANOVA with post-hoc Tukey test. Differences were considered significant when $P < 0.05$. For each figure, statistical analysis details are embedded in the figure legend.

References

1. Nagy N, Adori M, Rasul A, Heuts F, Salamon D, Ujvari D, et al. Soluble factors produced by activated CD4+ T cells modulate EBV latency. Proc Natl Acad Sci U S A. 2012 Jan 31;109(5):1512-7.

2. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol. 2005 May 15;174(10):6477-89.

3. Marits P, Wikstrom AC, Popadic D, Winqvist O, Thunberg S. Evaluation of T and B lymphocyte function in clinical practice using a flow cytometry based proliferation assay. Clin Immunol. 2014 Aug;153(2):332-42.

4. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012 Jun 28;9(7):676-82.

5. Westerberg LS, Meelu P, Baptista M, Eston MA, Adamovich DA, Cotta-de-Almeida V, et al. Activating WASP mutations associated with X-linked neutropenia result in enhanced actin polymerization, altered cytoskeletal responses, and genomic instability in lymphocytes. J Exp Med. 2010 Jun 7;207(6):1145-52.

Table S1. Flow-cytometric assay of specific cell-mediated immune response in activated whole blood.*

Stimuli	Reference donor‡			Ctrl			C1			HL0			HL1			HL2		
	CD4+	CD8+	CD19+	CD4+	CD8+	CD19+	CD4+	CD8+	CD19+	CD4+	CD8+	CD19+	CD4+	CD8+	CD19+	CD4+	CD8+	CD19+
Medium	65	10	5	108	77	14	143	15	8	28	70	9	35	113	6	30	68	15
Phytohemagglutinin	93	34		602	598					171	370		119	189				
Pokeweed mitogen	10138	2653	886	16724	1496	2971	34620	1629	612	2421	8970	62	1256	5361	37	3092	7211	108
Concanavalin A	11882	6164		11467	5324		16127	3380		5847	7084		805	635		2007	6947	
Prevenar vaccine	520	42		794	95		109	51		70	108		32	55		16	73	
Tetanus toxin	85	20		83	27		17211	464		50	48		32	72		190	113	
Influenza A vaccine	1638	95		1540	237		1730	177		199	53		1079	328		103	93	
PPD	2149	165		665	131		7656	272		6988	452		2873	194		40	110	
Candida	6650	64		14077	238		8965	152		1504	103		1781	222		6477	218	
Staphylococcal enterotoxin A/B	34350	16542		52044	12633		53077	7461		14260	23689		3058	3250		8705	22103	
Varicella zoster virus	190	61		91	44		701	51		170	59		130	154		197	84	

*Numbers indicate proliferating lymphocytes as determined by increased size in forward scatter by flow cytometry. Whole blood was stimulated for 7 d in medium alone or supplemented with indicated stimuli. At d 7, numbers of $CD4^+$, $CD8^+$, and $CD19^+$ blast cells were determined. ‡Age-matched reference donor. The results should be interpreted in relation to infection and vaccination history.

Figure S1

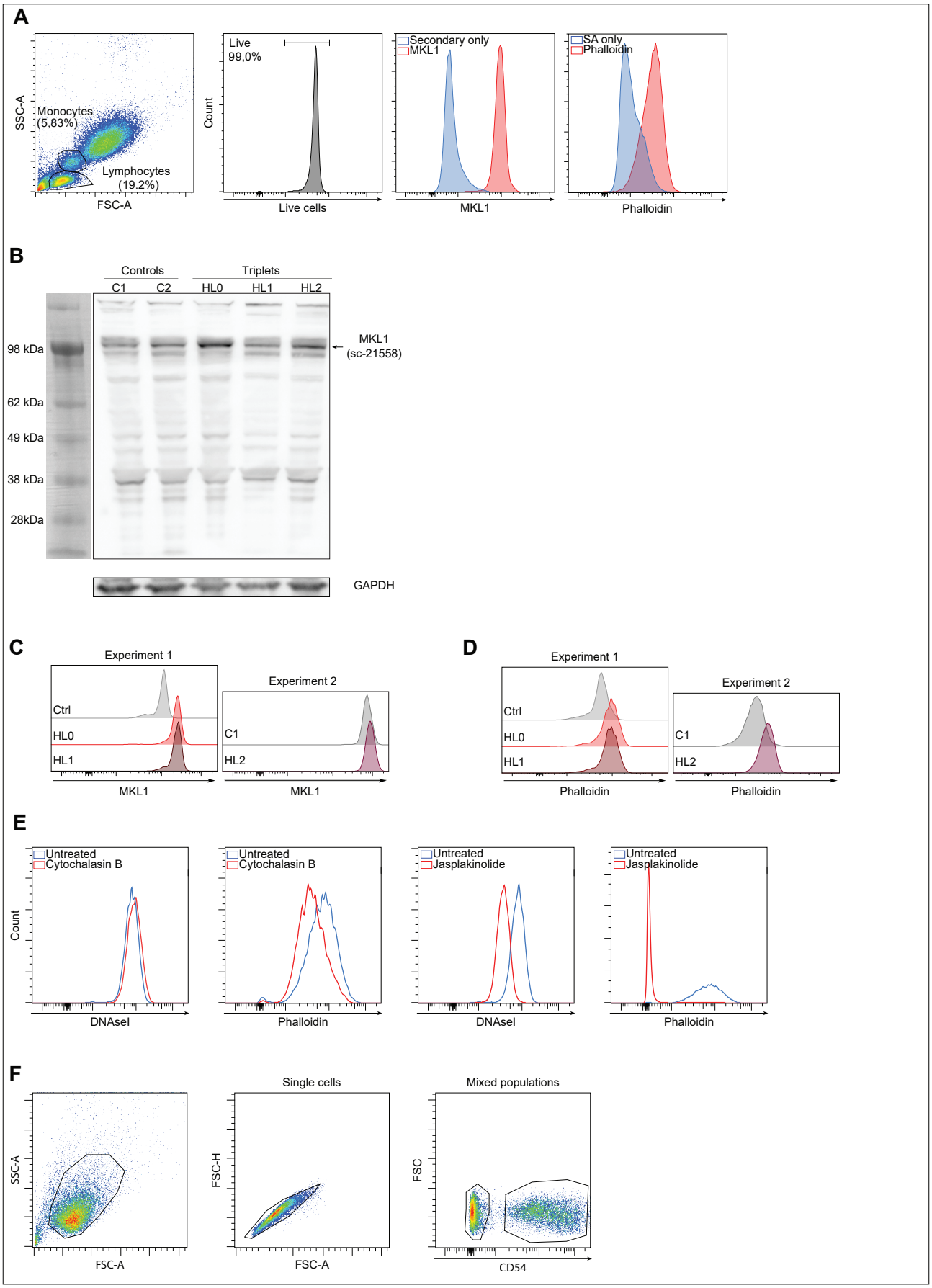

A
SSC-A
Monocytes
(5,83%)
Lymphocytes
(19.2%)
FSC-A
Live
99,0%
Count
Live cells
Secondary only
MKL1
MKL1
SA only
Phalloidin
Phalloidin
B
Controls
Triplets
C1
C2
HL0
HL1
HL2
98 kDa
62 kDa
49 kDa
38 kDa
28kDa
MKL1
(sc-21558)
GAPDH
C
Experiment 1
Experiment 2
Ctrl
HL0
HL1
C1
HL2
MKL1
MKL1
D
Experiment 1
Experiment 2
Ctrl
HL0
HL1
C1
HL2
Phalloidin
Phalloidin
E
Untreated
Cytochalasin B
Count
DNAseI
Untreated
Cytochalasin B
Phalloidin
Untreated
Jasplakinolide
DNAseI
Untreated
Jasplakinolide
Phalloidin
F
SSC-A
FSC-A
Single cells
FSC-H
FSC-A
Mixed populations
FSC
CD54

Figure S1. (A) FACS gating strategy for analysis of immune cells in PBMCs. Subpanels (left to right): gating of monocytes and lymphocytes using forward vs side scatter; gating on living cells using Aqua Dead Cell Stain; fluorescence intensity of goat Alexa568 conjugated anti-rabbit only (secondary; blue) and together with rabbit anti-human MKL1 (red); fluorescence intensity of streptavidin-APC/Cy7 only (SA; blue) and together with phalloidin-biotin (Ph; red). (B) Full Western Blot of LCLs from controls (C1 and C2) and triplets (HL0, HL1 and HL2) using the anti-human MKL1 antibodies sc-21558. (C) MKL1 expression in primary monocytes by flow cytometry. Subpanels represent experiments conducted at separate time points. (D) Phalloidin expression in primary monocytes by flow cytometry. Subpanels represent experiments conducted at separate time points. (E) Controls for detection of G-actin and F-actin using DNAseI and phalloidin staining, respectively. Flow cytometry analysis of G-actin and F actin content in LCLs from C2 treated or untreated for 30 min with 2 μM of cytochalasin B (CB; to depolymerize F-actin) or 0.5μM of jasplakinolide (Jas.; to stabilize F-actin). Note that cytochalasin B induced a small increase in DNAseI (labeling G-actin) and a decrease in Phalloidin (labeling F-actin, left panels). Jasplakinolide induced a decrease in DNAseI (right panels). Since jasplakinolide competes with phalloidin binding sites on F-actin, changes in F-actin content can't be visualized. (F) Gating strategy for analysis of F and G-actin content in LCLs. Subpanels (left to right): gating of LCLs using forward and side scatter; gating on single cells using FCS-Area vs FCS-Height; gating of the CD54-Alexa647 positive and negative populations.

Figure S2

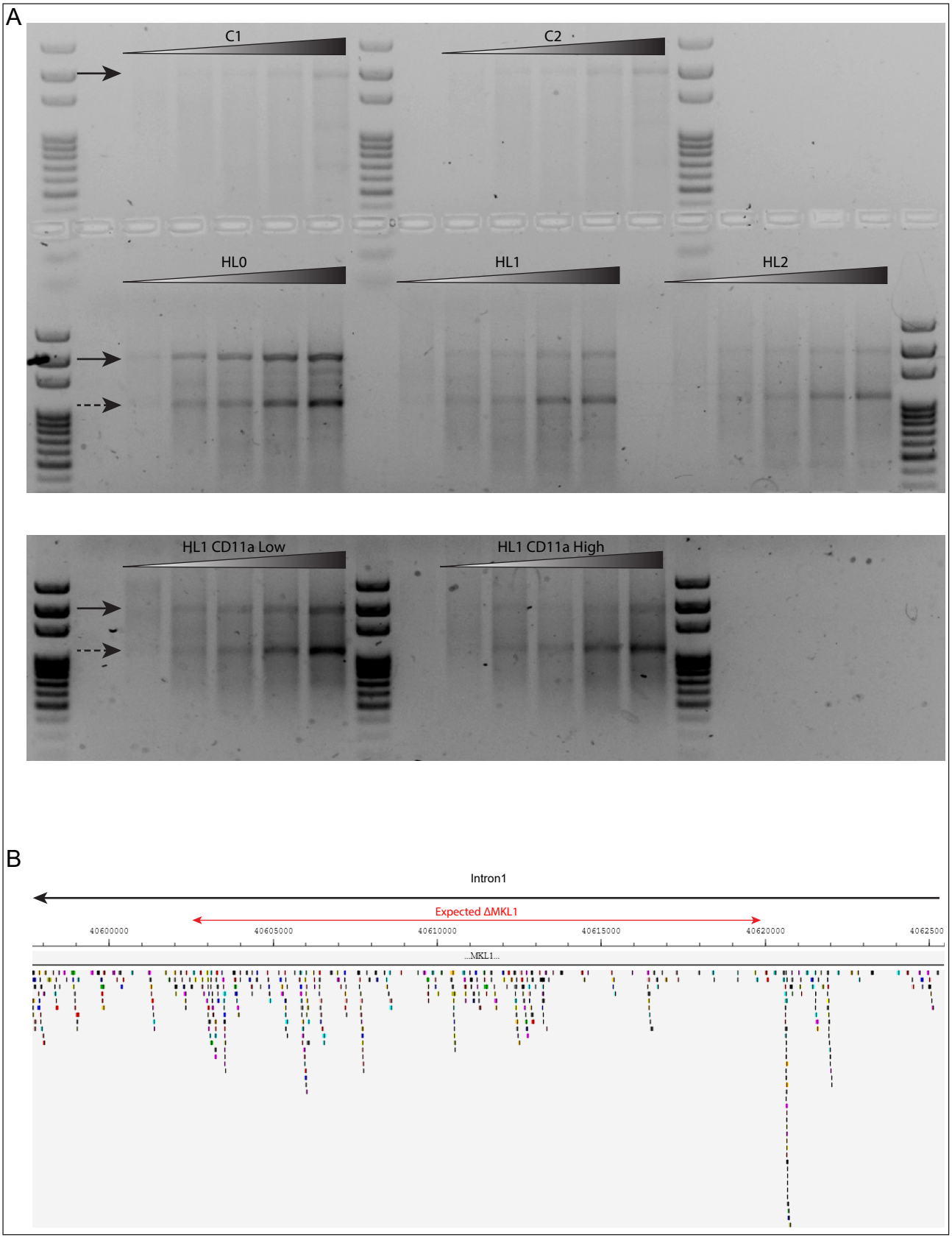
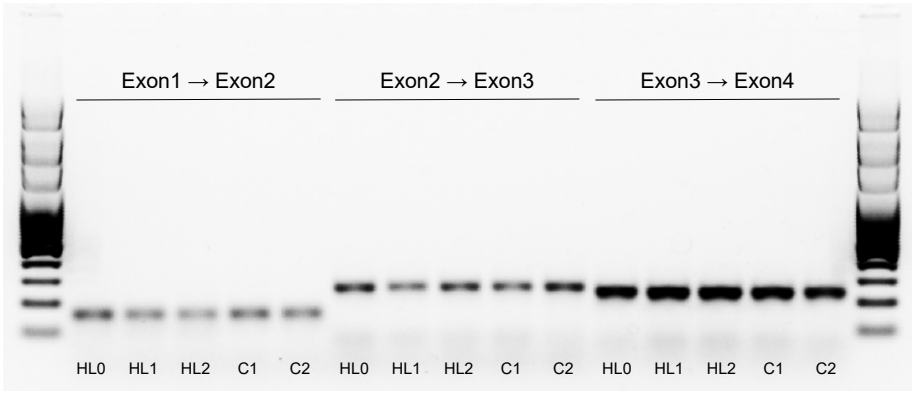

A
C1
C2
HL0
HL1
HL2
HL1 CD11a Low
HL1 CD11a High
B
Intron1
Expected ΔMKL1
40600000
40605000
40610000
40615000
40620000
40625000
...MKL1...

Figure S2. (A) PCR products using primer pair flanking the expected ΔMKL1. The PCR revealed a band present in all samples (upper band, plain arrow) and a band of lower molecular weight specific to the triplets indicating the intronic deletion (lower band, dashed arrow). The genomic DNA from each sample was subjected to 20, 25, 30, 35, 40 or 42 PCR cycles as seen on the gel from left to right. Upper image, top row: healthy donors C1 and C2; bottom row: triplets HL0, HL1 and HL2; Bottom image HL1 cells after been sorted for CD11a low and CD11a high populations. (B) Schematic of MKL1 first intron showing the transcription factors sites in the expected ΔMKL1 and flanking regions (modified from Gene Transcription Regulation Database, http://gtrd.biouml.org/).

Figure S3

A


Exon1 → Exon2
Exon2 → Exon3
Exon3 → Exon4
HL0 HL1 HL2 C1 C2 HL0 HL1 HL2 C1 C2 HL0 HL1 HL2 C1 C2

B

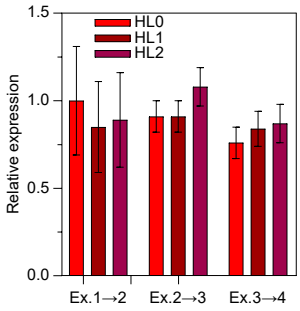

HL0
HL1
HL2
Relative expression
1.5
1.0
0.5
0.0
Ex.1→2
Ex.2→3
Ex.3→4

Figure S3. Detection of exon boundaries between exons 1 and 2, 2 and 3, 3 and 4, 4 and 5 and 5 and 6 in controls C1 and C2 and in triplets HL0, HL1 and HL2. (A) Exon boundaries were amplified y PCR and run on agarose gel. (B) Exon boundaries were detected by RT-qPCR. Results were normalized to C1 and C2 mean value.

Figure S4

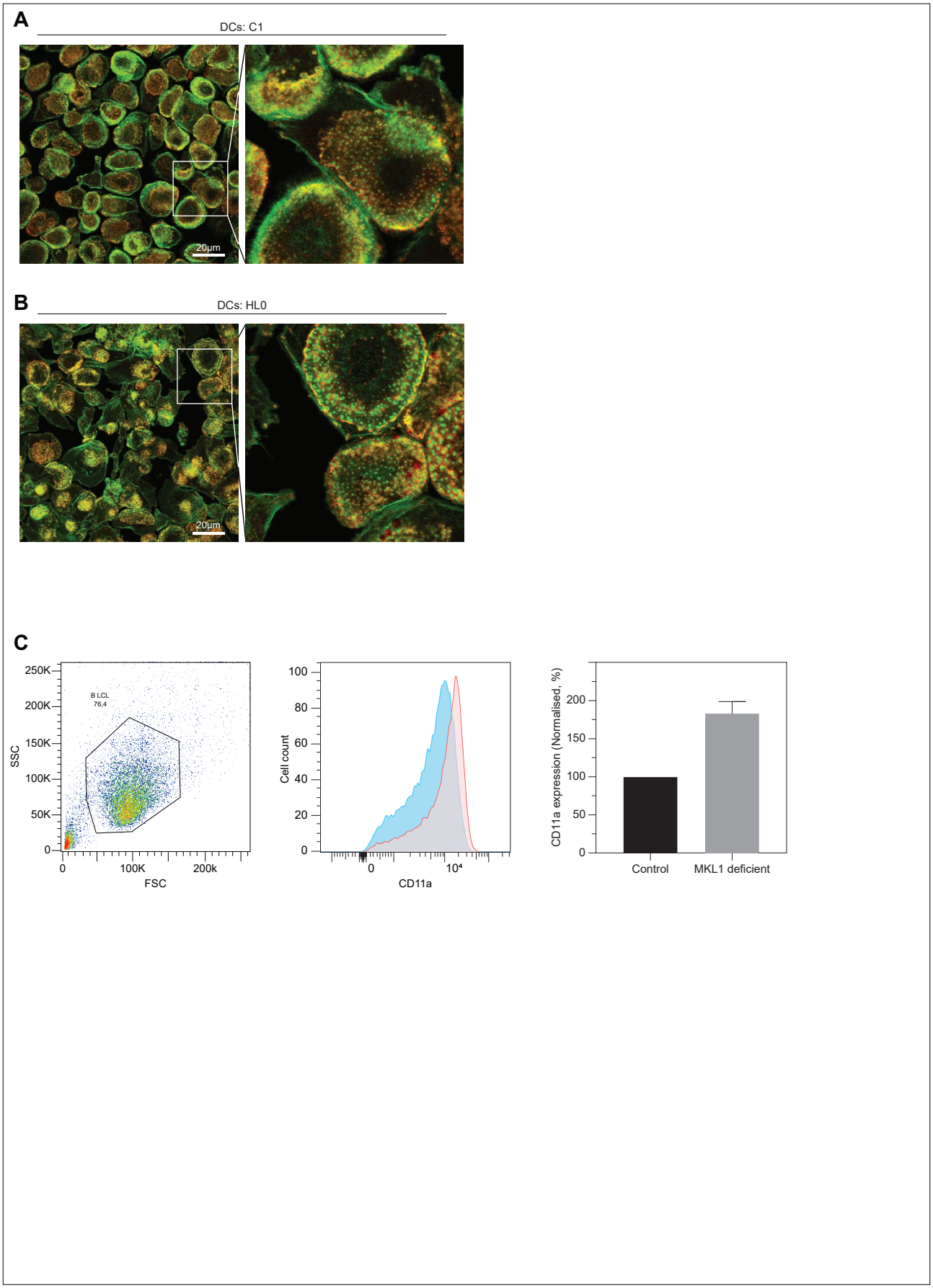
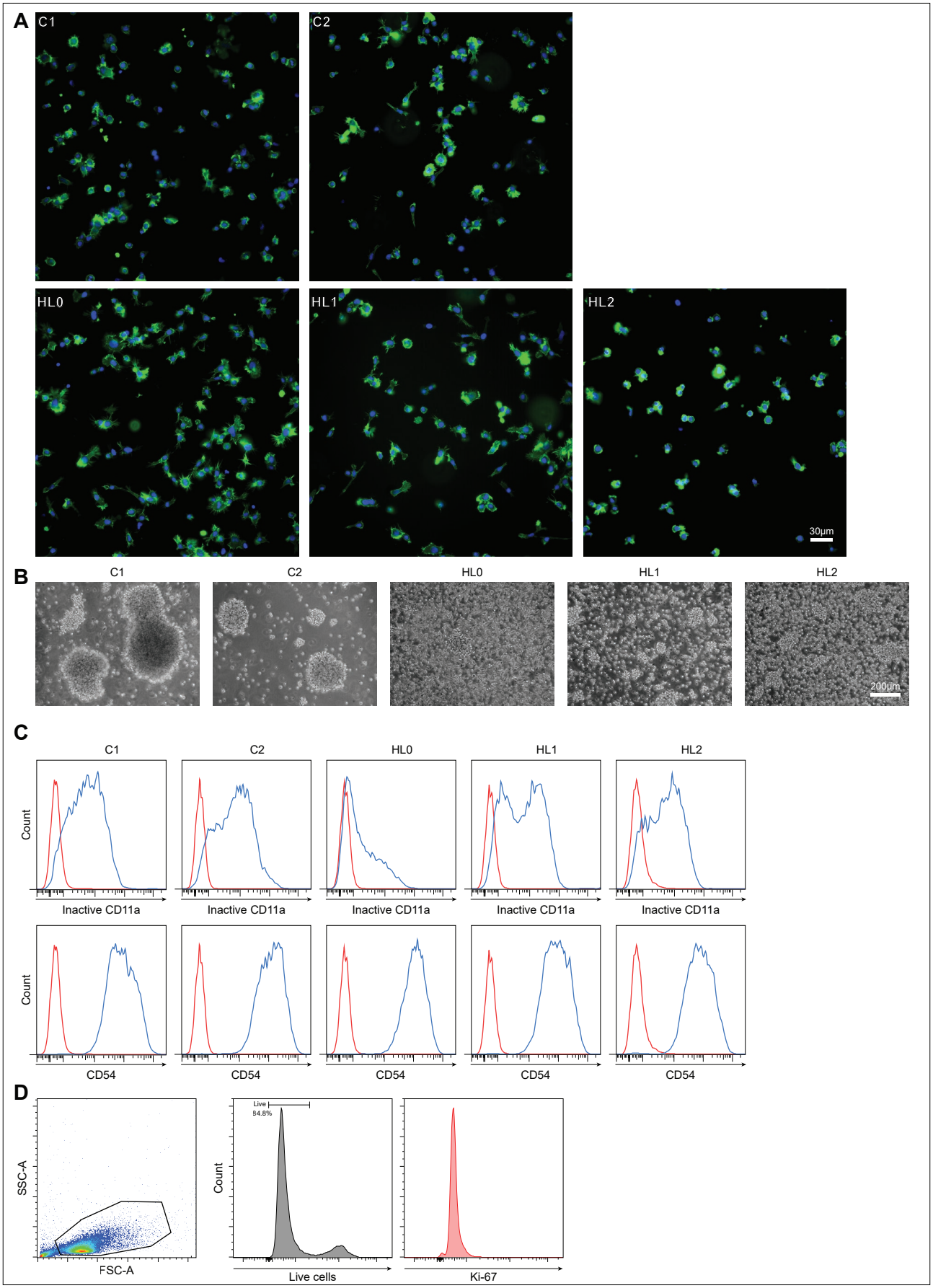

A
DCs: C1
20µm
B
DCs: HL0
20µm
C
B LCL
76.4
SSC
FSC
250K
200K
150K
100K
50K
0
100K
200k
Cell count
CD11a
10⁴
CD11a expression (Normalised, %)
Control
MKL1 deficient

Figure S4. (A) and (B) Representative images of monocytes derived dendritic cells from C2 control and triplet HL0, respectively . Cells were stained with AlexaFluor-488 tagged Phalloidin (green) together with a mouse anti-vinculin antibody followed by an anti-mouse AlexaFluor-568 antibody (red). Cells were images using Leica SP5 confocal microscope equipped with an HCX PL APO CS 63.0x N.A. 1.40 Oil UV objective. Original magnification: x630. (C) CD11a expression levels on control (blue histogram) and MKL1 deficient (red histogram) B LCLs. First graph, gating of the LCLs; second graph, CD11a expression (histograms); third graph, quantification of CD11a expression.

Figure S5


A
C1
C2
HL0
HL1
HL2
30μm
B
C1
C2
HL0
HL1
HL2
200μm
C
C1
C2
HL0
HL1
HL2
Count
Inactive CD11a
Count
CD54
D
SSC-A
FSC-A
Live
84.8%
Count
Live cells
Ki-67

Figures S5. (A) Representative immunocytochemistry images of B cells adhering to glass slides coated with fibronectin and anti-CD19. Microscope: Leica DMRE; objective: 25x/0.75 PL-FL. Cells stained with phalloidin-Alexa488 (green) and mounted in Vectashield Antifade Mounting Medium with DAPI (blue). Images taken at room temperature using the Hamamatsu C4880 camera and LAS AF acquisition software; brightness and contrast adjusted using ImageJ. Original magnification: 250X. (B) Representatives images of LCLs aggregates after 24hs in culture at 37oC. Images taken at room temperature using a Motic AE30 microscope equipped with 10x/0.25 Plan Achromat Phase lens and a Moticam 580. Original magnification: 100X. (C) Surface expression of inactive CD11 and CD54 in the controls and triplets LCLs. (D) FACS gating strategy for cultured B cells. Subpanels (left to right): gating of B cells using forward vs side scatter; live/dead staining using AmCyan; fluorescence intensity of Ki67-APC.

Figure S6

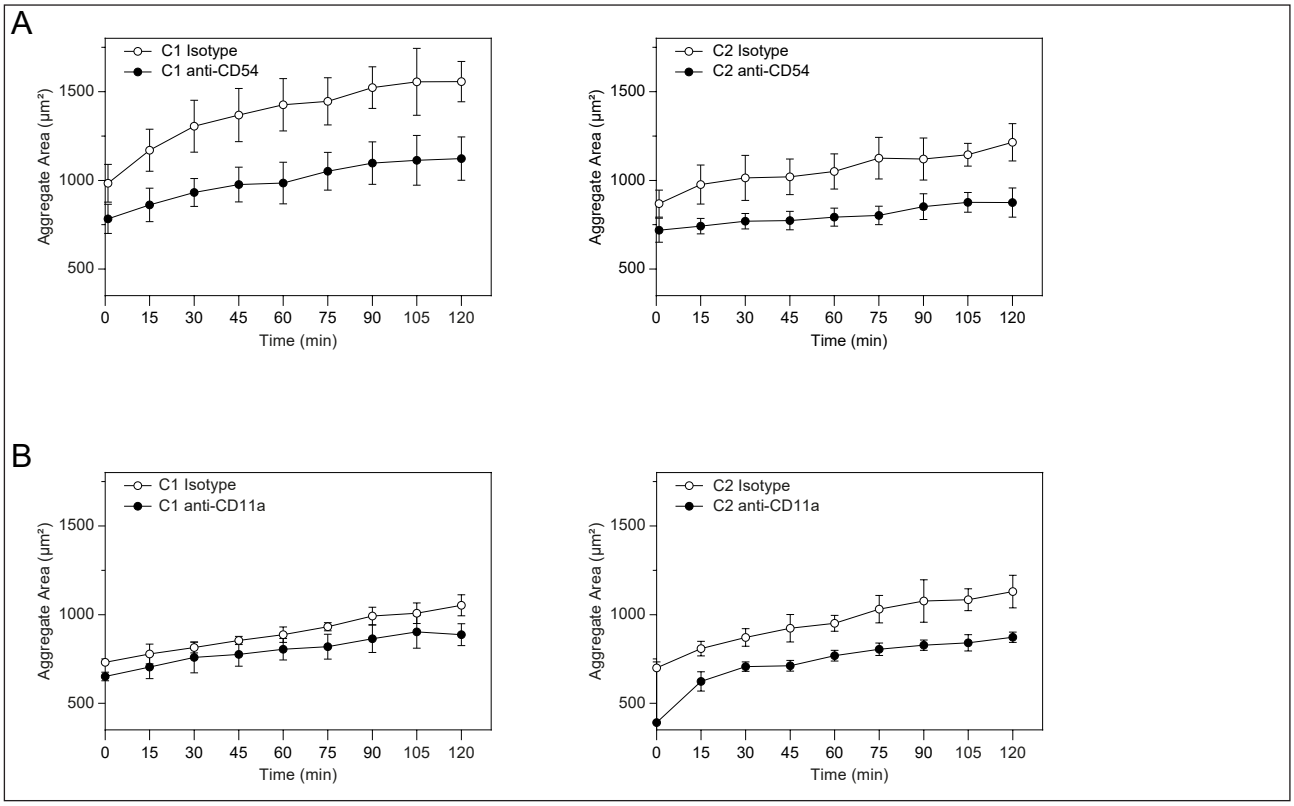

A
C1 Isotype
C1 anti-CD54
C2 Isotype
C2 anti-CD54
B
C1 Isotype
C1 anti-CD11a
C2 Isotype
C2 anti-CD11a
Aggregate Area (µm²)
Time (min)
500
1000
1500
0
15
30
45
60
75
90
105
120

Figure S6. Aggregation of C1 and C2 cell was monitored over 2 hours by time lapse microscopy in presence of blocking antibodies directed against CD54 and CD11a or in presence of isotype control antibody. (A) Average area of C1 and C2 cells aggregates in presence of 10µg/ml of anti-CD54 antibody or (B) anti-CD11a antibody.

Figure S7

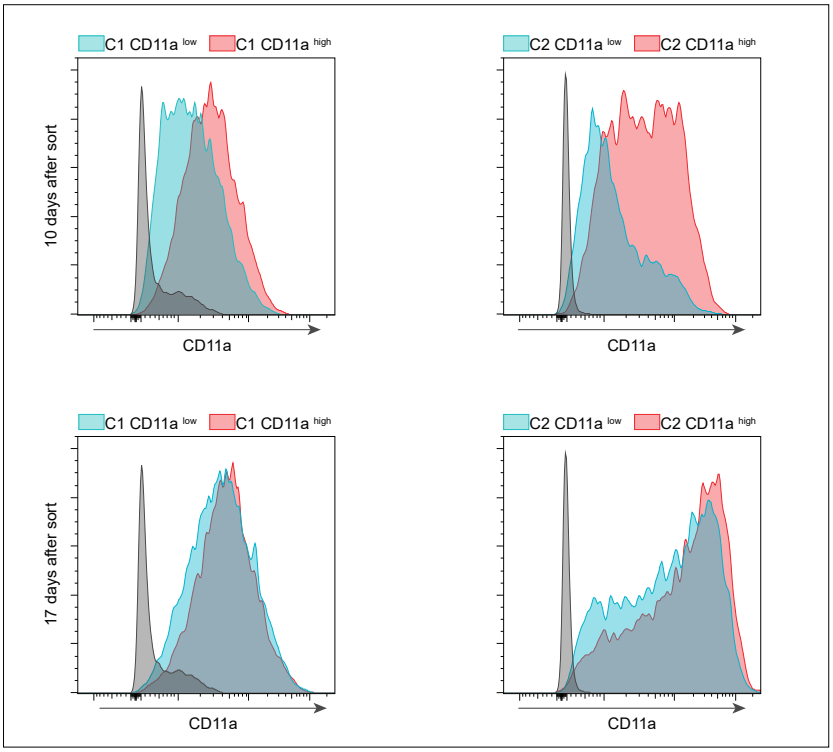

C1 CD11a low
C1 CD11a high
C2 CD11a low
C2 CD11a high
10 days after sort
17 days after sort
CD11a

Figure S7. CD11a expression in C1 and C2 cells sorted for CD11a low and high expression. Expression of CD11a was assessed by flow cytometry 10 and 17 days after sort.

Figure S8

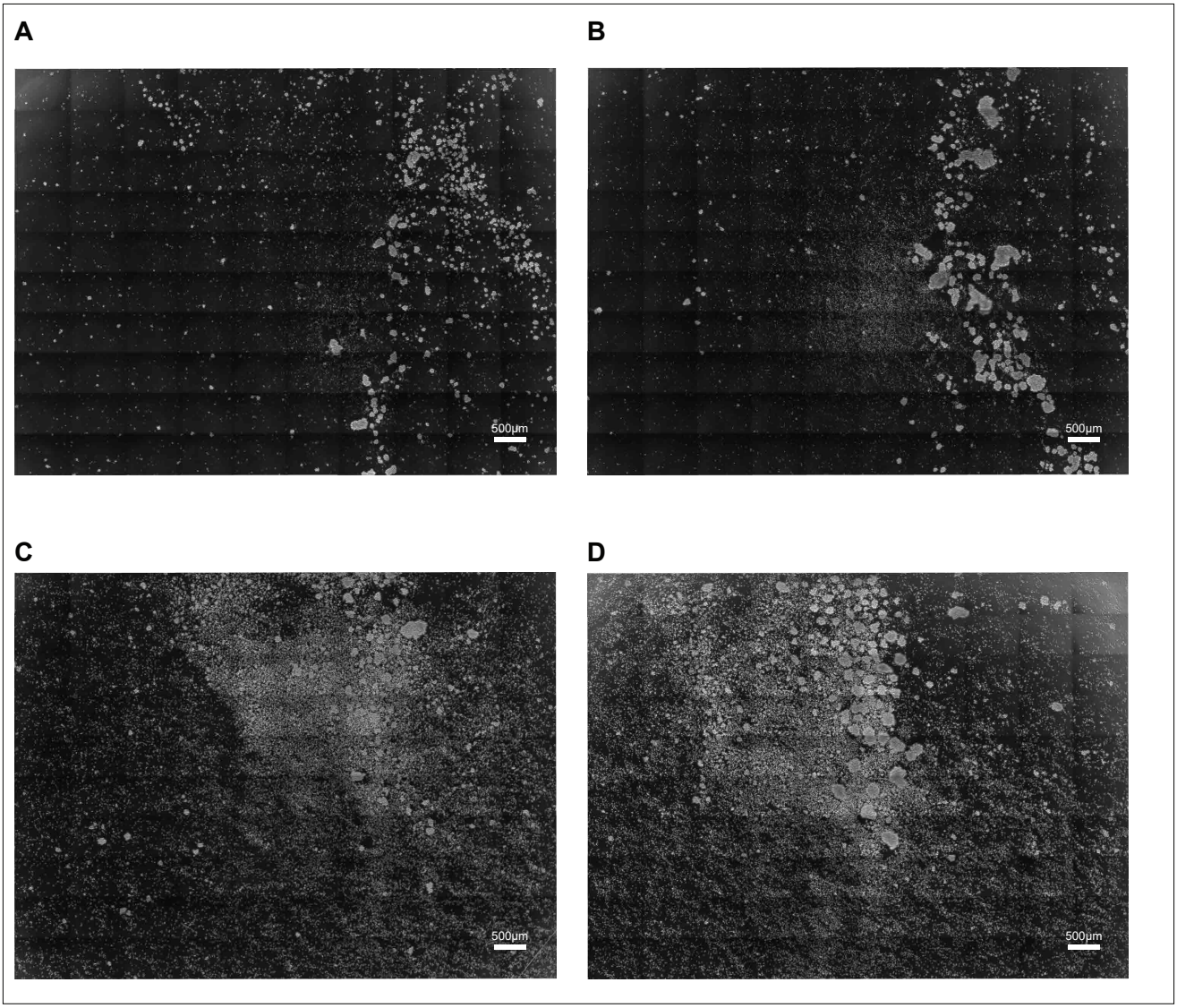


Figure S8. Aggregation of C1 and C2 sorted for CD11a low and high expression. On day 16, 200 000 cells of (A) C1 CD11a^{low}, (B) C1 CD11a^{high}, (C) C2 CD11a^{low} and (D) C2 CD11a^{high} were plated in a 24 wells plate and left at 37^{o}C for 24 hours before being imaged using a 10x objective. Image composed of 100 images stitched together.

Figure S9

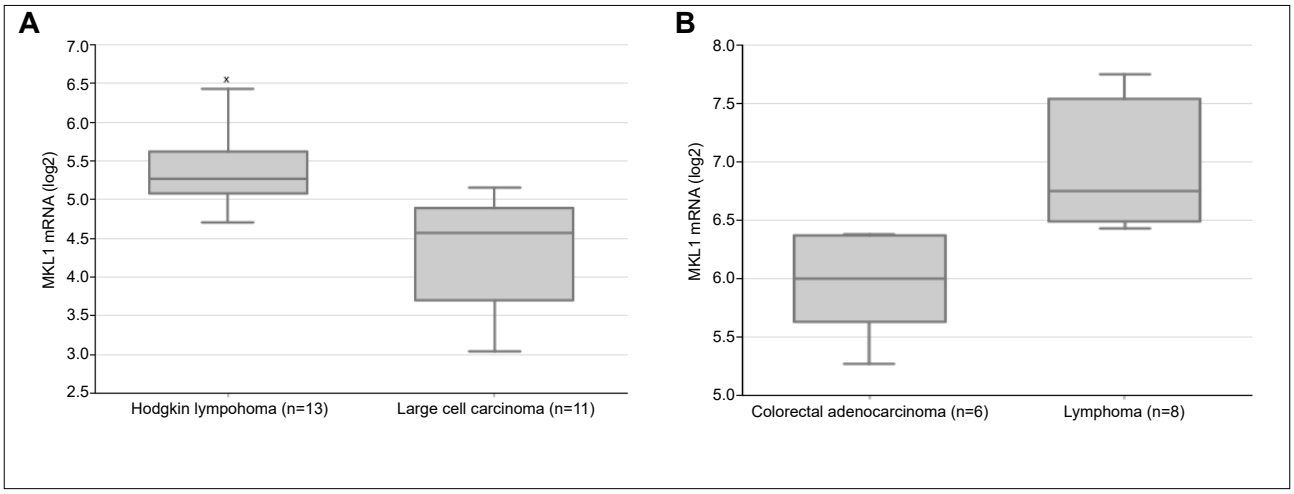

A
MKL1 mRNA (log2)
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
Hodgkin lympohoma (n=13)
Large cell carcinoma (n=11)
B
MKL1 mRNA (log2)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
Colorectal adenocarcinoma (n=6)
Lymphoma (n=8)

Figure S9. Expression of MKL1 in (A) HL and carcinoma and (B) in lymphoma and colorectal adenocarcinoma patients from the R2 database (R2: Genomics Analysis and Visualization Platform; http://r2.amc.nl).