SUPPLEMENTARY APPENDIX

Attenuated measles virus controls pediatric acute B-lineage lymphoblastic leukemia in NOD/SCID mice

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Online Supplementary Methods

ALL cell lines, xenografts and patient samples. The ALL cells lines Jurkat, CCRF-CEM, MOLT-4, REH, RS4;11 and NALM-6 were obtained from DSMZ (Braunschweig, Germany).

Xenograft ALL cells propagated in mice were procured from spleen tissue, processed and analyzed as described below for the human ALL NOD/SCID mouse model. Purity of samples was greater 90%, as determined by FACS analysis after staining for $CD45^{+}Ly5^{-}$ leukemic cells.

Patient ALL samples were obtained at diagnosis from pediatric patients with *de novo* ALL. The majority of patients was enrolled into the ALL-BFM study protocols. Patient characteristics for the xenografts treated with MV-Edm are listed in *Supplementary Table S1*. This study was approved by the ethical review board of the University Medical Center Ulm in accordance with the declaration of Helsinki.

For *in vitro* studies cells lines, xenografts and patient cells were cultured in RPMI 1640 (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS; Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Biochrom, Berlin, Germany).

Human PBMC. Human PBMC were isolated from buffy coats of healthy donors younger than 25 years by density gradient centrifugation (Biocoll separating solution; Biochrom). Untouched B and T lymphocytes were isolated using MACS isolation kits (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Lymphocytes were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Biochrom), 1 mM sodium pyruvate, 2 mM L-glutamin, 100 U/ml, penicillin and 100 µg/ml streptomycin (all from Biochrom).

Measles immune serum. Serum with anti-measles IgG at a protective concentration (index 7.01, as determined by the Institute for Virology, University Medical Center Ulm using standard procedures) was procured from a healthy donor. Serum was isolated by centrifugation at 3400 g for 20 min and inactivated at 56 °C for 30 min.

Virus production, titration and infection procedures. MV-Edm was propagated in Vero cells (ATCC, Wesel, Germany), infected at a MOI of 0.05 in OptiMEM (Life Technologies) at 37 °C. After 3 h medium was changed to DMEM supplemented with 5% FCS (Life Technologies) 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Biochrom). Cells were incubated at 37 °C for 1 d before being transferred to 32 °C for another 2 d. Cells were scraped and viral particles released by two cycles of snap freezing in liquid nitrogen and subsequent thawing. Viral titers were determined by 50% end-point dilution assays ($TCID_{50}$) on Vero cells.

ALL cell lines, xenografts, patient samples, PBMC, HSC, and isolated B and T cells were infected at a MOI of 1 in serum-free RPMI 1640 (Life Technologies), HSC in serum-free IMDM (Life Technologies) at 37 °C for 3 h. Medium was then changed depending on the experiment.

Cell death assays. To assess MV-Edm-induced cytotoxicity 4 x 10^4 cells from cell lines and 8 x 10^4 PBMC, patient cells or xenograft cells were seeded in 96-well plates and incubated with MV-Edm at a MOI of 1, with heat-inactivated MV-Edm or with medium. Cell viability was determined by counting small and granular cells, i.e. cells showing decreased forward scatter (FSC) and increased side scatter (SSC) by FACS analysis.

To determine dose-dependency of MV-Edm-induced cell death Jurkat cells were seeded in 96-well plates (4 x 10^4 cells per well) and infected with MV-Edm at increasing MOIs. Cell viability was determined 3 and 72 h after infection by FACS FSC/SSC.

To assess for syncytia formation Jurkat cells were seeded in 96-well plates (4 x 10^4 cells per well), infected with MV-Edm at a MOI of 1 and analysed 48 h and 72 h later by light microscopy.

Early apoptosis and non-apoptotic cell death were simultaneously assessed in 4 x 10^4 cells from ALL cell lines and 8 x 10^4 xenograft cells per well in 96-well plates after treatment with MV-Edm at a MOI of 1. Cells were stained with FITC-labeled Annexin V according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) and propidium iodide (PI) and analyzed by FACS. T lineage ALL cell lines and xenografts were assessed after 72 h while B lineage ALL cell lines were assessed at 96 h because of their protracted cell kill kinetics.

Colony-forming assay of ALL cell lines. REH and Jurkat cells infected with MV-Edm at a MOI of 1 were seeded in 6-well plates (1 x 10^3 cells per well) in methylcellulose-containing medium MethoCult H4100 (Stemcell Technologies, Grenoble, France) supplemented with 10% FCS (Life Technologies), 2 mM L-glutamin, 200 U/ml penicillin and 200 µg/ml streptomycin (Biochrom). Colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to the manufacturer's instructions (Roche) and counted microscopically after 14 d of incubation.

Replication of MV-Edm in Jurkat cells and PBMC. 2.5 x 10^6 Jurkat cells were seeded in 25 cm^2 flasks and infected with MV-Edm at a MOI of 1 or were left uninfected. Cells were harvested after 3, 24, 48 and 72 h. Viral particles were released by two cycles of snap freezing in liquid nitrogen and subsequent thawing. Viral titers were determined by 50% end-point dilution assays ($TCID_{50}$) on Vero cells after 72 h of incubation.

To compare replication in Jurkat cells and PBMC 4 x 10^4 cells per well were seeded in 96-well plates and infected with MV-Edm at a MOI of 1 or were left uninfected. After 72 h viral particles were released by two cycles of snap freezing and thawing. 15 µl of cleared lysate was added to 2.25 x 10^5 Vero cells in a 6-well plate and syncytia formation was documented after staining with crystal violet.

Spread and bystander effect of MV-Edm. To determine spread of MV-Edm Jurkat and REH cells were seeded in 25 cm^2 flasks (2 x 10^6 cells per flask) and infected with MV-Edm at a MOI of 1. 3 h after infection cells were washed. Infected Jurkat or REH cells were mixed with non-infected Jurkat or REH cells at increasing ratios of 1:20, 1:10, 1:5 and 1:2. The mixtures were seeded in 96-well plates at 4 x 10^4 cells per well. Cell viability was determined after 72 h by FACS FSC/SSC.

To assess spread of MV-Edm in the presence of antibodies against MV Jurkat and REH cells were seeded in 25 cm^2 flasks (2 x 10^6 cells per flask) and infected with MV-Edm at a MOI of 1. 3 h after infection cells were washed. Non-infected cells were seeded in 96-well plates (4 x 10^4 cells per well), infected cells were added at increasing ratios of 1:20, 1:10, 1:5 and 1:2 in the presence or

absence of 10% measles immune serum in the culture medium. Cell viability was determined by FACS FSC/SSC.

Flow cytometry of cell surface antigens. To assess HSC frozen human cord blood cells were simultaneously labeled with phycoerythrin (PE)-conjugated mouse monoclonal antibody against human CD34 (BD, Heidelberg, Germany) and with flourescein (FITC)-conjugated mouse anti-human CD38 (BD) or with unspecific PE- or FITC-conjugated anti-IgM as isotype control (both from BD). The $CD34^+CD38^-$ cell fraction was considered to represent the HSC compartment.

To determine CD46 expression on ALL cell lines (CCRF-CEM, Jurkat, MOLT 4, NALM-6, REH and RS4;11), PBMC (from buffy coats of 16 donors), HSC (3 donors) and xenografts #6, #13, #15 and #19 were stained with Pacific Blue-conjugated anti-CD46 (Biozol, Eching, Germany) or with Pacific Blue-conjugated anti-isotype antibody as control (BD).

Bone marrow cells from ALL patients at diagnosis were probed using Pacific Blue-conjugated anti-CD46 (Biozol), AmCyan-conjugated anti-CD19, PE-Cy7-conjugated anti-CD10 and PerCP-conjugated anti-CD45 (all from BD). $CD19^+CD10^+$ cells were considered as ALL blasts, $CD34^+CD19^-$ cells as HSC and $CD45^+CD19^-$ cells as non-leukemic leukocytes.

To determine $CD150^+$ cells ALL cell lines, PBMC, HSC and xenografts #6, #13, #15 and #19 were labeled with PE-conjugated anti-human CD150 (BD) or with PE-conjugated anti-isotype as control (BD).

To define ALL blasts in mouse peripheral blood or tissues cells were simultaneously stained with peridinin-chlorophyll (PerCP)-conjugated anti-human CD45 and with PE-conjugated monoclonal rat anti-mouse CD45 (Ly5) antibody (BD). The ALL cell line REH was used as positive control.

All antibodies were incubated for 20 min in PBS (Biochrom), containing 5% BSA (Serva, Heidelberg, Germany) and 0.5% sodium azide (Sigma). Data were acquired on a FACScan Flow Cytometer or a LSRII Flow Cytometer (both from BD) and analyzed with FloJo software (Tree Star, Olten, Switzerland) or CellQuest software (BD).

Colony-forming cell assay of hematopoetic cells. $CD34^+CD38^-$ cells were sorted from 2 x 10^6 frozen human cord blood cells. Cells were infected for 3 h (MOI of 1) or were left uninfected at 37 °C in IMDM (Life Technologies) supplemented with Fms-related tyrosine kinase 3 ligand 1 ng/ml, stem cell factor 1 ng/ml, interleukin-3 0.2 ng/ml, interleukin-6 0.2 ng/ml and granulocyte colony-stimulating factor 0.2 ng/ml (all from Immunotools, Friesoythe, Germany). 1 x 10^3 infected cells were seeded in 3 cm dishes containing methylcellulose-based medium MethoCult H4434 (Stemcell Technologies). Colonies (CFU-GEMM = Colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte; CFU-GM = Colony forming unit-granulocyte, macrophage; BFU-E = Burst forming unit-erythroid) were counted microscopically after 14 d of incubation.

Quantitative RT-PCR. RNA was isolated using the RNeasy mini kit (Quiagen, Hilden, Germany) and treated with DNase I (Life Technologies). cDNA was generated using 1 µg of RNA, random hexamers and the SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative real-time PCR was performed using LightCycler Fast Start DNA Master SYBR Green I (Roche) and a LightCycler PCR machine (Roche). Primer sequences were as follows ("s" indicates sense and "a" denotes antisense primer):

RIG-I s 5'- ACCAGAGCACTTGTGGACGCT-3'

RIG-I a 5'- TGCCGGGAGGGTCATTCCTGT-3'

MDA-5 s 5'- GGCACCATGGGAAGTGATT-3'

MDA-5 a 5'- ATTTGGTAAGGCCTGAGCTG-3'

MAVS s 5'- GAGACCAGGATCGACTGCGGGC-3'

MAVS a 5'- AGAGGCCACTTCGTCCGCGA-3'

IRF-3 s 5'- GGACGCTCACCACGCTATGCC-3'

IRF-3 a 5'- GGCACGTGGGCACAACCTTGAC-3'

IFNAR1 s 5'- AGTTCAGTGGCTCCACGCCT-3'

IFNAR1 a 5'- TCCATCAGATGCTTGTACGCGG-3'

IFNAR2 s 5'- TGCTCACACAATTTCTGGCTGGC-3'

IFNAR2 a 5'- ACTGCTTGCTCATCACTGTGCTCT-3'

IFN-β s	5'- TCGAAGCCTTTGCTCTGGCACA-3'
IFN-β a	5'- TGCGGCGTCCTCCTTCTGGA-3'
β-actin s	5'- TCACCCTGAAGTACCCCATC-3'
β-actin a	5'-TAGCACAGCCTGGATAGCAA-3'
IRF-1 s	5'-ACACAGGCCGATACAAAGCA-3'
IRF-1 a	5'-TGGAATCCCCACATGACTTCC-3'
ISG-15 s	5'-GCGAACTCATCTTTGCCAGT-3'
ISG-15 a	5'-AGCATCTTCACCGTCAGGTC-3'
IFIT1 s	5'-AGCTTACACCATTGGCTGCT-3'
IFIT1 a	5'-TGCTGTAAATTAGGCAGCCGT-3'

cDNA was denaturated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 58 °C (IFNAR1, IFNAR2, MDA-5, RIG-I, β-actin) or 60 °C (IFN-β, MAVS, IRF-1, ISG-15 and IFIT1) for 10 s and 72 °C for 10 s. Expression of genes was normalized to expression of β-actin.

Human ALL NOD/SCID mouse model. Unconditioned NOD/SCID mice (Charles River, Kißlegg, Germany) with a median age of 8-10 weeks (w) were used. Housing and treatment of animals were in accordance with state guidelines. Mice were kept in a specific pathogen-free environment and provided with autoclaved water and food without restriction.

1 x 10^7 primary ALL cells propagated in NOD/SCID mice were injected into a lateral tail vein. After grafting blood samples were evaluated regularly at 1- or 2-w intervals for the presence of human leukemia cells by determining $CD45^+Ly5^-$ cells using flow cytometry. The proportion of leukemic cells, reflecting the leukemic burden, was calculated as percent of nucleated cells.

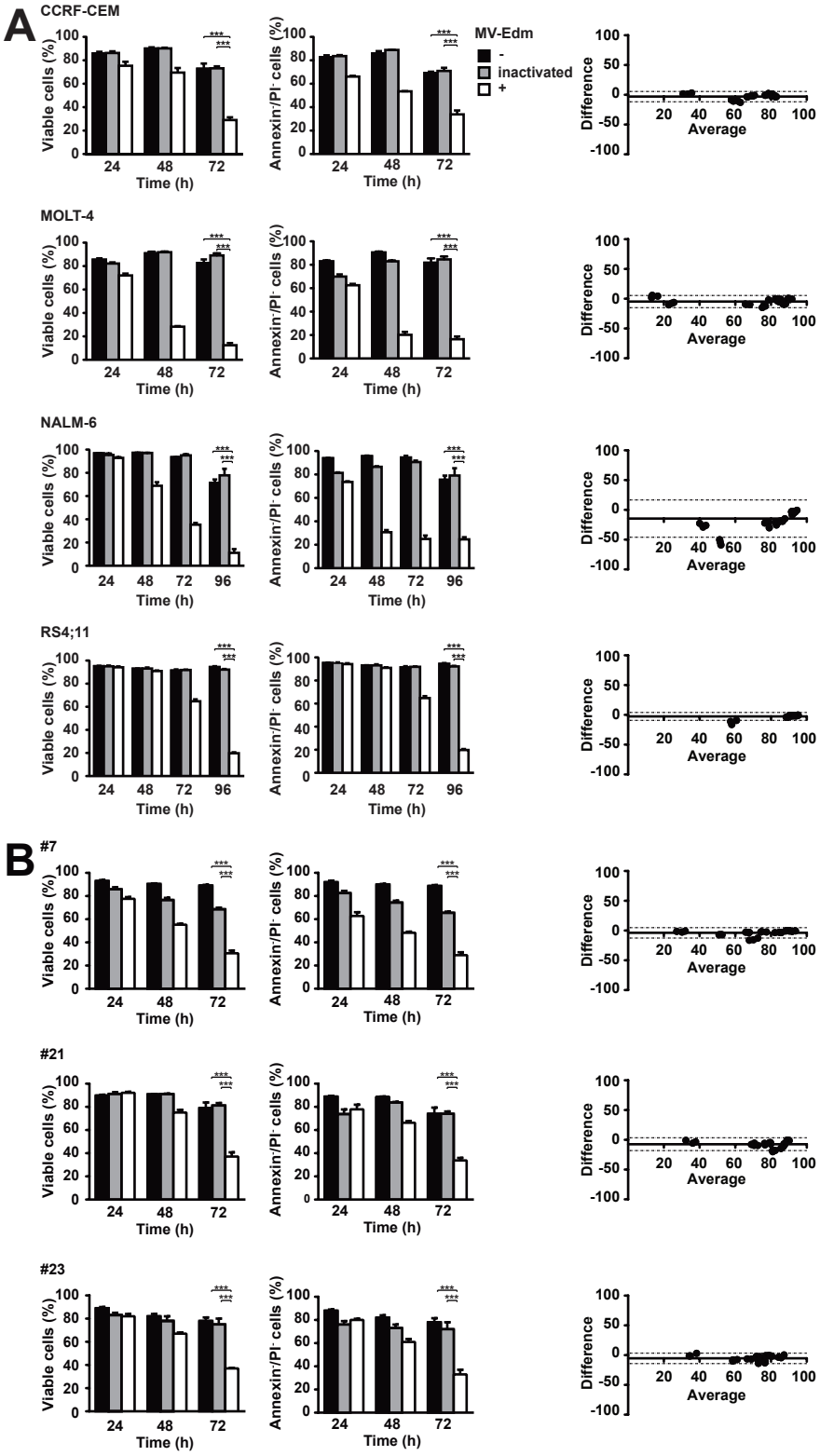
At necropsy cell suspensions from spleen, bone marrow and meninges were prepared and brains were procured. Spleen and meningeal tissue was minced and strained through 70 µm nylon cell sieves (Falcon, BD) in Hank's balanced salt solution (HBSS, Gibco Invitrogen, Karlsruhe, Germany), mononuclear cells of the spleen were isolated by density gradient centrifugation (Biocoll separating solution; Biochrom). Bone marrow cells were isolated flushing the femoral cavity with HBSS. Meningeal cells were procured from the meninges of the skull. After erythrocyte lysis cell

suspensions were analyzed for the presence of $CD45^{+}Ly5^{-}$ leukemic cells by FACS analysis.

***In vivo* treatment.** NOD/SCID mice were transplanted with B-cell precursor ALL xenograft cells from patient #6, #13, #15 and #19. Mice were monitored as described above for the human ALL NOD/SCID mouse model. Once peripheral blast counts reached 5-20%, mice received injections into the tail vein of 2 x 10^5 pfu MV-Edm ("+"), 2 x 10^5 pfu heat-inactivated MV-Edm ("inactivated") or PBS ("-"), all at a volume of 200 µl, given each day (d) for 5 d. Mice of control groups ("-" or "inactivated") were sacrificed for analysis when they clearly showed clinical signs of disease (i.e. impaired general condition, lethargy, ruffled fur or abnormal posture) or they died spontaneously of their disease, precluding histological analysis because of autolysis. Mice of the treatment group were killed and analyzed when their survival time after therapy was three times the survival time after injection of the animals in the "- / inacitivated" control groups or when they reached the end of their natural lifespan of 250 d.

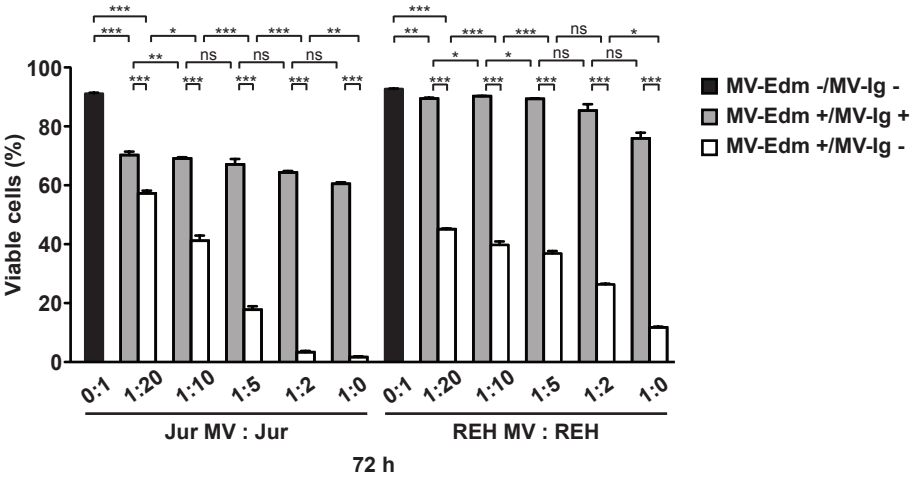
Histology. Hematoxylin eosin stainings were performed on serial sections of formalin-fixed, paraffin-embedded tissue. Briefly, paraffin sections were dewaxed with xylene and passed through decreasing concentrations of acetone (100%, 70%, 40%; 10 min each). For immunohistochemistry the following antibodies were used according to standard protocols: Ki-67 (clone MIB-1, DAKO, Carpinteria, CA; 1:200), cleaved caspase-3 (Cell Signaling, Danvers, MA; 1:100) and MV-H protein (clones CV1, CV4, Merck Millipore former Chemicon, Billerica, MA, dilution 1:200). For antigen retrieval, sections were treated by microwave irradiation in citrate buffer (pH 6) for 20 min and then incubated with the primary antibody. For detection of bound antibodies the Envision kit was used (DAKO).

Statistical analysis. Statistical analysis was performed using GraphPad Prism (La Jolla, CA).



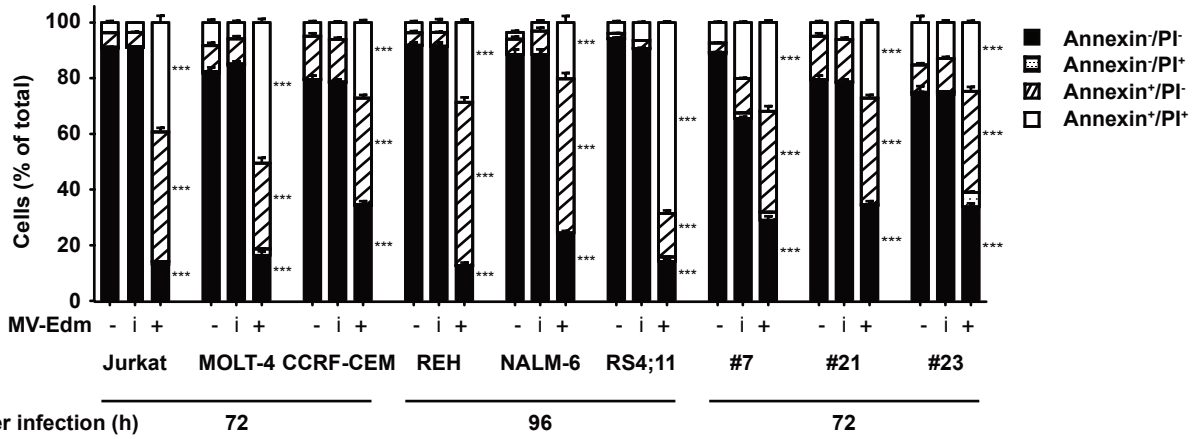
Online Supplementary Figure S1. **ALL cell lines and ALL xenografts are efficiently killed by MV-Edm**

ALL cell lines **(A)** and human ALL xenografts isolated from spleens of NOD/SCID mice **(B)** were incubated with MV-Edm at a MOI of 1 ("+"), with heat-inactivated MV-Edm ("inactivated") or with medium ("-"). Cell viability was determined by FACS using forward/side scatter analysis, left panels, and Annexin V staining and uptake of propidium iodide (PI), middle panels. Xenograft #23 refers to primary ALL #2 (Figure 2A). Results are means +SD of triplicates and are expressed as percentage of total cells. *** $p < 0.001$ using the unpaired t-test. For cell lines, three independent experiments were performed, with similar results. Agreement between forward/side scatter and Annexin V/PI analyses was determined using Bland-Altman plots (right panels). Mean difference between the methods (bias) is indicated by a solid line, ± 1.96 standard deviations of the differences (95% limit of agreement) by dashed lines. Average denotes average of results of both methods.

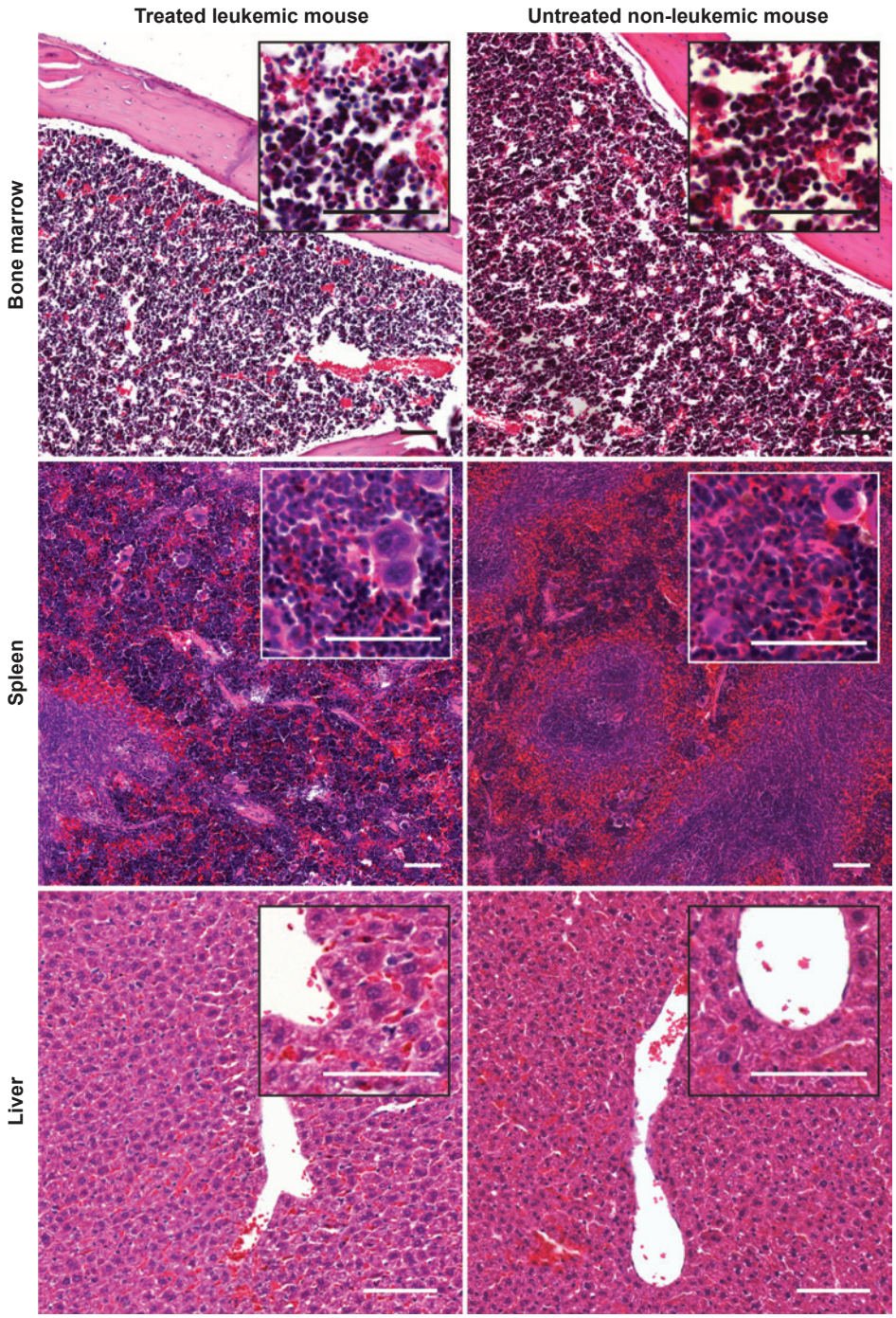


Online Supplementary Figure S2. **Strong bystander effect of MV-Edm in Jurkat and REH ALL cells in the absence of neutralizing antibodies**

Jurkat and REH cells infected with MV-Edm at a MOI of 1 were mixed with uninfected Jurkat and REH cells at increasing ratios. 4 x 10^4 cells of the mixtures were seeded per well of a 96-well plate in the presence or absence of 10% measles immune serum in the culture medium. Cell viability was determined after 72 h by FACS FSC/SSC. Results are means +SD of triplicates and are expressed as percentage of total cells. Similar results were obtained in three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant using the unpaired t-test.



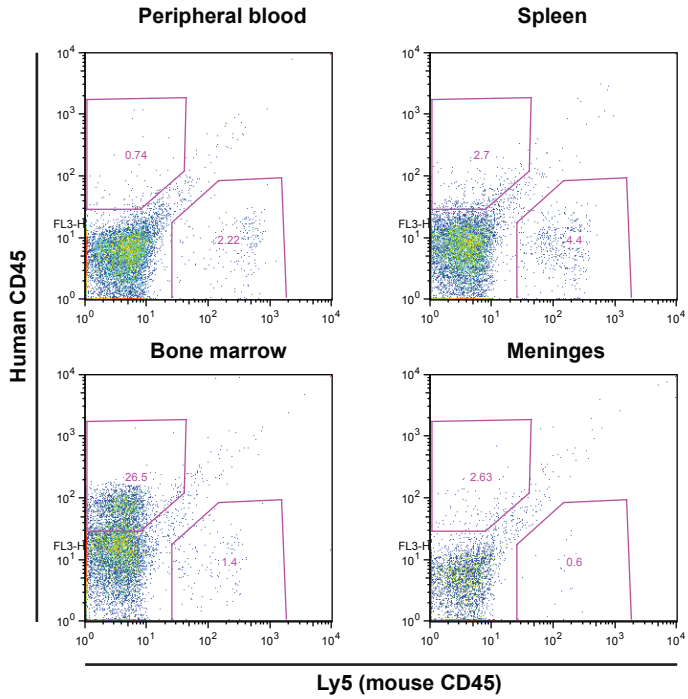
Online Supplementary Figure S3. **MV-Edm induces apoptosis in ALL cell lines and xenografts**
4 x 10^4 cells from ALL cell lines and 8 x 10^4 xenograft cells were seeded per well of 96-well plates. Cells were infected with MV-Edm ("+") and inactivated MV ("i") at a MOI of 1 or were treated with medium ("-"). T lineage ALL cell lines and xenografts were assessed after 72 h while B lineage ALL cell lines were assessed after 96 h because of their protracted cell kill kinetics. Cells were analyzed by Annexin V staining and propidium iodide (PI) uptake using FACS. Results are means +SD of triplicates. Similar results were obtained in three independent experiments. *** $p < 0.001$ using the unpaired t-test.



Online Supplementary Figure S4. **Restitutio ad integrum of hematpoetic organs initially infiltrated by ALL after treatment with MV-Edm**

Bone marrow, spleen and liver from a leukemic mouse of xenograft #15 treated with MV-Edm and sacrificed at advanced age are compared to the organs of an untreated non-leukemic mouse. Organs were formalin-fixed, paraffin-embedded and stained with HE. Inserts show higher magnification. Scale bars equal 100 µm.

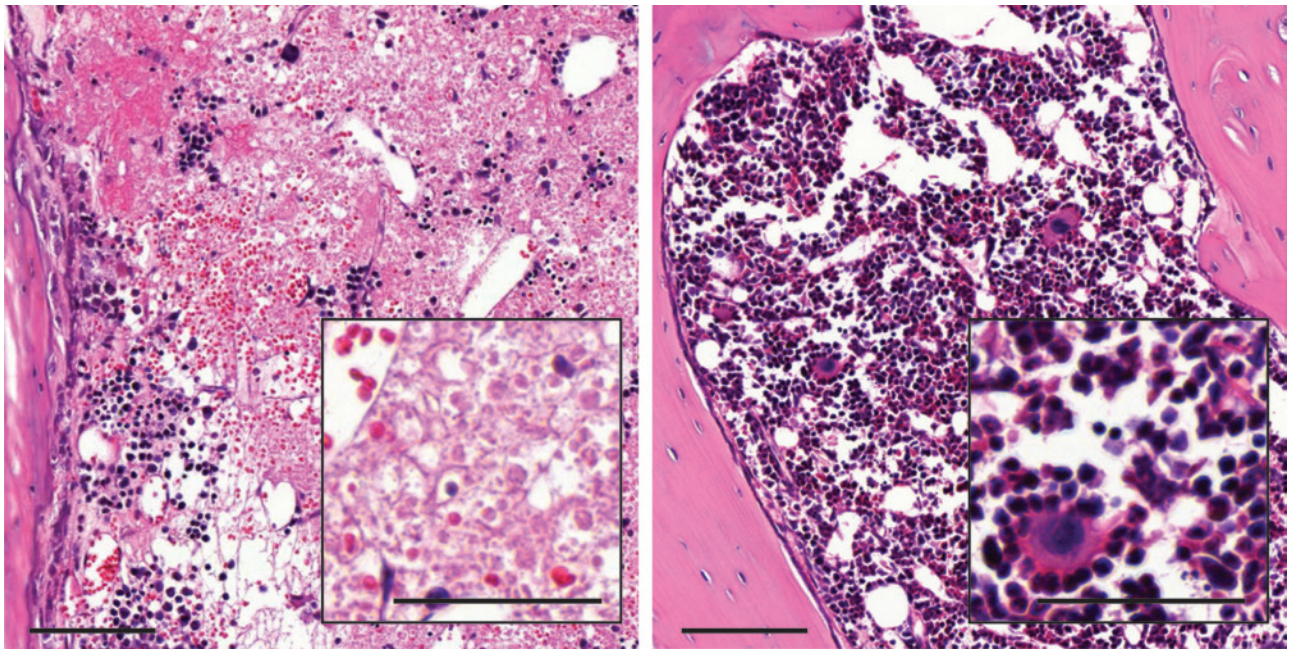
A

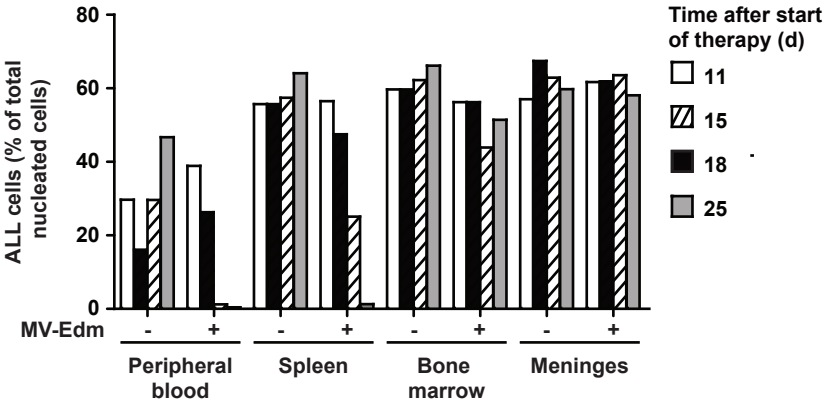


Online Supplementary Figure S5. **Moderate leukemic infiltration, extensive necrosis and severe depression of hematopoesis in the bone marrow of a mouse with xenograft #13 treated with MV-Edm**

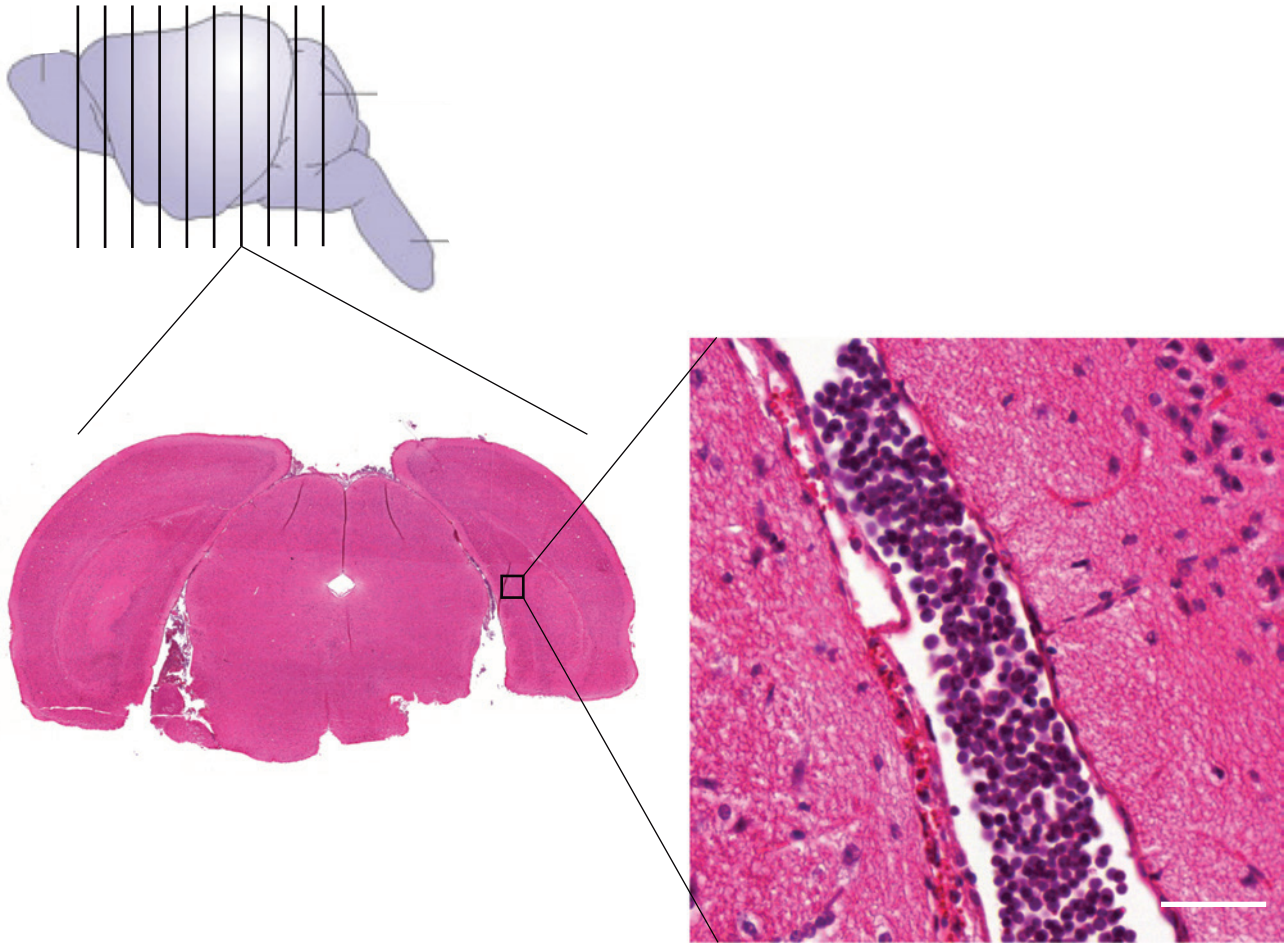
The mouse died 20 d after start of therapy and analysis was performed immediately post mortem. **(A) Leukemic load in the hematopoetic system and the meninges is limited while hematopoesis is nearly absent.** FACS analysis of bone marrow, spleen, peripheral blood and meninges was performed for human CD45^{+} leukemic cells and Ly5^{+} murine hematopoetic cells. **(B) Moderate leukemic infiltration, marked necrosis and pronounced depression of hematopoesis in the bone marrow.** HE-stained bone marrow of the mouse (left panel) is compared to bone marrow of a healthy mouse (right panel). Scale bars equal 100 µm for lower magnification and 50 µm for higher magnification (inserts).

B





Online Supplementary Figure S6. **Kinetics of blast clearance by MV-Edm in vivo: Rapid clearance of peripheral blood and spleen, and delayed impact in bone marrow and meninges**
1 x 10^7 ALL cells from xenograft #15 were intravenously transplanted into NOD/SCID mice. Once peripheral blasts reached 5-20 % mice were injected with 200 µl PBS containing 2 x 10^5 pfu MV-Edm or an equal volume of PBS with inactivated MV-Edm each day for 5 d. 11, 15, 18 and 25 d after the last application of MV-Edm one pair of mice (treated and untreated) was killed and blood, spleen, bone marrow and meninges were procured and stained for $CD45^+Ly5^-$ leukemic blasts.



Online Supplementary Figure S7. **Scheme of assessing extent of CNS disease**
Formalin-fixed, paraffin-embedded brains were serially sectioned in coronal orientation. 4-6 sections were made every 200 µm (upper left panel). Sections were stained by HE (lower left panel). The presence of leukemic blasts was recorded for each section A representative section of an untreated leukemic mouse is shown (right panel, scale bar equals 100 µm).

Online Supplementary Table S1. **Clinical characteristics of primary ALL and xenografts treated with MV-Edm**

		N	%
Total		21	100
Age (years)	<1	1	4.8
	1-17 (mean 7.33)	20	95
Gender	female	7	33
	male	13	62
	not available [A]	1	4.8
Immunophenotype	pro-B-ALL	3	14
	c-ALL	12	57
	pre-B-ALL	4	19
	pre-T-ALL	1	4.8
	cortical T-All	1	4.8
Genetic abnormalities	TEL/AML1-fusion	5	24
	BCR/ABL-fusion	1	4.8
	MLL-rearrangement	2	9.5
	t(1;19)	1	4.8
	none	12	57
WBC at diagnosis (per µl)	< 50000	12	57
	< 100000	5	23.8
	< 150000	1	4.8
	not available [A]	3	14
Prednisone response	poor	1	4.8
	good	17	81
	not available [A]	3	14
Remission d 33	yes	17	81
	no	1	4.8
	not available [A]	3	14
Risk group	high-risk	3	14
	non-high-risk	14	67
	not available [A]	4	19
CNS disease	yes	0	0
	no	17	81
	not available [A]	4	19
Relapse	yes	9	43
	no	10	48
	not available [A]	2	9.5

[A] As these patients were not enrolled in the ALL-BFM study protocol.