Supplemental Methods

In vitro peptide stimulation

PBMC harvested from AML patients who were in remission after chemotherapy were peptide stimulated in vitro following three different strategies. In strategy 1, PBMC were stimulated with HLA-A*02:01-positive allogeneic irradiated EBV-LCL that were pulsed with 10 µM CLAVEEVSL for 2 hrs at 37°C in IMDM supplemented with 2% FBS, 1.5% L-glutamine and 1% penicillin/streptomycin at a ratio of 5:1 (PBMC:EBV-LCL). PBMC were cultured in TCM without cytokines at a concentration of $0.5*10^6$ cells/ml in 48-wells flat-bottom culture plates. After 2 and 5 days, 100 IU/ml IL-2 and 10 ng/ml IL-15 (Miltenyi Biotec) were added. On day 11, bulk cultures were separately analyzed by flow cytometry. In strategy 2, PBMC were stimulated with 20 µM CLAVEEVSL at a concentration of 10^7 cells/ml in IMDM supplemented with 2% FBS, 1.5% L-glutamine, 1% penicillin/streptomycin. After 2 hrs of incubation at 37°C, PBMC were diluted to $0.5*10^6$ cells/ml in 48-wells flat-bottom culture plates in TCM with 20 IU/ml IL-2. After 3 and 6 days, 100 IU/ml IL-2 and 10 ng/ml IL-15 were added. On day 11, PBMC were re-stimulated with HLA-A*02:01-positive allogeneic irradiated PBMC that were pulsed with 10 µM CLAVEEVSL as described above at a ratio of 5:1 (allogeneic PBMC:PBMC) in TCM with 100 IU/ml IL-2. On day 21, bulk cultures were separately analyzed by flow cytometry. In strategy 3, PBMC were stimulated as described for strategy 2 with the exception that PBMC were re-stimulated on day 11 with HLA-A*02:01-positive allogeneic PBMC in the presence of 0.8 µg/ml PHA but absence of CLAVEEVSL. For all 3 strategies flow cytometric analysis was performed on PBMC directly after thawing before peptide stimulation and on day 11 or 21 after peptide stimulation. For FACS analysis, all bulk cultures of approximately $1*10^6$ peptide-stimulated PBMC each were stained with FITC-conjugated antibodies against CD4, CD14, CD16 (Cat. No. 335035) and CD19, a Pacific Blue-conjugated antibody against CD8 (Cat. No. 558207) (BD Biosciences) and ΔNPM1-CLA tetramers conjugated to APC and PE to allow for combinatorial coding. Zombie Aqua was included as live/dead dye. Cells were measured on a BD LSR II using BD FACSDiva v6 software and analysis was performed with FlowJo software.

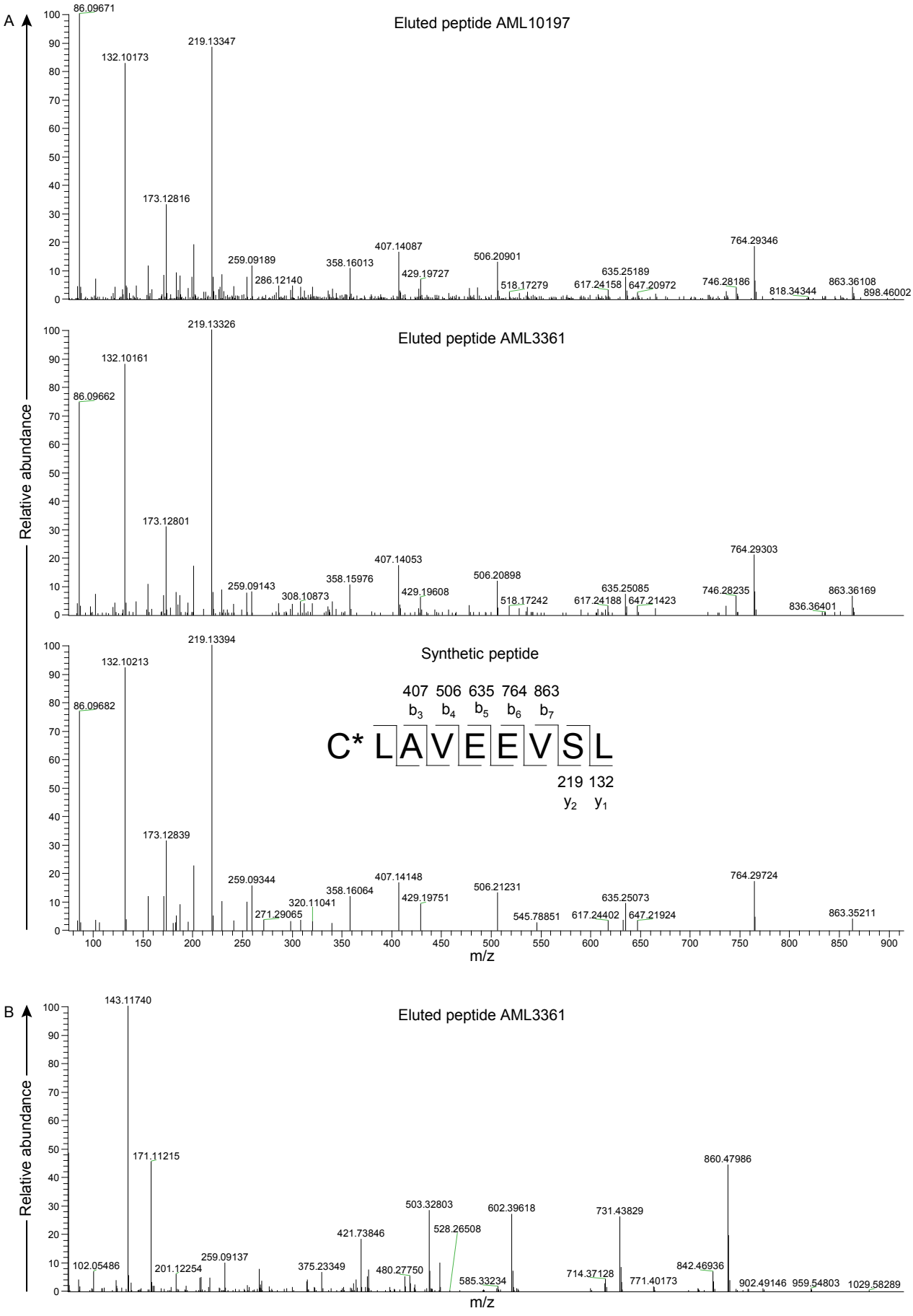
Antibodies and FACS analysis of primary AML

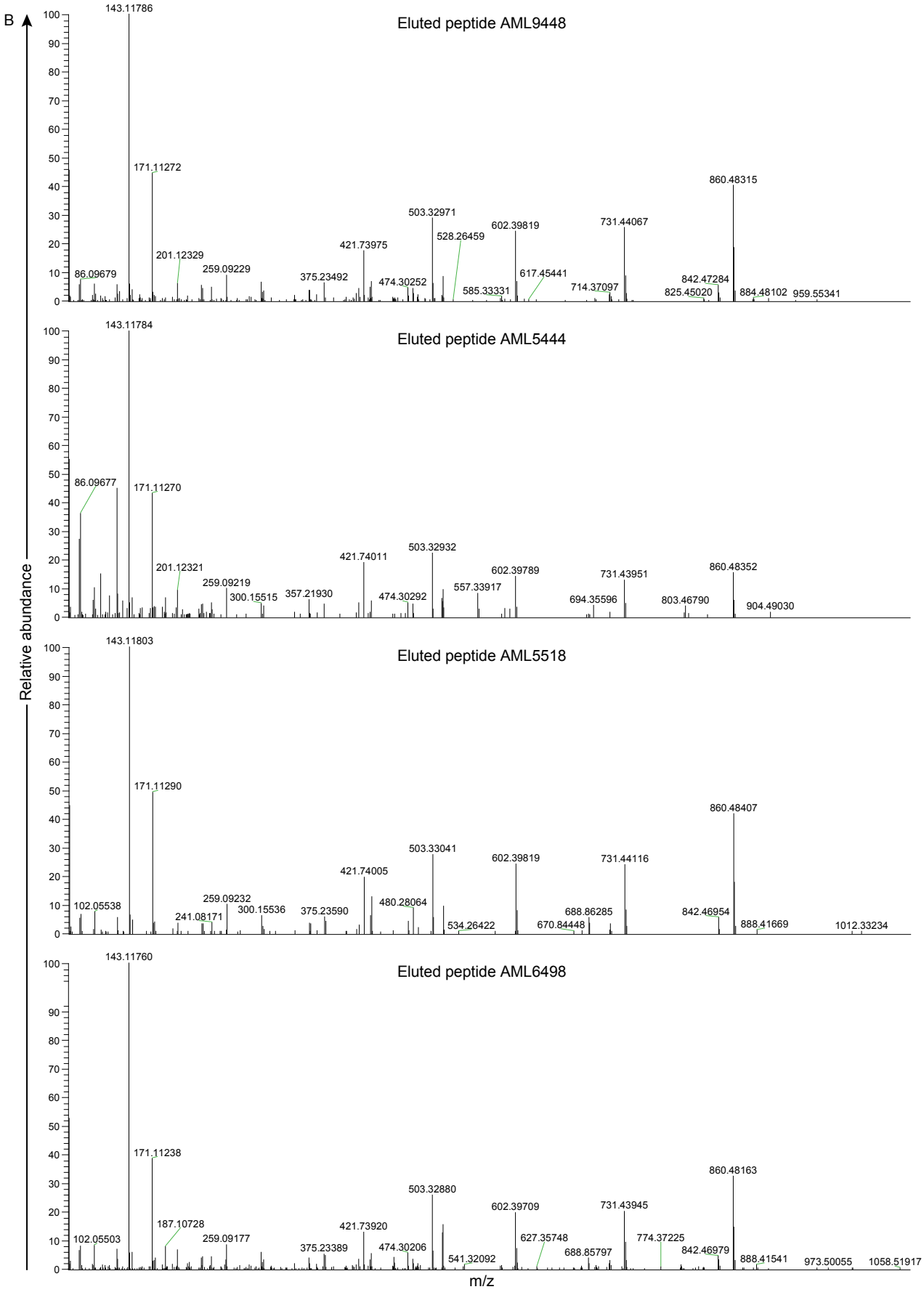
Primary AML were stained with Alexa Fluor 700-conjugated anti-CD33 (Cat. No. 561160), Brilliant Violet 421-conjugated anti-CD34 (Cat. No. 562577), FITC-conjugated anti-CD58 (Cat. No. 555920), PE-conjugated anti-CD80 (Cat. No. 557227), PE-CF594-conjugated anti-CD86 (Cat. No. 562390) (BD Biosciences) and APC-conjugated anti-CD54 (Cat. No. 353112,

Biolegend). HLA-A*02:01 staining was performed indirectly with a mouse antibody against human HLA-A*02:01 (BB7.2) and FITC-conjugated goat anti-mouse Ig (Cat. No. 349031, BD Biosciences). After HLA-A*02:01 staining, cells were blocked with 5% mouse serum, washed and stained with Alexa Fluor 700-conjugated anti-CD33 or Brilliant Violet 421-conjugated anti-CD34. Zombie Aqua was included as live/dead dye. Cells were measured on a BD LSR II using BD FACSDiva v6 software and analysis was performed with FlowJo software.

Supplemental Figures

Figure S1

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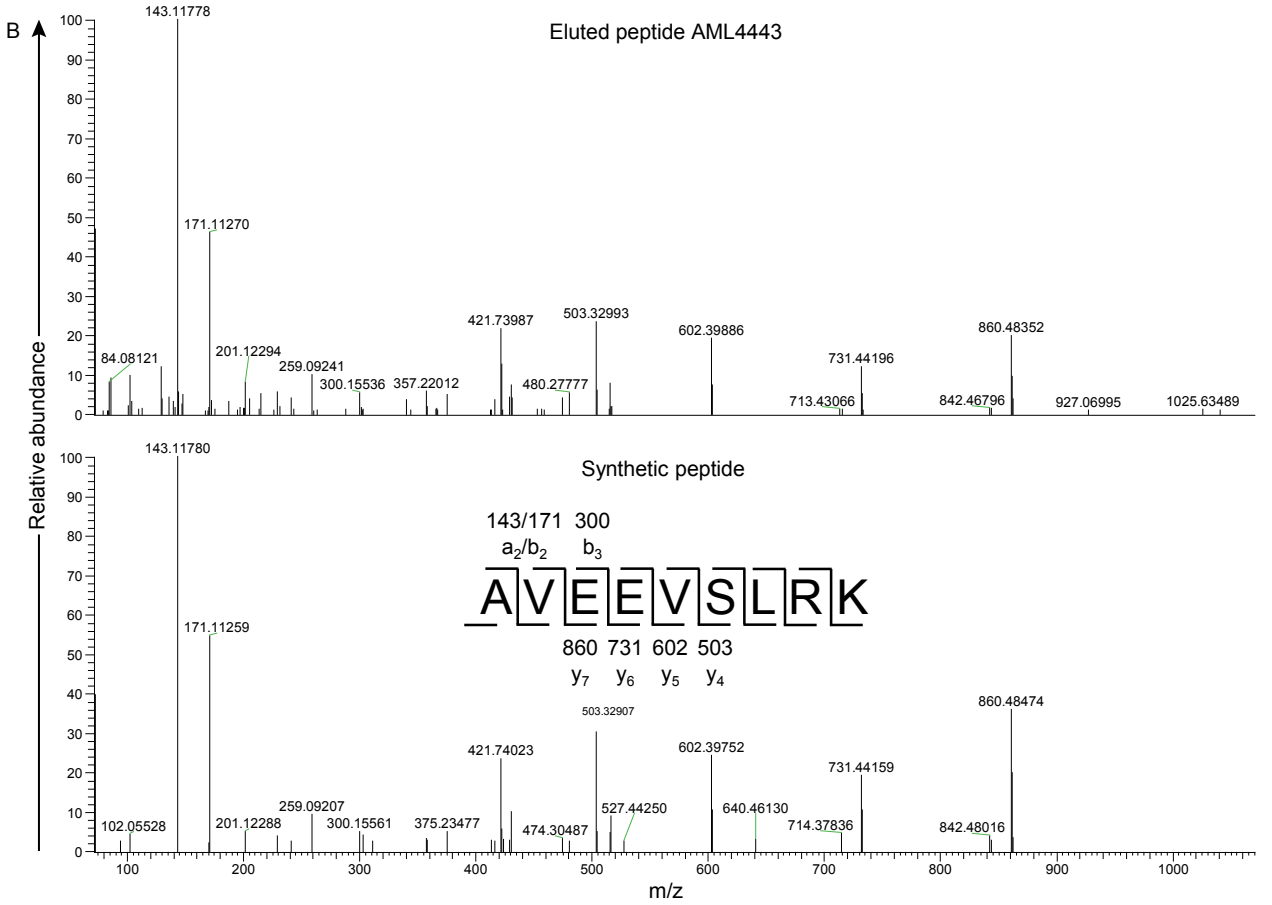
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y7 y6 y5 y4
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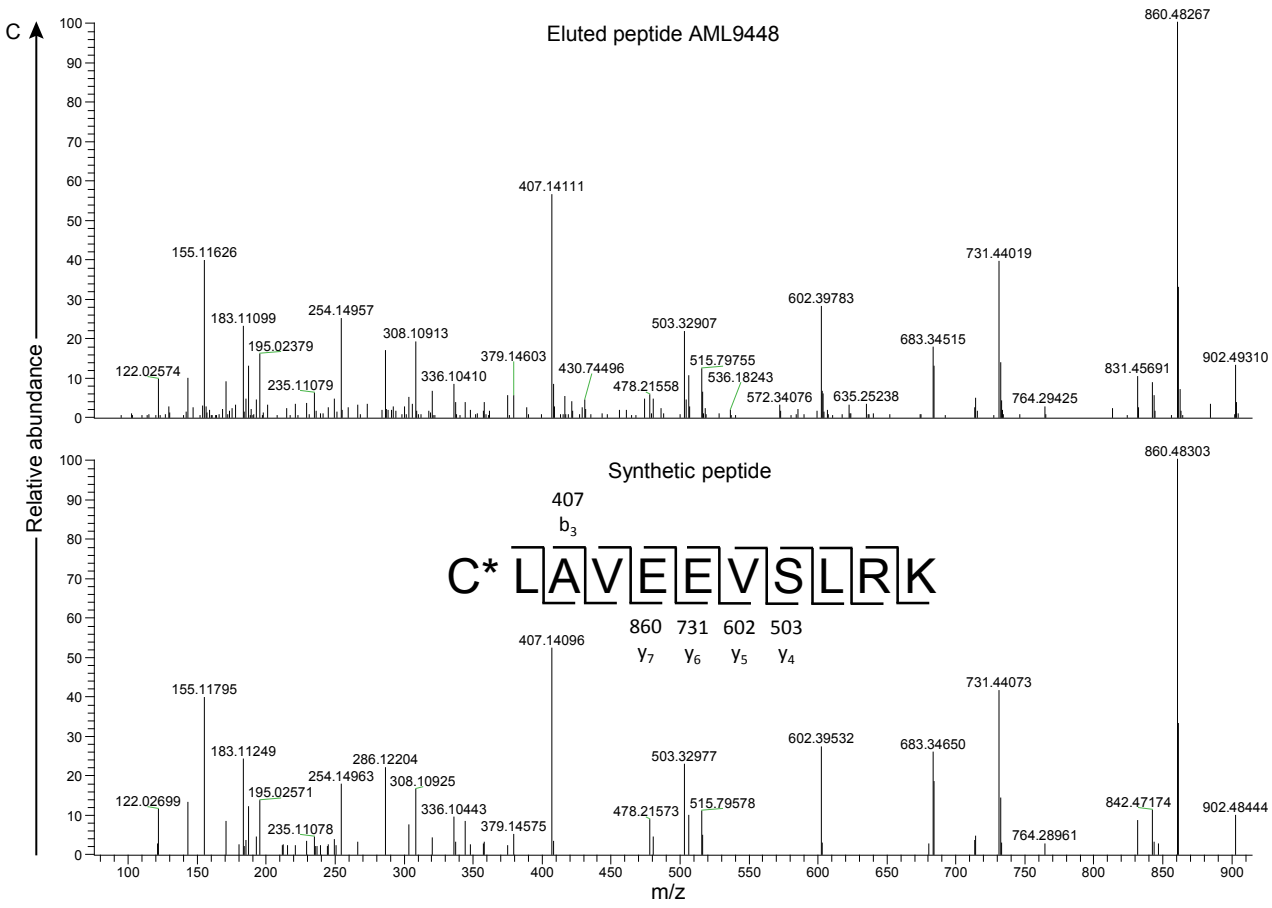
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407
b3
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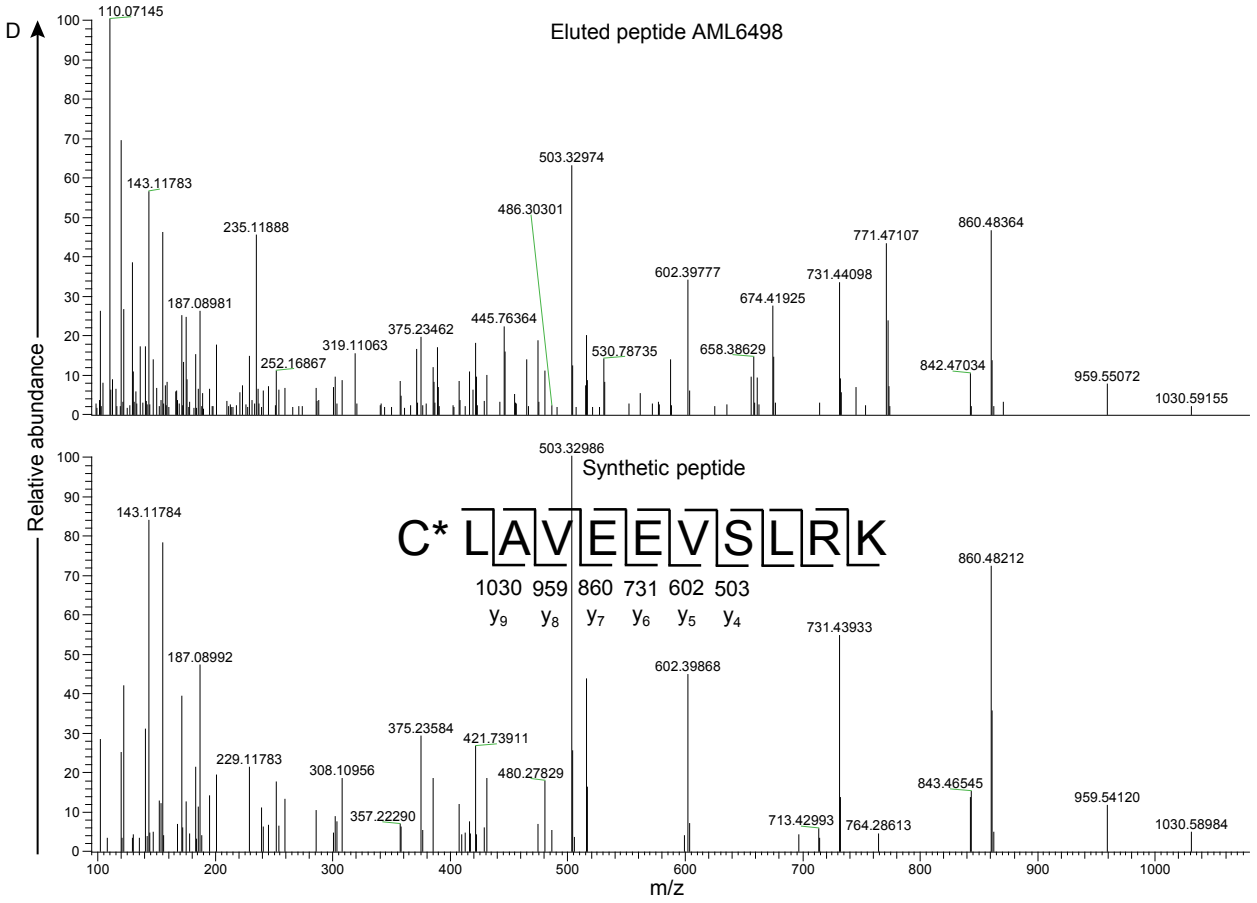
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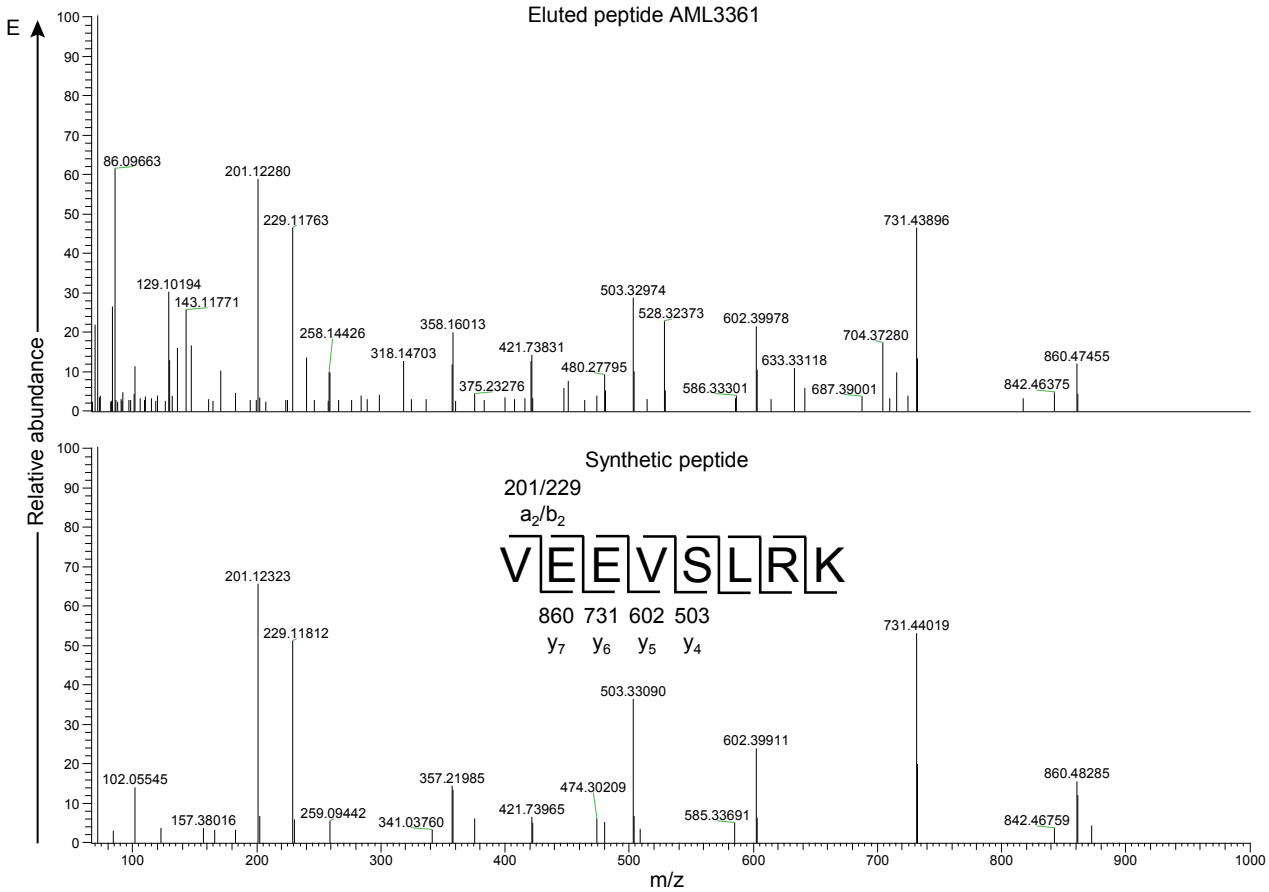
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y7 y6 y5 y4
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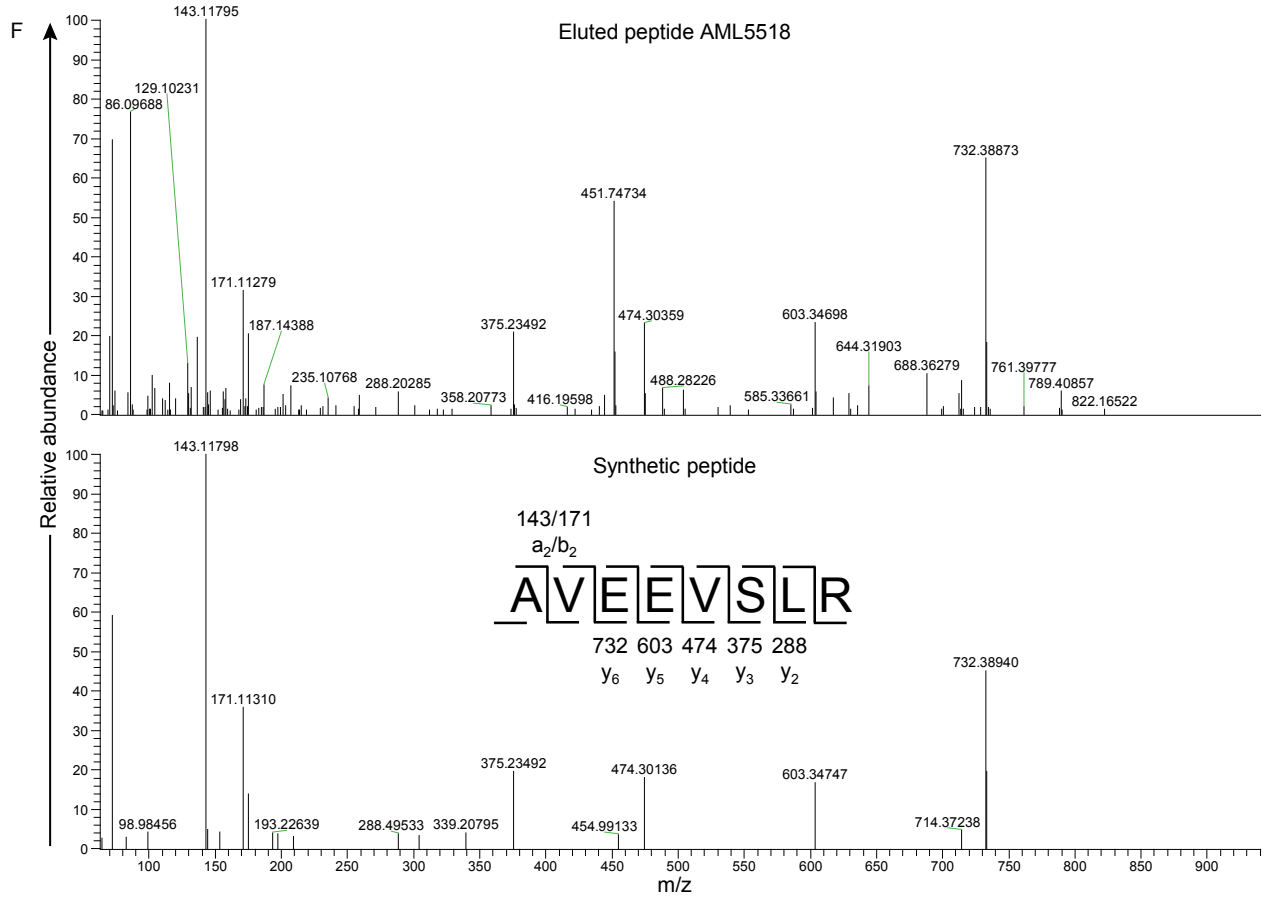

Figure S1. Validation of ΔNPM1-derived peptides eluted from primary AML. ΔNPM1-derived peptides were validated as HLA-binding peptides on primary AML by comparing tandem mass spectra between eluted peptides from AML and synthetic peptides. Matching tandem mass spectra are shown. C*=cysteinylation of the Cys-residue. (A) Tandem mass spectra for eluted peptides from AML10197 (upper) and AML 3361 (middle) and synthetic peptide C*LAVEEVSL (lower). (B) Tandem mass spectra for eluted peptides from AML3361 (first), AML9448 (second), AML5444 (third), AML5518 (fourth), AML6498 (fifth) and AML4443 (sixth) and synthetic peptide AVEEVSLRK (last). (C) Tandem mass spectra for an eluted peptide from AML9448 (upper) and synthetic peptide C*LAVEEVSLRK (lower). (D) Tandem mass spectra for an eluted peptide from AML6498 (upper) and synthetic peptide C*LAVEEVSLRK (lower). (E) Tandem mass spectra for an eluted peptide from AML3361 (upper) and synthetic peptide VEEVSLRK (lower). (F) Tandem mass spectra for an eluted peptide from AML5518 (upper) and synthetic peptide AVEEVSLR (lower).

Figure S2

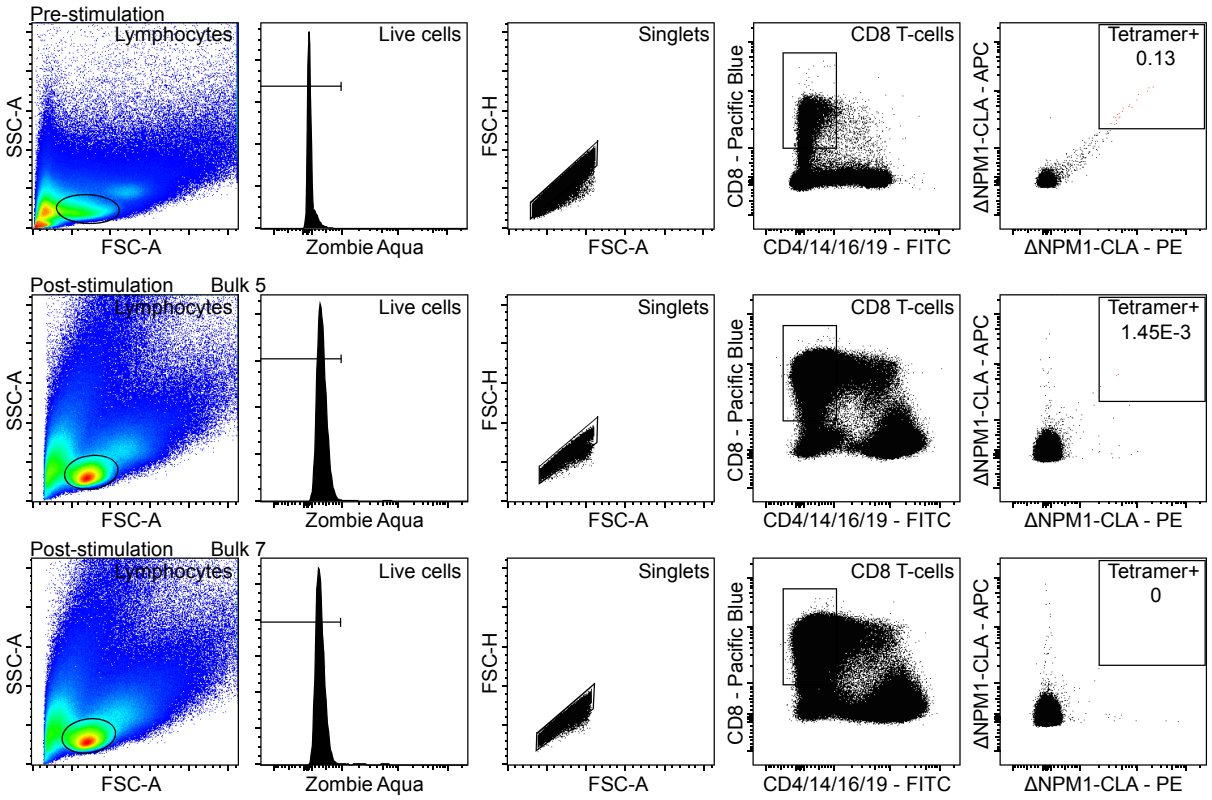

Figure S2. Screening for ΔNPM1-specific T-cells in a representative patient with AML after in vitro peptide stimulation. PBMC from AML patients who were in remission after chemotherapy were thawed and stimulated in vitro with CLAVEEVSL. A minimum of $1*10^6$ total events were analyzed by flow cytometry before (pre-stimulation) and after (post-stimulation) peptide stimulation. Cells were stained with a Pacific Blue anti-CD8 antibody, a mix of FITC-conjugated anti-CD4, anti-CD14, anti-CD16 and anti-CD19 antibodies and ΔNPM1-CLA tetramers conjugated to APC and PE. Zombie Aqua was used as live/dead dye. A representative screening for ΔNPM1-specific T-cells using combinatorial coding APC- and PE-conjugated ΔNPM1-CLA tetramers is shown for patient AML11282. Viable Zombie Aqua-negative single cells were selected and gated for Pacific Blue-positive FITC-negative CD8 T-cells. Within this CD8 population, T-cells that were double positive for APC- and PE-conjugated ΔNPM1-CLA tetramers were counted. Data are shown for PBMC before peptide stimulation (upper) and two of the 14 bulk cultures after peptide stimulation (culture 5: middle; culture 7: lower).

Figure S3

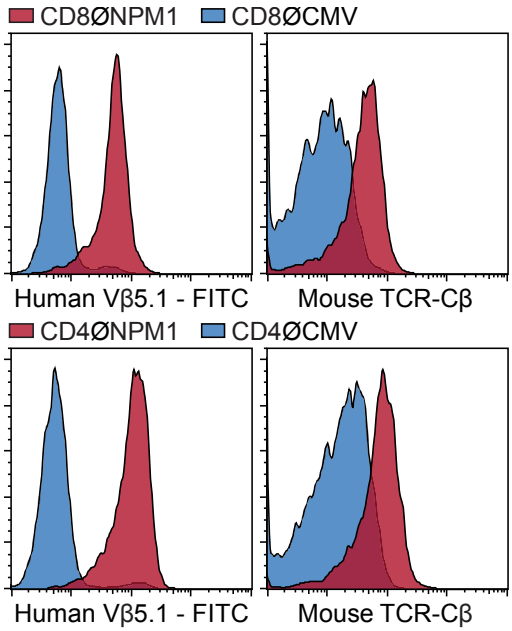

Figure S3. TCR staining after ΔNPM1 TCR gene transfer. TCR-transduced T-cells were analyzed by flow cytometry at day 7 after transduction using anti-mouse TCR-Cβ - APC and anti-human TCR-Vβ5.1 - FITC antibodies. All TCR-transduced T-cells stained positive for mouse TCR-Cβ. T-cells transduced with the TCR for ΔNPM1 (CD8ØNPM1 and CD4ØNPM) were also positive for human TCR-Vβ5.1 (left panels). Results are shown for donor 1.

Figure S4

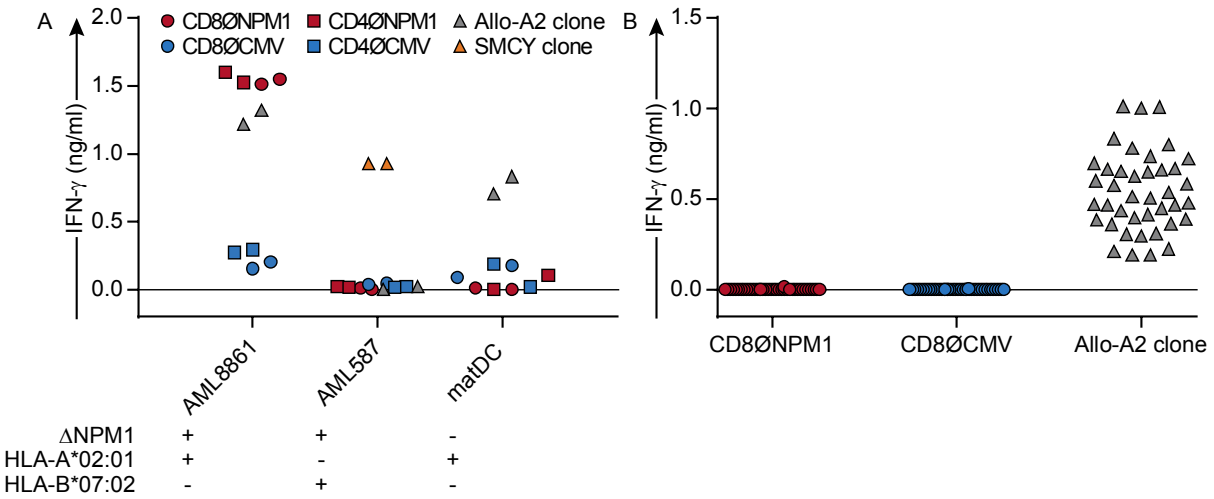

Figure S4. Lack of T cell reactivity against control samples. (**A**) TCR-transduced T-cells were tested for reactivity against autologous monocyte-derived mature DC (matDC) and AML with ΔNPM1 that were HLA-A*02:01-positive (AML8861) or -negative (AML587) by IFN-γ ELISA. CD8 (CD8ØNPM1; red circles) and CD4 (CD4ØNPM1; red squares) T-cells with the TCR for ΔNPM1 reacted against AML8861, but not against AML587 or autologous mature DC, whereas CD8 (CD8ØCMV; blue circles) and CD4 (CD4ØCMV; blue squares) T-cells with the CMV-specific TCR failed to recognize both AML samples and autologous mature DC. The allo-A2 clone (grey triangles) is included as a positive control for the autologous matDC and AML8861. The HLA-B*07:02-restricted SMCY-specific CD8 T-cell clone (orange triangles) is included as a positive control for the HLA-A*02:01-negative, but HLA-B*07:02-positive AML587 which was loaded with an HLA-B*07:02-binding SMCY peptide. Release of IFN-γ (ng/ml) in duplicate wells is shown for donor 2. (**B**) TCR-transduced CD8 T-cells were tested for recognition of a panel of 40 HLA-A*02:01-positive allogeneic EBV-LCL by IFN-γ ELISA. No reactivity against EBV-LCL was observed for either CD8ØNPM1 (red circles) or CD8ØCMV (blue circles). The allo-A2 clone (grey triangles) is included as positive control. Mean release of IFN-γ (ng/ml) in duplicate wells is shown for donor 1.

Supplemental Tables

Table S1. HLA class I binding prediction by NetMHCpan 3.0

Peptide	Predicted HLA class I binding affinity (nM)				
	A*01:01	A*02:01	A*03:01	A*11:01	Other HLA class I alleles[A]
CLAVEEVSL	27798.0	592.2	NA	33678.3	≥3562.6
AVEEVSLRK	16147.9	36154.8	534.4	58.2	≥1755.9
CLAVEEVSLRK	NA	NA	498.2	1111.8	≥5404.5
AVEEVSLR	31753.2	NA	NA	NA	≥39787.9
VEEVSLRK	NA	44597.1	NA	15474.5	≥38325.9

[A] Predicted binding affinity (nM) for all other HLA class I alleles as expressed by primary AML from which the indicated peptide has been eluted.

Table S2. Direct isolation of ΔNPM1-specific T-cells from patients with AML

Patient	Days after diagnosis[A]	PBMC[B] (*10^6)	Sorted T-cells[C]	Growing clones	Tetramer clones[D]
AML10833	89	8.6	21	1	0
AML9559	107	6.4	2	2	0
AML6395	69	10.6	6	2	0
AML11282	81	8.0	12	0	-
AML9423	98	9.0	0	-	-
AML10418	108	7.5	0	-	-

[A] Number of days after diagnosis when peripheral blood for T-cell isolation was collected.

[B] Indicated are numbers of PBMC from HLA-A*02:01-positive AML patients with ΔNPM1 that are used for T-cell isolation.

[C] PBMC were stained with anti-CD8-Alexa Fluor 700 and a mix of PE-conjugated pHLA tetramers for CLAVEEVSL and its cysteinylated variant C*LAVEEVSL. Indicated are numbers of tetramer-positive CD8 T-cells that are sorted.

[D] Number of growing T-cell clones positive for the ΔNPM1-CLA or ΔNPM1-C*LA tetramer.

Table S3. ΔNPM1-specific T-cells in patients with AML after in vitro peptide stimulation

IVS[A]	Patient	Days after diagnosis[B]	PBMC[C] (*10^6)	# bulks	CD8 T-cells	
					% Tetramer[D] pre-stimulation	% Tetramer[E] post-stimulation
1	AML10833	89	4.9	10	0.051	0.000-0.005
	AML9559	107	3.2	7	0.044	0.000-0.009
2	AML10833	89	4.9	10	0.180	0.000-0.061
	AML9559	107	2.9	6	0.170	0.004-0.120
3	AML11282	81	7.0	14	0.130	0.000-0.001
	AML10418	108	6.5	13	0.083	0.000-0.002

[A] IVS, in vitro peptide stimulation strategy. Three different strategies for in vitro peptide stimulation have been followed as described in the supplemental methods.

[B] Number of days after diagnosis when peripheral blood for T-cell isolation was collected.

[C] Indicated are numbers of PBMC from HLA-A*02:01-positive AML patients with ΔNPM1 that are used for peptide stimulation.

[D] Percentage of CD8 T-cells that are positive for combinatorial coding ΔNPM1-CLA-APC and ΔNPM1-CLA-PE tetramers prior to peptide stimulation.

[E] Percentage of CD8 T-cells that are positive for combinatorial coding ΔNPM1-CLA-APC and ΔNPM1-CLA-PE tetramers after in vitro peptide stimulation. For each patient, the range of tetramer-positive CD8 T-cells in different bulk cultures is shown.

Table S4: Flow cytometric analysis of primary AML

AML	Leukemic cells (%)[A]	HLA-A*02:01 (ΔMFI[B])	CD80 (MFI)	CD86 (MFI)	CD54 (MFI)	CD58 (MFI)
9559	93	1126	455	3821	8918	2135
7170	92	1558	201	1178	5786	1379
8861	98	380	55	198	186	369
10418	99	159	46	92	152	319
10594	27	444	81	491	2451	524
10833	92	1437	262	1440	3438	1413
3361	100	1236	108	294	1218	720
6395	84	1195	233	1254	11287	2061
10197	90	331	71	439	3896	451
1775	80	514	58	91	1682	414
2250	61	880	54	115	1954	774
2467	40	895	77	281	8722	612
4716	80	690	61	100	1563	589
EBV-LCL	-	4245	7555	22823	24005	5072

[A] Percentages of leukemic cells as determined by CD33 or CD34 expression in peripheral blood samples used for flow cytometric analysis.

[B] HLA-A*02:01 staining was performed indirectly with a mouse antibody against human HLA-A*02:01 (BB7.2) and FITC-conjugated goat anti-mouse Ig. ΔMFI indicates the difference in geometric mean fluorescence intensity between AML stained with BB7.2 and goat anti-mouse Ig and AML stained with goat anti-mouse Ig only.

Table S5. ΔNPM1 gene expression and variant allele frequencies by RNA-sequencing[A]

AML	NPM1 CDS[B]	VAF[C]	ΔNPM1 Protein	NPM1 reads	Total reads	NPM1/total reads
9559	c.859_860insTCTG	34.47	p.Trp288CysfsTer12	9498	103961552	0.0000913607
7170	c.859_860insTCTG	35.28	p.Trp288CysfsTer12	12988	111732282	0.000116242
8861	c.860_861insCTGC	27.21	p.Trp288CysfsTer12	21735	101868266	0.000213364
10418	c.859_860insTCTG	37.08	p.Trp288CysfsTer12	30907	139712362	0.000221219
10594	c.859_860insTCTG	33.29	p.Trp288CysfsTer12	17041	148624447	0.000114658
10833	c.859_860insTCTG	32.17	p.Trp288CysfsTer12	10129	128443731	0.0000788594
3361	c.859_860insTCTG	46.33	p.Trp288CysfsTer12	21222	118571640	0.00017898
6395	c.859_860insTCTG	31.97	p.Trp288CysfsTer12	6310	160372609	0.0000393459
10197	c.860_861insCTGC	35.74	p.Trp288CysfsTer12	15039	105485637	0.000142569

[A] Paired-end RNA sequencing with a read length of 126 bp was performed on an Illumina HiSeq 2500 v4 sequencer to at least 12.5 Gbp per case. Mutations were detected by VARSCAN.

[B] Indicated is the position and type of *NPM1* mutation in the coding sequence (CDS).

[C] VAF, variant allele frequency.