Supplementary Figures

for

Immune surveillance of acute myeloid leukemia is mediated by HLA-presented antigens on leukemia progenitor cells

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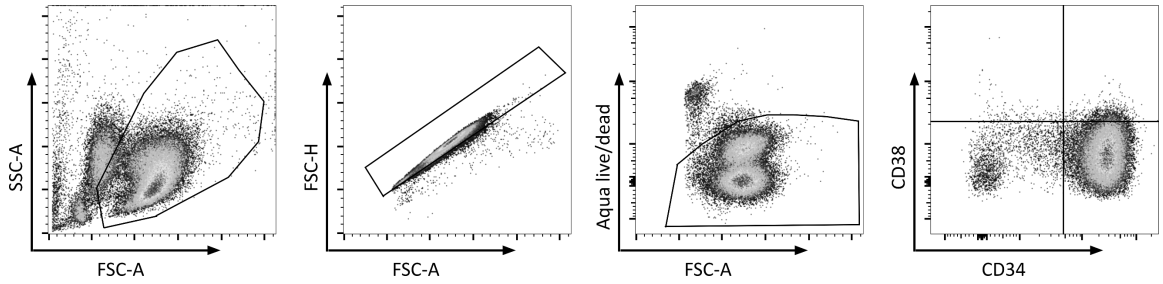

Figure S1: Gating strategy for the evaluation of LSC frequencies pre and post enrichment. Exemplary sample showing the gating strategy for the evaluation of $CD34^{+}CD38^{-}$ LSC frequencies pre and post enrichment. The first gate identifies the lymphocytes (FSC-A vs. SSC-A), which are further gated for single cells (FSC-A vs. FSC-H) and viable cells (FSC-A vs. Aqua live/dead). The viable cells are analyzed for their frequency of $CD34^{+}CD38^{-}$ cells. This gating strategy was applied for the plots presented in Figure 1A and B. Abbreviations: FSC, forward scatter; SSC, side scatter.

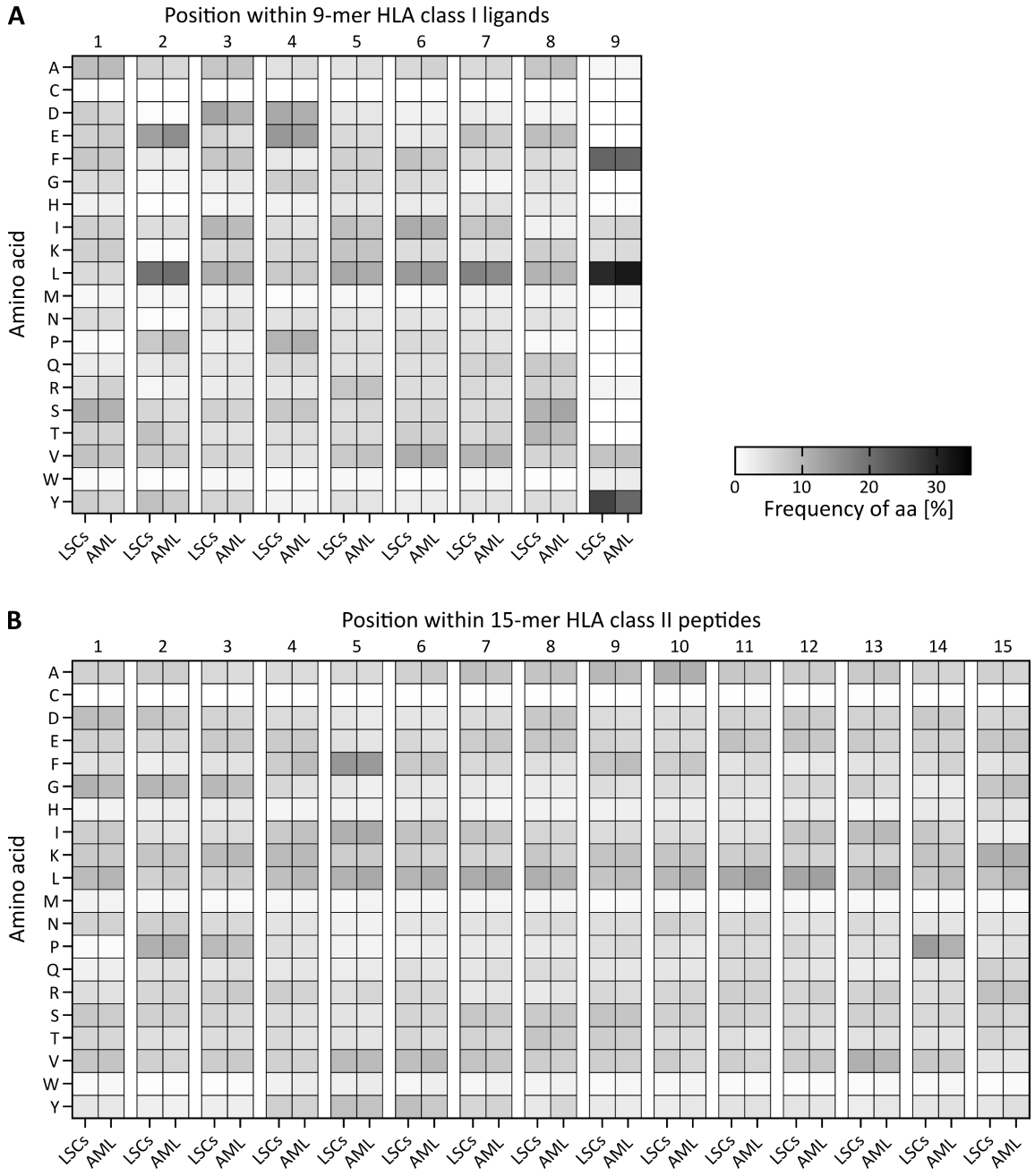

Figure S2: Amino acid distribution within LSC and AML bulk immunopeptidomes. Position-specific amino acid distribution within the LSC- and corresponding AML bulk cell-derived (A) HLA class I and (B) HLA class II immunopeptidomes. The abundance of each amino acid within the 9-mer HLA class I ligands and 15-mer HLA class II peptides was calculated on a sample-by-sample basis for each respective position within the peptides followed by the calculation of the mean amino acid frequency within the LSC and AML bulk cohort, respectively. Abbreviations: aa, amino acid; LSCs, leukemia stem and progenitor cells.

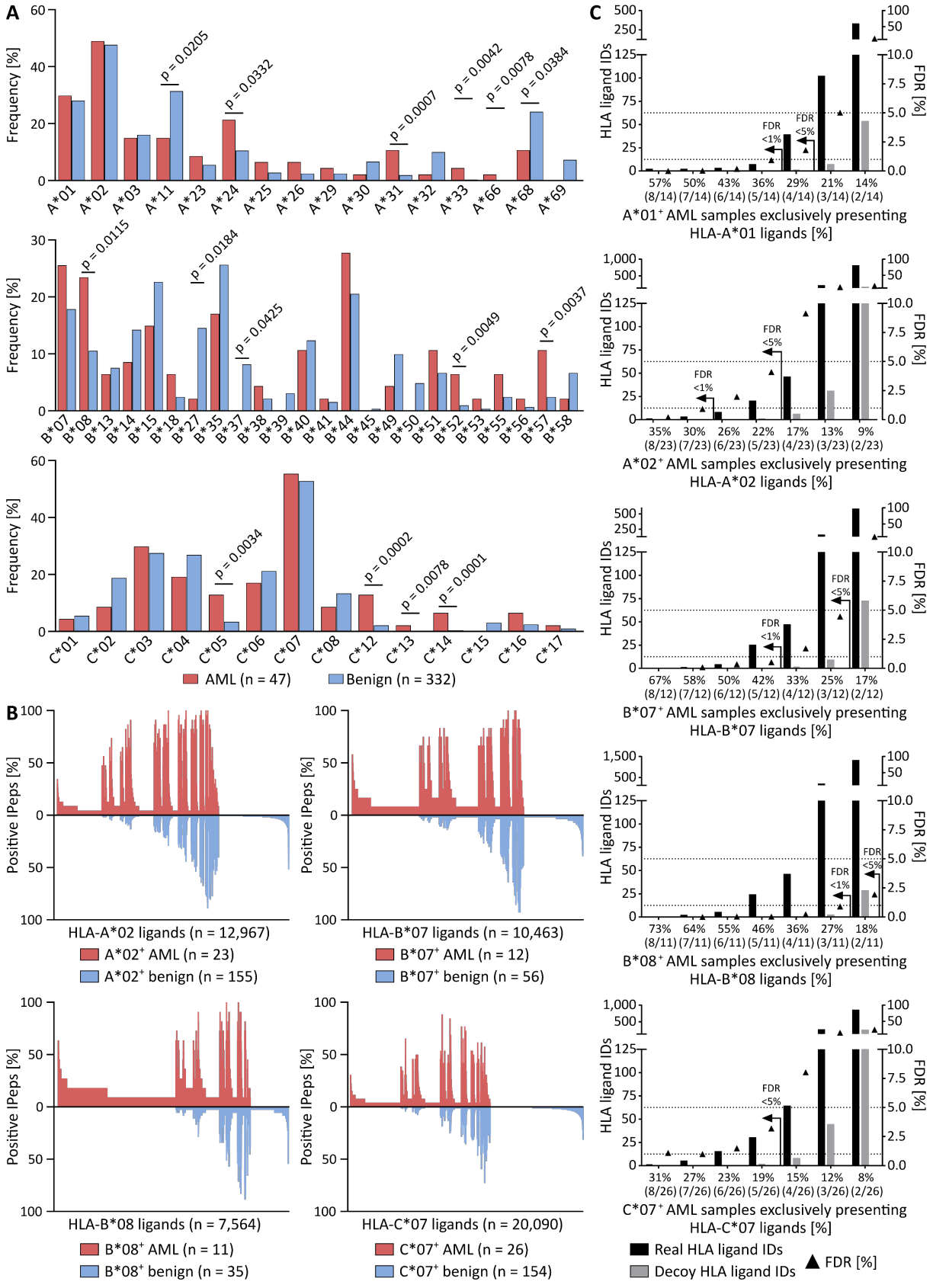
A
Frequency [%]
p = 0.0205
p = 0.0332
p = 0.0007
p = 0.0042
p = 0.0078
p = 0.0384
A*01 A*02 A*03 A*11 A*23 A*24 A*25 A*26 A*29 A*30 A*31 A*32 A*33 A*66 A*68 A*69
p = 0.0115
p = 0.0184
p = 0.0425
p = 0.0049
p = 0.0037
B*07 B*08 B*13 B*14 B*15 B*18 B*27 B*35 B*37 B*38 B*39 B*40 B*41 B*44 B*45 B*49 B*50 B*51 B*52 B*53 B*55 B*56 B*57 B*58
p = 0.0034
p = 0.0002
p = 0.0078
p = 0.0001
C*01 C*02 C*03 C*04 C*05 C*06 C*07 C*08 C*12 C*13 C*14 C*15 C*16 C*17
AML (n = 47)
Benign (n = 332)
B
Positive IPeps [%]
HLA-A*02 ligands (n = 12,967)
A*02+ AML (n = 23)
A*02+ benign (n = 155)
HLA-B*07 ligands (n = 10,463)
B*07+ AML (n = 12)
B*07+ benign (n = 56)
HLA-B*08 ligands (n = 7,564)
B*08+ AML (n = 11)
B*08+ benign (n = 35)
HLA-C*07 ligands (n = 20,090)
C*07+ AML (n = 26)
C*07+ benign (n = 154)
C
HLA ligand IDs
FDR [%]
FDR <1%
FDR <5%
57% 50% 43% 36% 29% 21% 14%
(8/14) (7/14) (6/14) (5/14) (4/14) (3/14) (2/14)
A*01+ AML samples exclusively presenting HLA-A*01 ligands [%]
35% 30% 26% 22% 17% 13% 9%
(8/23) (7/23) (6/23) (5/23) (4/23) (3/23) (2/23)
A*02+ AML samples exclusively presenting HLA-A*02 ligands [%]
67% 58% 50% 42% 33% 25% 17%
(8/12) (7/12) (6/12) (5/12) (4/12) (3/12) (2/12)
B*07+ AML samples exclusively presenting HLA-B*07 ligands [%]
73% 64% 55% 46% 36% 27% 18%
(8/11) (7/11) (6/11) (5/11) (4/11) (3/11) (2/11)
B*08+ AML samples exclusively presenting HLA-B*08 ligands [%]
31% 27% 23% 19% 15% 12% 8%
(8/26) (7/26) (6/26) (5/26) (4/26) (3/26) (2/26)
C*07+ AML samples exclusively presenting HLA-C*07 ligands [%]
Real HLA ligand IDs
Decoy HLA ligand IDs
FDR [%]

Figure S3: HLA class I immunopeptidomics. (A) Frequencies of HLA-A (upper panel), HLA-B (middle panel), and HLA-C (lower panel) allotypes within the AML (n = 47) and benign (n = 332) immunopeptidome cohorts. (B) Allotype-specific comparative immunopeptidome profiling based on the frequency of HLA-restricted presentation in HLA-A*02 (upper left panel), B*07 (upper right panel), B*08 (lower left panel), and C*07 (lower right panel) positive AML and benign immunopeptidomes, respectively. Frequencies of positive immunopeptidomes for the respective HLA ligand (x-axis) are indicated on the y-axis. HLA ligands are depicted on the x-axis, sorted according to the frequency of AML and benign samples presenting the respective ligand. Comparative profiling was performed with curated AML immunopeptidome data. (C) Statistical analysis of the proportion of false-positive AML-associated antigen identifications at different representation frequencies. The numbers of identified HLA-A*01-, A*02-, B*07-, B*08-, and C*07-restricted peptides based on immunopeptidome analysis of AML (n = 47) and benign (n = 332) cohorts were compared with random virtual (HLA-matched) AML-associated peptides (left y-axis), respectively. Virtual immunopeptidomes of AML and benign samples were generated *in silico* based on random weighted sampling from the entirety of peptide identifications in both original cohorts. These randomized virtual immunopeptidomes were used to define AML-associated antigens based on simulated cohorts of AML versus benign tissue samples. The process of peptide randomization, cohort assembly, and AML-associated antigen identification was repeated 1,000 times and the mean value of resultant virtual AML-associated antigens was calculated and plotted for the different threshold values. The corresponding FDRs (right y-axis) for any chosen threshold (x-axis) were calculated and the 1% and 5% FDRs are indicated within the plot (dotted lines and arrows). (A) Chi-square test. Abbreviations: n, number; IPeps, immunopeptidomes; IDs, identifications; FDR, false discovery rate.

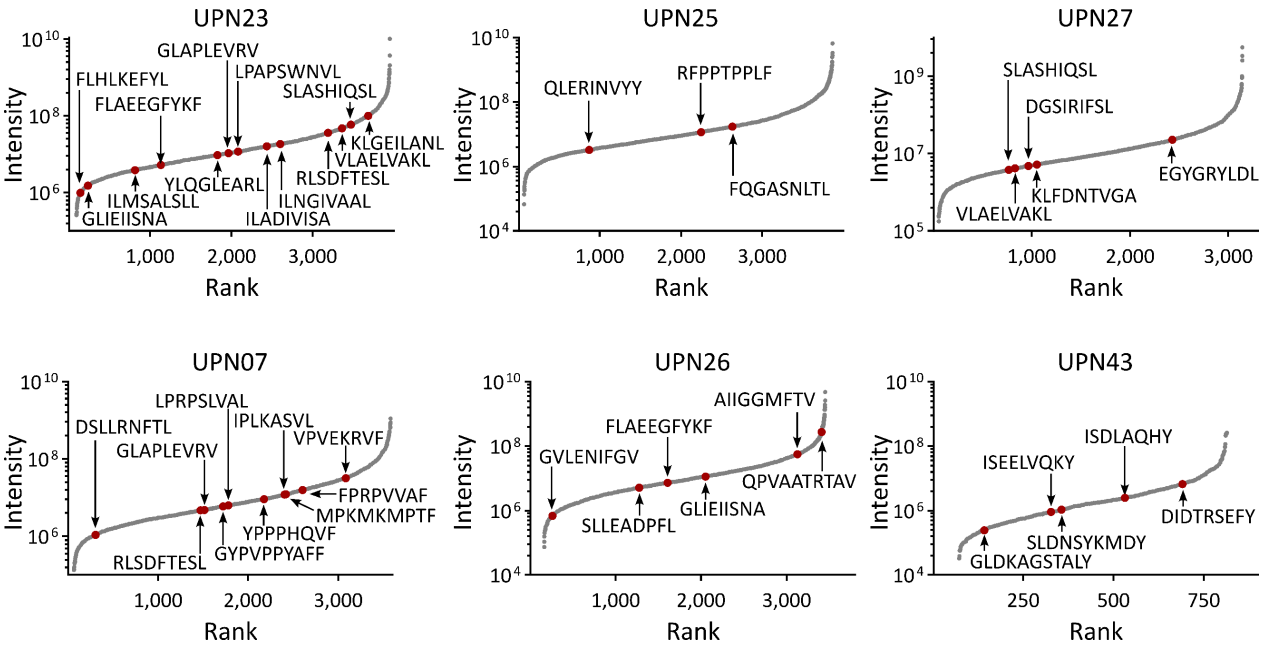

Figure S4: Abundance of AML- and AML/LSC-associated HLA class I ligands within the immunopeptidome. Ranked intensity values of MS data derived from HLA class I immunopeptidomes of representative patients with identified HLA class I AML- and AML/LSC-associated peptides, respectively. Positions of the AML- and AML/LSC-associated peptides are projected on the curve. Abbreviation: UPN, uniform patient number.

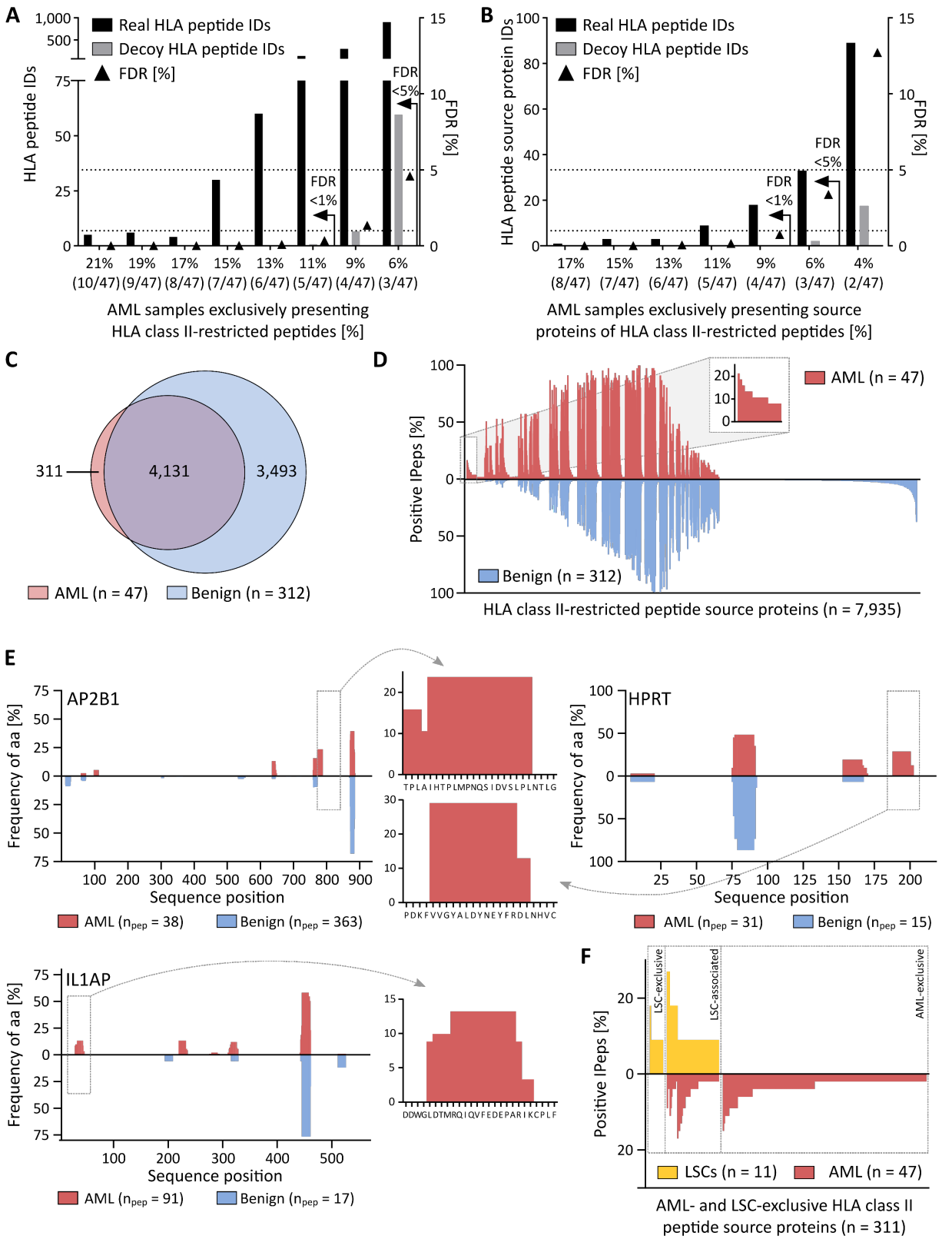
A
Real HLA peptide IDs
Decoy HLA peptide IDs
FDR [%]
HLA peptide IDs
FDR [%]
FDR <1%
FDR <5%
21% (10/47)
19% (9/47)
17% (8/47)
15% (7/47)
13% (6/47)
11% (5/47)
9% (4/47)
6% (3/47)
AML samples exclusively presenting HLA class II-restricted peptides [%]
B
Real HLA peptide IDs
Decoy HLA peptide IDs
FDR [%]
HLA peptide source protein IDs
FDR [%]
FDR <1%
FDR <5%
17% (8/47)
15% (7/47)
13% (6/47)
11% (5/47)
9% (4/47)
6% (3/47)
4% (2/47)
AML samples exclusively presenting source proteins of HLA class II-restricted peptides [%]
C
311
4,131
3,493
AML (n = 47)
Benign (n = 312)
D
AML (n = 47)
Benign (n = 312)
Positive IPeps [%]
HLA class II-restricted peptide source proteins (n = 7,935)
E
AP2B1
Frequency of aa [%]
Sequence position
TPLAIHTPLMPNQSIDVSLPLNTLG
PDKFVVGYALDYNEYFRDLNHVC
AML (n_pep = 38)
Benign (n_pep = 363)
HPRT
Frequency of aa [%]
Sequence position
AML (n_pep = 31)
Benign (n_pep = 15)
IL1AP
Frequency of aa [%]
Sequence position
DDWGLDTMRQIQVFEDEPARIKCPLF
AML (n_pep = 91)
Benign (n_pep = 17)
F
LSC-exclusive
LSC-associated
AML-exclusive
Positive IPeps [%]
LSCs (n = 11)
AML (n = 47)
AML- and LSC-exclusive HLA class II peptide source proteins (n = 311)

Figure S5: Identification of AML- and AML/LSC-associated HLA class II antigen targets by comparative immunopeptidome profiling. (A, B) Statistical analysis of the proportion of false-positive AML-associated antigen identifications at different representation frequencies. The numbers of identified HLA class II-restricted peptides (A) and peptide source proteins (B) based on immunopeptidome analysis of AML (n = 47) and benign (n = 312) samples were compared with random virtual AML-associated peptides and source proteins (left y-axis), respectively. Virtual immunopeptidomes of AML and benign samples were generated *in silico* based on random weighted sampling from the entirety of identifications in both original cohorts. These randomized virtual immunopeptidomes were used to define AML-associated antigens based on simulated cohorts of AML versus benign samples. The process of peptide randomization, cohort assembly, and AML-associated antigen identification was repeated 1,000 times and the mean value of resultant virtual AML-associated antigens was calculated and plotted for the different threshold values. The corresponding FDRs (right y-axis) for any chosen threshold (x-axis) were calculated and the 1% and 5% FDRs are indicated within the plot (dotted lines and arrows). (C) Overlap analysis of HLA class II peptide source protein identifications of AML (n = 47) and benign samples (n = 312). (D) Comparative HLA class II immunopeptidome profiling on HLA peptide source protein level based on the frequency of HLA-restricted source protein presentation in AML and benign immunopeptidomes. Frequencies of positive immunopeptidomes for the respective HLA peptide source protein (x-axis) are indicated on the y-axis. HLA class II peptide source proteins (n = 7,935) are depicted on the x-axis, sorted according to the frequency of AML and benign samples presenting the respective peptide source protein. The box on the left and its magnification highlight the subset of AML-associated antigens showing AML-exclusive, high frequent presentation. (E) Hotspot analysis of the proteins AP2B1, HPRT, and IL1AP by HLA class II-presented peptide clustering. Identified peptides were mapped to their amino acid positions within the source protein. Representation frequencies of amino acid counts within each cohort for the respective amino acid position (x-axes) are indicated on the y-axes. The boxes and their magnifications highlight the identified hotspots with the respective amino acids on the x-axes. (F) Comparative profiling of AML- and LSC-exclusive HLA class II source protein identifications (not identified on benign tissue samples) based on the frequency of HLA-restricted source protein presentation in the immunopeptidomes of LSC (n = 11) and AML (n = 47) samples. Frequencies of positive immunopeptidomes for the respective HLA peptide source protein (x-axis) are indicated on the y-axis. HLA class II peptide source proteins (n = 311) are depicted on the x-axis, sorted according to the frequency of LSC and AML samples presenting the respective peptide source protein. The boxes mark subsets of LSC-exclusive antigens, LSC-associated antigens showing presentation on both LSCs and AML bulk cells, and AML-exclusive antigens. Overlap analysis, comparative profiling, and hotspot analysis were performed with curated AML immunopeptidome data. Abbreviations: IPep, immunopeptidome; n, number; aa, amino acid; n_{pep}, number of peptides; LSC, leukemia stem and progenitor cells; IDs, identifications; FDR, false discovery rate.

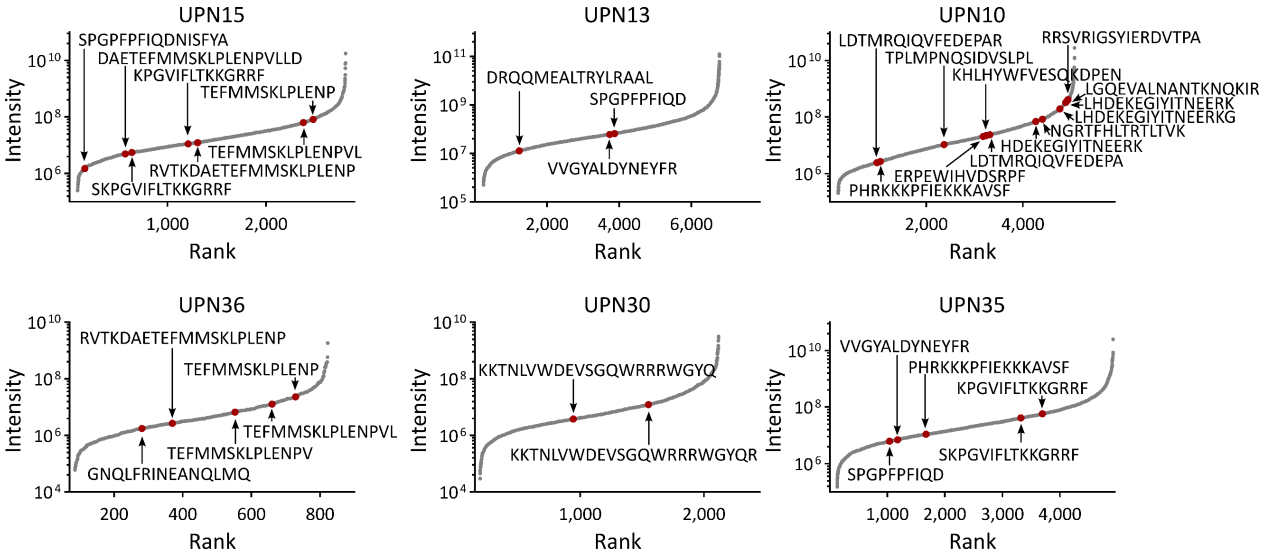

Figure S6: Abundance of AML- and AML/LSC-associated HLA class II peptides within the immunopeptidome. Ranked intensity values of MS data derived from HLA class II immunopeptidomes of representative patients with identified HLA class II AML- and AML/LSC-associated peptides, respectively. Positions of the AML- and AML/LSC-associated peptides are projected on the curve. Abbreviation: UPN, uniform patient number.

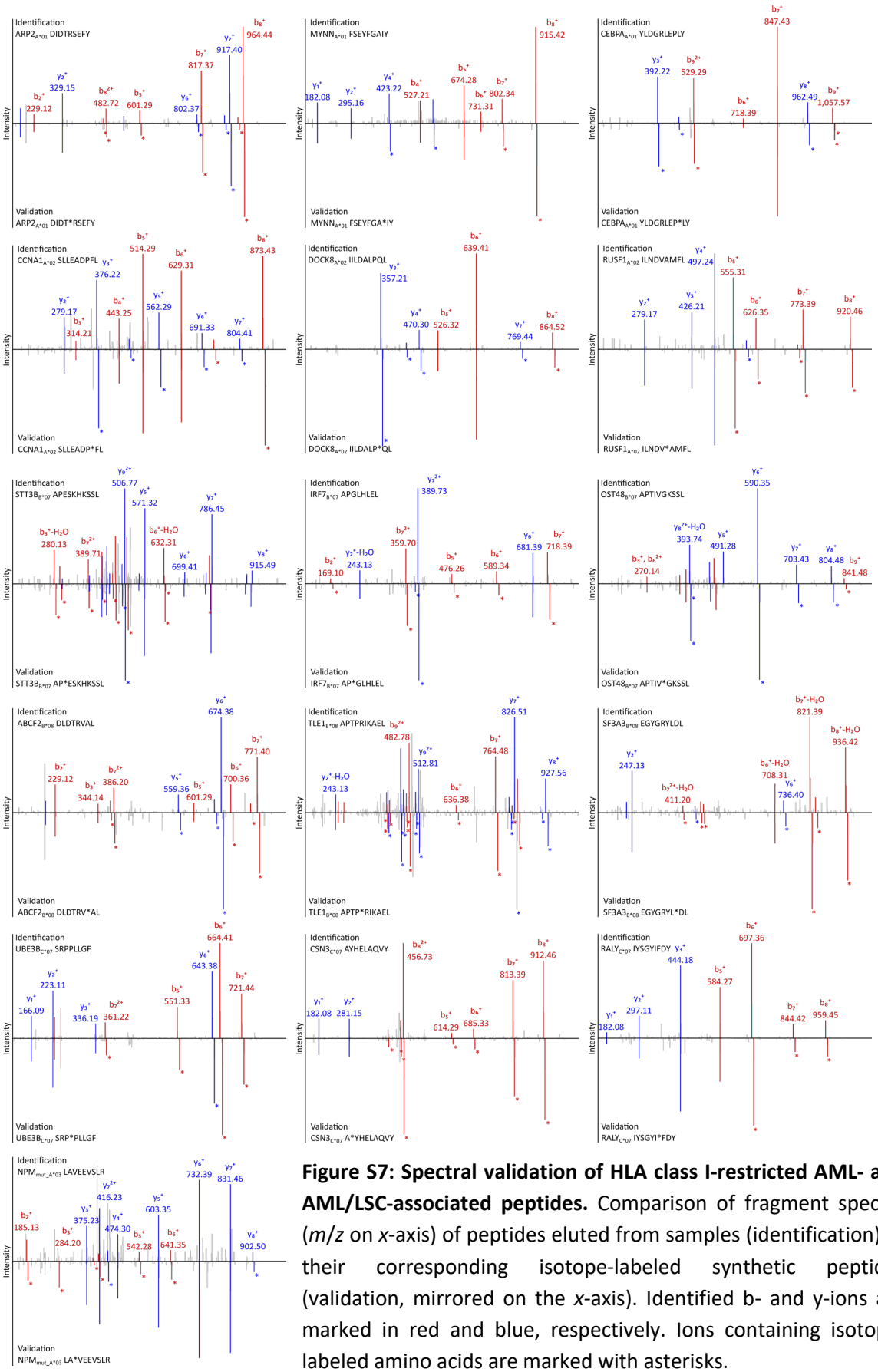

Figure S7: Spectral validation of HLA class I-restricted AML- and AML/LSC-associated peptides. Comparison of fragment spectra (*m/z* on *x*-axis) of peptides eluted from samples (identification) to their corresponding isotope-labeled synthetic peptides (validation, mirrored on the *x*-axis). Identified b- and y-ions are marked in red and blue, respectively. Ions containing isotope-labeled amino acids are marked with asterisks.

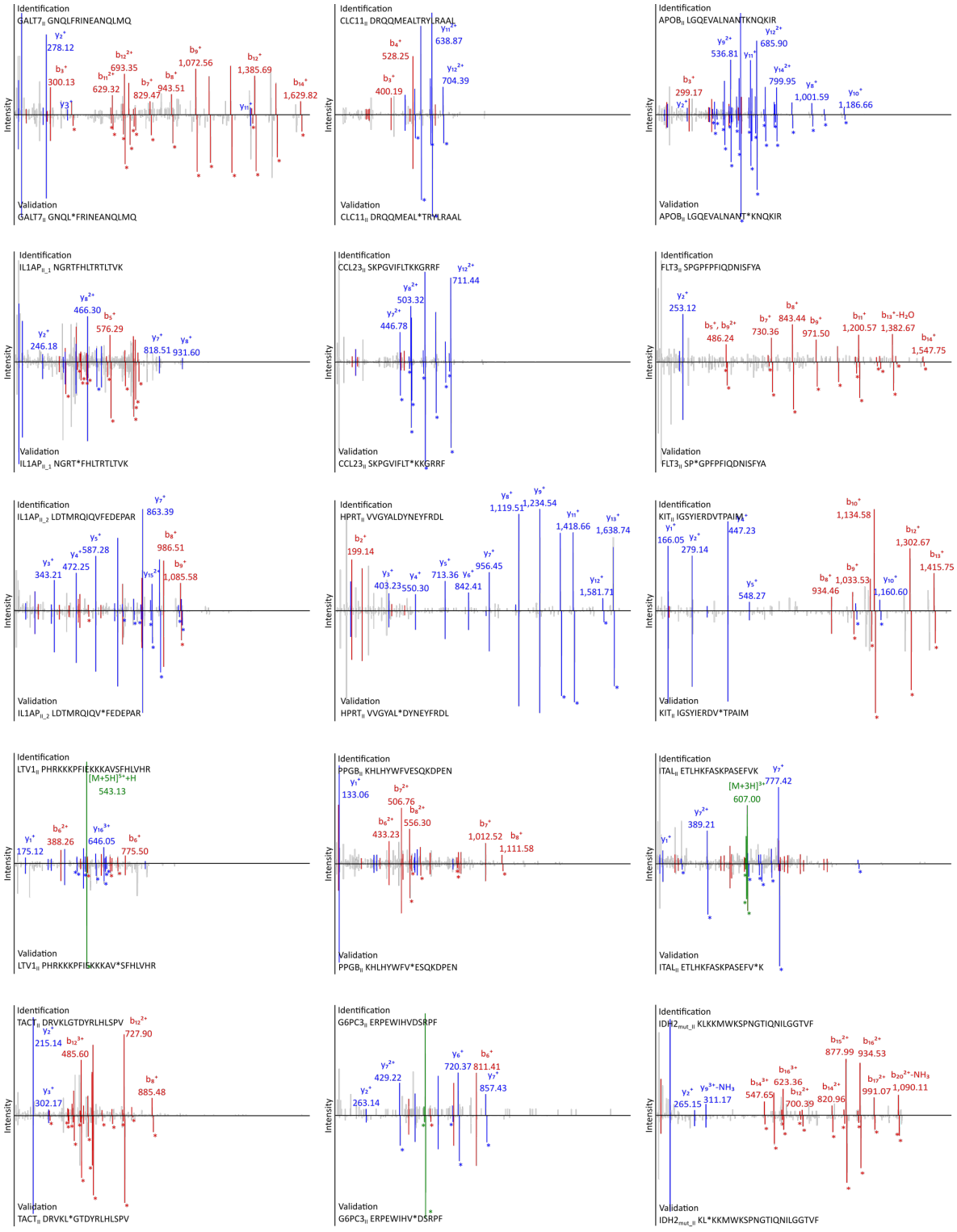

Figure S8: Spectral validation of HLA class II-restricted AML- and AML/LSC-associated peptides. Comparison of fragment spectra (*m/z* on *x*-axis) of peptides eluted from samples (identification) to their corresponding isotope-labeled synthetic peptides (validation, mirrored on the *x*-axis). Identified b-, y-, and precursor ions are marked in red, blue, and green, respectively. Ions containing isotope-labeled amino acids are marked with asterisks.

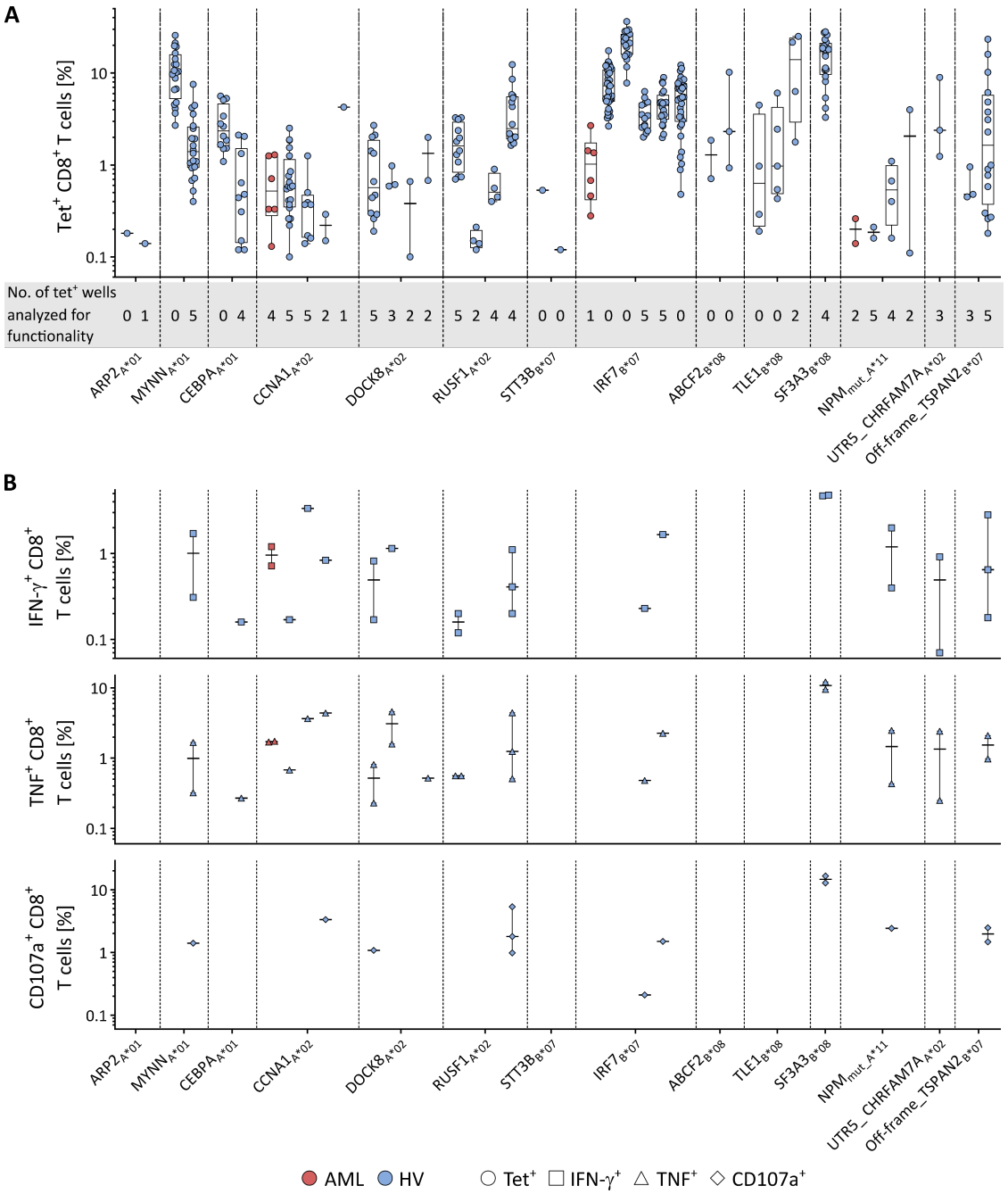

Figure S9: Magnitude and functionality of *in vitro* primed CD8⁺ T cells. (A) Frequency of *de novo* induced peptide-specific CD8⁺ T cells in terms of calculated frequency of tetramer⁺ CD8⁺ T cells after aAPC-based *in vitro* priming experiments with HLA-matched PBMC samples of HVs (blue) and AML patients (red). The depicted calculated frequency refers to the frequency of peptide-specific tetramer⁺ T cells within the single, viable CD8⁺ T cells after staining for CD8 and PE-conjugated tetramer of indicated specificity minus the tetramer⁺ CD8⁺ population in the negative control (staining of T cells from the same donor primed with an HLA-matched control peptide). Each box plot depicts the results of a specific HV or AML patient. Priming was performed in up to 50 wells per donor with 1 x 10^6 CD8⁺ T cells per well. Each dot represents the frequency in a single well. Boxes represent median and 25th to 75th percentiles, whiskers are minimum to maximum. Only positive wells are shown. Up to five tetramer-positive wells were

further analyzed for functionality. (B) Functional characterization of peptide-specific $CD8^+$ T cells after in *vitro* aAPC-based priming by intracellular cytokine (IFN-γ, TNF) and degranulation marker (CD107a) staining. Calculated frequency of IFN-γ^+ $CD8^+$ (upper panel), TNF^+ $CD8^+$ (middle panel) or $CD107a^+$ $CD8^+$ (lower panel) for the respective HV and AML patient samples shown in panel A. Each box plot depicts the results of a specific HV or AML patient. Boxes represent median and 25^{th} to 75^{th} percentiles, whiskers are minimum to maximum. Each dot represents the calculated frequency for one test well. Abbreviations: Tet, tetramer; no., number; HV, healthy volunteer.

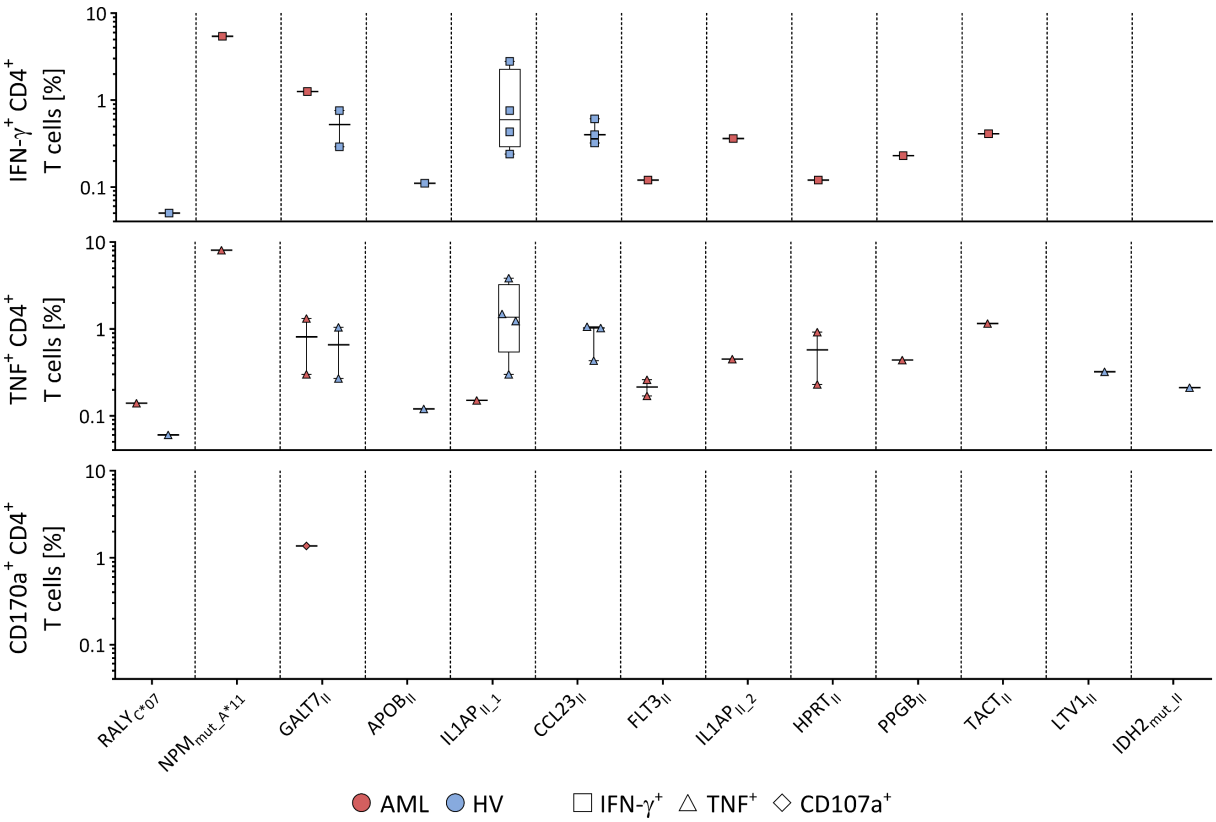

Figure S10: Characterization of memory T cell responses detected in ELISpot assays. Functional characterization of peptide-specific IFN-γ^+ (upper panel), TNF$^+$ (middle panel) or CD107a$^+$ (lower panel) T cells in terms of calculated frequency after IFN-γ ELISpot assays after 12-day stimulation (see Figure 6C) by intracellular cytokine (IFN-γ, TNF) and degranulation marker (CD107a) staining using PBMC samples of HVs (blue) and AML patients (red). All analyzed samples showed a CD4$^+$ T cell-mediated response against the AML/LSC-associated HLA class I and HLA class II peptides. Dots represent data from individual donors. Data is shown for donors with a response. Boxes represent median and 25th to 75th percentiles, whiskers are minimum to maximum. Abbreviation: HV, healthy volunteer.

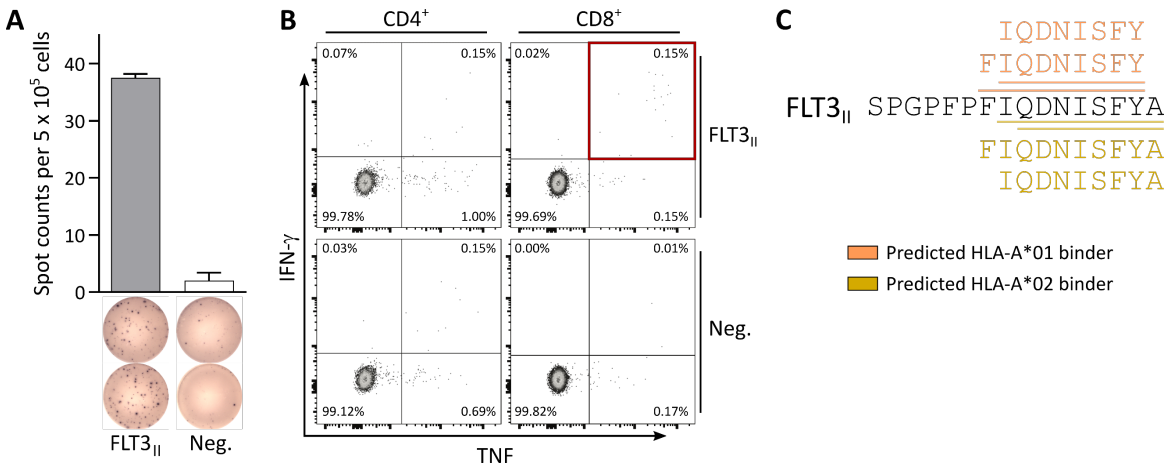

Figure S11: Further characterization of HLA class II-restricted antigens. (A) $FLT3_{II}$-specific T cell responses as assessed by IFN-γ ELISpot assay after 12-day stimulation using PBMC samples of an AML patient. (B) Functional characterization of $FLT3_{II}$-specific T cells by flow cytometry-based intracellular cytokine and surface marker staining revealed a $CD8^+$ T cell-driven $FLT3_{II}$-specific response. (C) *In silico* peptide prediction revealed four possible embedded HLA class I-restricted peptides for the HLA allotypes of the patient (A*01, A*02, B*08, B*13, C*06, C*07). Abbreviations: neg., negative.

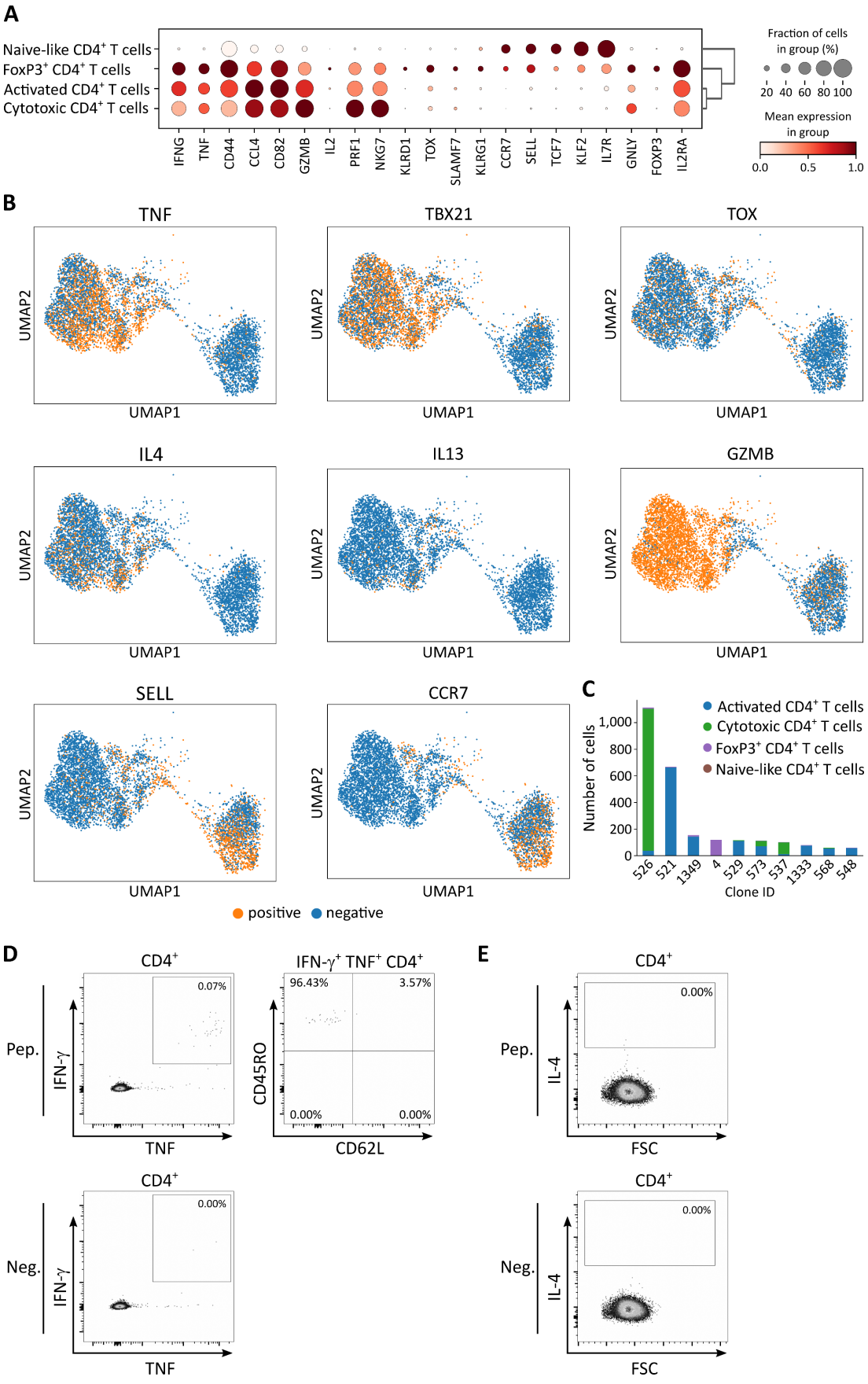
A
Naive-like CD4+ T cells
FoxP3+ CD4+ T cells
Activated CD4+ T cells
Cytotoxic CD4+ T cells
IFNG
TNF
CD44
CCL4
CD82
GZMB
IL2
PRF1
NKG7
KLRD1
TOX
SLAMF7
KLRG1
CCR7
SELL
TCF7
KLF2
IL7R
GNLY
FOXP3
IL2RA
Fraction of cells in group (%)
20 40 60 80 100
Mean expression in group
0.0 0.5 1.0
B
TNF
TBX21
TOX
IL4
IL13
GZMB
SELL
CCR7
UMAP1
UMAP2
positive
negative
C
Activated CD4+ T cells
Cytotoxic CD4+ T cells
FoxP3+ CD4+ T cells
Naive-like CD4+ T cells
Number of cells
1,000
800
600
400
200
0
526
521
1349
4
529
573
537
1333
568
548
Clone ID
D
CD4+
IFN-γ+ TNF+ CD4+
Pep.
Neg.
IFN-γ
TNF
CD45RO
CD62L
0.07%
96.43%
3.57%
0.00%
0.00%
0.00%
E
CD4+
Pep.
Neg.
IL-4
FSC
0.00%
0.00%

Figure S12: In-depth characterization and phenotyping of HLA class II AML/LSC-specific CD4$^+$ T cells by single-cell RNA sequencing and multi-color flow cytometry. (A - C) Single-cell RNA sequencing analysis of CD4$^+$ T cells of HLA class II AML/LSC peptide-stimulated and IFN-γ secretion-based sorted PBMCs of AML patients (n = 3) after 12-day *in vitro* expansion. (A) Dot plot of different marker genes across the CD4$^+$ T cell subclusters showing scaled mean expression by colors and their fraction of cells in group (%) by size. (B) Uniform Manifold Approximation and Projection (UMAP) plots showing the Boolean representation of different marker genes. (C) Distribution of the ten largest T cell receptor (TCR) clonotypes across the cell types of the CD4$^+$ T cell subclustering. (D, E) Representative example of the multi-color flow cytometry-based *ex vivo* phenotyping of HLA class II-restricted AML/LSC peptide-specific CD4$^+$ T cell responses using PBMC samples of AML patients. Graphs show single, viable CD4$^+$ T cells stained for the cytokines (D) IFN-γ and TNF as well as (E) IL-4. (D) IFN-γ$^+$TNF$^+$ CD4$^+$ T cells were further characterized with the markers CD62L and CD45RO. Abbreviations: UMAP, Uniform Manifold Approximation and Projection; ID, identifier; pep., peptide pool of HLA class II-restricted AML/LSC peptides; neg., negative; FSC, forward scatter.

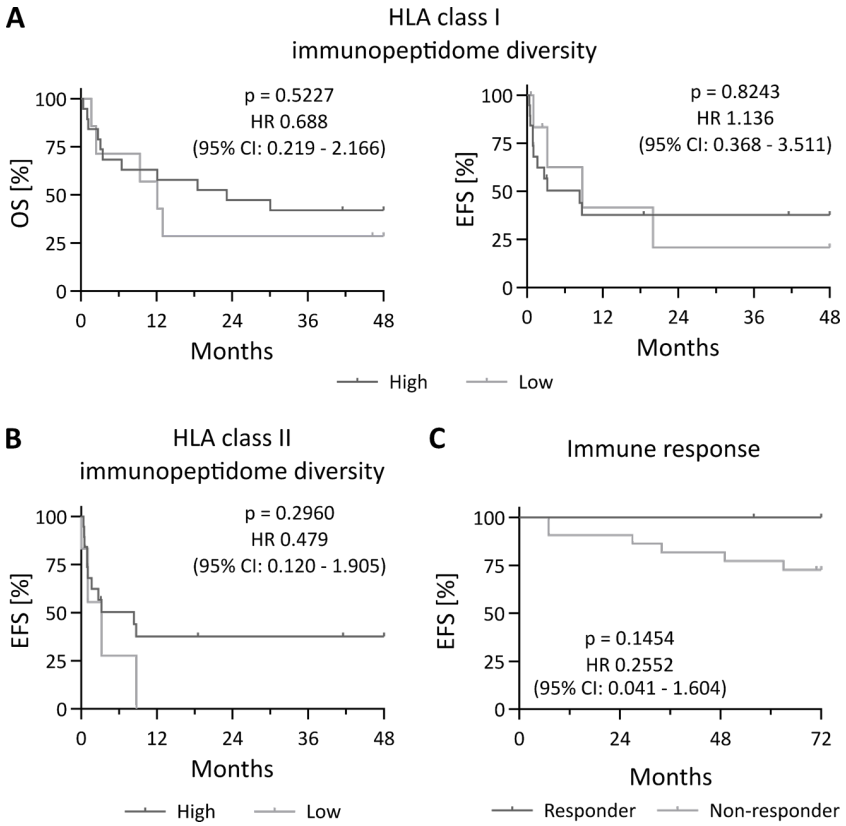

Figure S13: Impact of immunopeptidome diversity and peptide-specific immune responses on patient survival. (A) Impact of HLA class I-restricted immunopeptidome diversity in terms of unique AML-exclusive peptide presentation on OS (left) and EFS (right) survival of AML patients (n = 26, n = 19 high, n = 7 low). (B) Impact of HLA class II-restricted immunopeptidome diversity in terms of unique AML-exclusive peptide presentation on EFS of AML patients (n = 25 total, n = 19 high, n = 6 low). Immunopeptidome diversity was classified as low and high according to the median peptide yields in the AML immunopeptidome cohort. (C) Retrospective correlation analysis of preexisting antigen-specific T cell responses as assessed by *ex vivo* IFN-γ ELISpot assay against HLA class II-restricted AML/LSC-associated peptides with EFS of AML patients after allogeneic stem cell transplantation (n = 29 total, n = 7 responder, n = 22 non-responder). AML patients showing a positive T cell response against the pool of HLA class II-restricted AML/LSC-associated peptides in *ex vivo* IFN-γ ELISpot assays were classified as responders. T cell responses were considered positive when > 10 spots/500,000 cells were counted and the mean spot counts was at least three-fold higher than the negative control. Kaplan-Meier analysis, log-rank test. Abbreviations: OS, overall survival; EFS, event-free survival.