DATA SUPPLEMENT

Identification and validation of expressed HLA-binding breast cancer neoepitopes for potential use in individualized cancer therapy

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METHODS

In vitro peptide HLA-A2 binding assay

For the determination of the *in vitro* HLA-A2 binding affinity of synthesized peptides, T2A2 cells were cultured with 50 μg/mL of the indicated peptides overnight at 37°C. The binding of exogenous peptide stabilizes the HLA-A2 complex on the surface of the TAP-deficient T2A2 cells. The affinity of the peptide to bind the HLA-A2 molecule is assessed via flow cytometry as function of increased HLA-A2 mean fluorescent intensity. FITC-labeled anti-HLA antibodies were purchased from One Lamda, and data was acquired using a FACS Calibur Flow cytometer (BD Biosciences).

In vitro generation of neoepitope-specific T cells from healthy donors

Peripheral blood from healthy donors was obtained under the appropriate Institutional Review Board (IRB) approval and with informed consent. Neoepitope-specific T cells were generated from HLA-A2 expressing healthy donor peripheral blood mononuclear cells (PBMCs) using three rounds of *in vitro* stimulation. For each round of stimulation, dendritic cells (DCs) were prepared from PBMCs by plating 2 x 10^7 cells/well in 6-well plates in AIM V medium (Invitrogen). Cells were allowed to adhere for 2 to 4 hours at 37°C. Following incubation, non-adherent cells were removed, and remaining cells were washed once with PBS and incubated in AIM V media containing 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech) and 20 ng/mL interleukin-4 (IL-4; Peprotech). Cytokine-containing media was changed every 48 hours for 5 days. On day 5, DCs were matured for 1 to 2 days with CD40L (0.5 μg/mL; Enzo Life Sciences) in the presence of a cross-linking enhancer (1 μg/mL). After maturation, DCs were peptide-pulsed with MDA-MB-231 derived 9-mer neoepitopes (20 μg/mL) in RPMI media containing 10% (v/v) human AB serum for 1 hour. For the first round of *in vitro* stimulation, 2 x 10^6 autologous PBMCs were added per well following peptide incubation. After 48 hours, 100 ng/mL each of IL-15 (Peprotech) and IL-7 (Peprotech) were added to each well and cytokines were replenished every 2 to 3 days for 7 days. For subsequent rounds of *in vitro* stimulation, DCs were peptide pulsed for 1 hour as described above. T cells were harvested from the previous round of *in vitro* stimulation, counted, and added to new DCs in 2 mL media. IL-15 and IL-7 were added to media

every 2 days. Following the third round of *in vitro* stimulation, CD8+ T cells were isolated using a negative selection (Miltenyi Biotec). Purified CD8 cells rested in media containing IL-15 and IL-7 prior to assessment of either their cytotoxicity or antigen-dependent IFN-γ secretion.

Neoepitope prediction for two TILGen subjects

Preclinical validation of neoepitope prediction in BC patients

From two TILGen study[1,2] patients, peripheral blood and tumor tissue were obtained after approval by the internal Institutional Review Board (IRB) and with informed patient consent according to the Declaration of Helsinki. Briefly, the TILGen (TILs and genomics) study was a predefined substudy of the iMODE-B (imaging and molecular detection of breast cancer) study. iMODE-B is concerned with molecular markers at the time of breast cancer diagnosis or progression, molecular detection, and imaging detection of breast cancer. The TILGen study focused on the identification of antigen-specific TILs in TNBC and HER2-positive breast cancer patients in order to identify immunogenic targets that could help to improve cancer immunotherapy. Patients were eligible for inclusion in the iMODE-B study if an indication existed for a diagnostic biopsy because of a suspicious breast lesion.[1,2] Peripheral blood was obtained for the isolation of germline DNA and generation of antigen-presenting cells (APC) (Epstein-Barr Virus (EBV)–transformed B cells and monocyte-derived DCs). Tumor material was obtained by ultrasound guided needle-core biopsy for DNA extraction and expansion of TILs. One patient with HER2+ BC (TILGen 1) and another with TNBC (TILGen 2) were the initial patients tested for neoantigen-reactivity. Both received neoadjuvant chemotherapy according to standard of care and had pathological complete responses. For patient 1, resected tissue from the primary breast tumor site after neoadjuvant chemotherapy was available in addition to the initial biopsy and underwent analysis.

Generation of autologous dendritic cells (DCs)

DCs of the two breast cancer patients were generated from monocytes isolated from autologous PBMCs by magnetic separation as described in the Data Supplement. Monocytes (1 x 106 /mL RPMI) were incubated with 560 U/mL GM-CSF(Miltenyi Biotech) and 500 U/mL IL-4 (Peprotech). On day 5, 200 U/mL TNFα, 2000 U/mL IL-1β, 1600 U/mL IL-6, 560 U/mL GM-CSF, 500 U/mL IFN-γ (all Miltenyi Biotech) and 1 µg/ml PGE-2 (Enzo) were added. DCs were harvested on day 6 to 7 and subsequently used for stimulation.

Peptide pulsing of stimulator cells

Before pulsing, peptides were dissolved in DMSO and diluted in RPMI. Peptide pulsing was performed by incubating stimulator cells for 2 hours with synthetic peptides (1 µg/mL or as indicated) in RPMI containing 2% human serum. Peptide-pulsed cells were washed and subsequently used as stimulator cells. Alternatively, MCF-7 cells (5 x 10^6 / ml RPMI) were lysed in three subsequent freeze-thawing cycles (liquid nitrogen to 37°C)

followed by loading of 100 µL of those cell lysates onto EBV-LCLs. T-cell activation was measured either as cytokine release by IFN-γ ELISA (Invitrogen) according to the manufacturer's instructions or as upregulation of CD137 in flow cytometry. For blocking experiments, HLA class I (W6/32; Biolegend) and HLA class II (PdV5.2; Santa Cruz Biotechnology) blocking antibodies were added at 2 µg/mL to the co-culture 30 minutes before addition of the T-cell clone.

Retroviral transduction of BC-derived neoantigens and HLA alleles

To confirm processing and presentation of neoantigens when endogenously expressed, total RNA from patient-derived EBV-LCLs was obtained using RNeasy Mini columns (Qiagen) and transcribed into cDNA by reverse transcriptase using the One Taq RT-PCR Kit (New England Biolabs, Germany). HLA restriction molecules and RBMX, PNMAL1 and CARS2 wildtype (wt) were amplified with specific primers and PNMAL1 P100R, CARS2 Q171H and RBMX T55I were generated by a 2-step PCR with oligos encoding the single nucleotide mutation (see all oligos and plasmids in Table S4). HLA-DQ and -DP α- and β- chains were fused by a T2A linker. PCR products were cloned into retroviral vector MP71 or pLZRS, including the marker genes ΔNGFR or GFP. Inserted PCR products were verified by sequencing. Wild-type φnx A packaging cells were transfected with these vectors as described previously. [3] Viral supernatants were used for transduction of EBV-LCL, class II negative HeLa or MCF-7 cells on plates coated with 30 µg/ml recombinant human fibronectin CH296 (RetroNectin;Takara Bio). Expression of the transgenes was verified by marker gene expression and/or surface expression of HLA class II alleles.

Flow cytometry

For flow cytometric analysis and/or isolation of cells, the following antibodies were used: CD3 labelled with FITC or BV (Brilliant Violet) 510, APC-Cy7 or APC-labeled CD8, BV421-labelled CD4, PE-labelled CD137 and NGFR (all BD Sciences), as well as PE-labelled HLA-DP (Leinco). IFN-γ producing cells were PE-labelled by using the cytokine secretion assay (Miltenyl Biotech) followed by flow cytometric sorting (Aria II, BD). The IOTest Beta Mark TCR Vβ Repertoire Kit (Beckman Coulter), which enables the identification of 24 different specificities, was used to determine the T-Cell Receptor (TCR) variable beta chain of different T-cell clones.

TCR sequencing and clonotypic PCR

For TCR sequencing, RNA of T-cell clones was isolated using an RNeasy mini kit according to the manufacturer's protocol, including QIA shredder and DNase digestion (all Qiagen). cDNA was generated using SMARTScribe Reverse Transcriptase (Clontech) and 1 µM dNTPs (Roche) with an oligo in the constant β chain region (5'-CAGTATCTGGAGTCATTGA-3') and a target switching RNA anchor with added ribosomal guanine residues (5'-AAGCAGTGGTATCAACGCAGAGTACggg-3') as described previously. [4] In a second PCR step, amplification of the variable region was performed by using the Pwo SuperYield DNA Polymerase (Roche) according to the

manufacturer's instructions. Oligos included an anchor specific primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') used in combination with a more proximal primer within the constant region (5'-CACGTGGTCGGGGWAGAAGC-3'). PCR was performed with 33 cycles, an annealing temperature of 59°C, and 1 minute of extension time. Resulting PCR bands were purified via gel electrophoresis, excised, and sequenced. Sequencing was performed using 5 μL of DNA (100 ng/μL) mixed with 5 μL of the respective primer followed by Sanger sequencing at GATC Biotech (Germany). Analysis of the resulting sequences was performed using the International Immunogenetics Information Systems. [5]

For clonotypic PCR, forward primers were designed to bind in the highly variable CDR3 region, while reverse primers bound in a distance of 70 to 120 bp. The following primers were used: 3E1 fw: 5'-CAATGGCTACAATGTCTCCAGATTAA-3', 3E1 rv: 5'-AGGCACTGCTGGCACAGA-3'; E15 fw: 5'-TCAGGGCGCCAGTTCTCTAA-3', E15 rv: 5'-CCTAAGCTGCTGGCGCAAA-3'; G44 fw: 5'-CAGGAATGCCAAAGGAACGATT-3', G44 rv: 5'-CTGGTGAGCTGGCACAGAA-3'; 1A35 fw: 5'-ATCCGTCTCCACTCTGAAGATC-3', rv: 5'-TTCAGTGTTGCTCCCTAAGCT-3'. PCR was done in 10 µL reaction mix containing 100 ng of cDNA with 100 nM of each primer and 5 µL of the SYBR Select Master Mix (Thermo Scientific). Amplification was performed with 2 minutes at 50°C, 2 minutes at 95°C, followed by 65 cycles of 3 seconds at 95°C, 30 seconds at 59°C. β-Actin was used as a housekeeping gene: fw: 5'-CCGAGGACTTTGATTGCACA-3', rv: 5'-AGTGGGGTGGCTTTTAGGAT-3'. To verify specific amplification of qPCR products, they were Sanger sequenced using the described primers.

Immunoblotting

Cells (5×10^6 cells/sample) were lysed on ice in 70 µL RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 0,2 mM Na3VO4, pH 7.4) supplemented with protease inhibitors (Roche) for 30 minutes. Cytosolic proteins were isolated from supernatants after centrifugation at 16.000 x g (20 min, 4°C) and protein concentration was determined by Pierce BCA Protein Assay Kits (Thermo Fischer Scientific). Protein lysate (15 µg) was separated by SDS-PAGE for 1 hour at 120 V using precast Mini-PROTEAN TGX 4–15% gradient gels (Bio-Rad) and blotted on 0.2 µm Trans-Blot Turbo polyvinylidene difluoride membranes (Bio-Rad). Proteins were detected by rabbit anti-CARS2 antibody (Sigma-Aldrich), mouse IgG1 anti β-Aktin (Santa Cruz), and visualized by the WesternDot 625 goat anti-rabbit Western blot kit according to the manufacturer's protocol (Life Technologies).

Table S1. HLA-A2 restricted neoepitopes identified in the MDA-MB-231 cell line. Highlighted rows indicate the 20 peptides that were synthesized for further *in vitro* analysis.

Gene Name	Transcripts per million	Affinity (nM)	Neoepitope
RTTN	6.42	6	GLQDCLHSV
PIGO	18.88	7	LLIAHFLGV
SLC17A5	29.48	9	GTIGIFWFV
MUC4	3.92	15	LLVTSLSSV
ENTPD7	4.57	23	FLRQWVAFL
PITPNM2	7.14	25	NVFDTVMHV
ARFGAP1	48.51	27	SLLPHKHVV
KIF13B	8.47	28	TLTHILYDV
RTN4	422.86	31	AMAKIQAKV
ZNF703	50.98	34	LLNPHTLGL
NCAPD3	39.7	34	YLEYGLHAA
RANBP2	35.47	35	FLTNDETKV
ADGRG6	29.04	35	SLQGLFILI
HAUS3	17.76	39	SLVQHQLAV
SLC17A5	29.48	42	GIFWFVLWI
DNAH14	10.31	45	ILIQELEEI
YIPF1	19.35	64	AMLVFAIAI
IDUA	7.58	80	LLSNDNALL
PRR16	5.21	84	SVHHYAWVV
RFX2	4.45	93	SMVGITMDI
ZCCHC7	17.23	96	NLVGYENSV
KMT2C	17.49	98	QMYHYSCAA
TCF25	38.79	99	ILCEIKEAV
ATP13A1	36.77	105	ALASCHLLM
COMMD8	26.81	116	ALSSDTIAA
MUC4	3.92	129	LLVTNASSV
DYM	12.99	130	RLLQSGAEL
PIGO	18.88	150	TMDSGEWDL
ATP11A	20.46	173	HMQDYGLII
SLC17A5	29.48	175	WFVLWIWLV
C10orf88	8.52	187	HIDDNIALL
ADAM8	87.28	191	GLLGDSEEA
IDUA	7.58	198	ALLSNDNAL
HERC2	12.92	218	LLLLQLWYS
ENTPD7	4.57	218	RQWVAFLGL
RTN4	422.86	271	KIQAKVPGL
SLC17A5	29.48	288	ILLSLRNQL
TLN1	208.07	290	KQAAHTLEA
PRDM2	12.1	320	KIQDIQLKI
USP5	53.58	360	GLGGLPNIV
COMMD8	26.81	365	KLALSSDTI
GTF3C1	29.64	385	FIGRPWHVV
PANK2	26.55	410	RLLLRMGGV
PITPNM2	7.14	415	VMHVHYPSA
DPY19L4	14.75	428	KLIASILYQ
ASPM	40.59	430	MLKPSTLII
SLC18A2	5.14	433	FANMGIATL
YIPF1	19.35	445	WICAMLVFA
DPY19L4	14.75	464	QQMSLYPKL
RFC1	30.26	468	KQNWRLLPA

Table S2. All peptides used for screening T-cell reactivities in HER2+ TILGen patient 1 are shown. For HLA class I, all predicted neoepitopes were used as 9-mer peptides. For HLA class II, fusion peptides were generated for each mutation predicted to bind.

No.	Gene	Mutation	Sequence	HLA class
1	**RBMX**	p.T55I	SRGFAFVIF	I
2	**RAB1B**	p.L186V	RPNVKIDST	I
3	**GOLGA3**	p.H1486Tfs*35	PRGDPQRTV	I
4	**PARP9**	p.G383V	LVTKVFNLF	I
5	**BAIAP2**	p.N339K	DSYSKTLPV	I
6	**PTHLH**	p.D99del	FGSDEGRYL	I
7	**SLC1A4**	p.A516F	NPFGPVASA	I
8			PLVTHQNPF	I
9	**DOK7**	p.S56R	LRERSRLTL	I
10	**HELLS**	p.H535R	EREKNMRSF	I
11	**RRN3**	p.S199L	RYVPLTPWF	I
12			VPLTPWFLM	I
13			ARYVPLTPW	I
14			YVPLTPWFL	I
15	**CDK20**	p.I135M	LLMSASGQL	I
16			MSASGQLKI	I
17			KPANLLMSA	I
18	**ANAPC1**	p.T416M	LWTEMITNI	I
19			CIDHLWTEM	I
20			WTEMITNIR	I

No.	Gene	Mutation	Sequence	HLA class
21	**CDK20**	p.I135M	DLKPANLLMSASGQLKIADFG	II
22	**SLCO2B1**	p.R369C	QFIKVFPRVLLQTLCHPIFLLVVLSQVC	II
23	**ABAT**	p.R450Q	DSIQNKLILIARNKGVVL	II
24	**RRN3**	p.S199L	TCHRALQIIARYVPLTPWFLMPILVEKFP	II
25	**FAM204A**	p.D141E	WKELTQYFGVNDRFEP	II
26	**SLC1A4**	p.A516F	EETSPLVTHQNPFGPVAS	II
27	**PARP9**	p.G383V	AKQFQRSQLVLVTKVFNLFCKYIYHVLWH	II
28	**RBMX**	p.T55I	DRETNKSRGFAFVIFESPADAKDAA	II

Table S3. All peptides used for screening T-cell reactivities in TNBC TILGen patient 2 are shown. For HLA class I (left), all predicted neoepitopes were used as 9-mer peptides. For HLA class II (right), fusion peptides were generated for each mutation predicted to bind.

No.	Gene	Mutation	Sequence	HLA class
1	HEBP2	p.F142S	RSSDGFSSA	I
2	DCUN1D2	p.D150A	ATAKFKDFY	I
3	BHLHE40	p.T163S	SRDLKSSQL	I
4			AKHENSRDL	I
5	CARS2	p.Q171H	RVTENIPHI	I
6	SLC30A5	p.M748I	ITKQMESMK	I
7	CSF1R	p.D698G	GYKNIHLEK	I
8	ECI1	p.Y136D	LRLDQSNLV	I
9	NUP160	p.L308V	KVRMWSYKE	I
10	MTMR12	p.R642H	KVWAQHYLR	I
11			HYLRWIPEA	I
12	PUS7	p.E223K	KTKDREGKK	I
13	PNMAL1	p.P100R	RTQDAEFLK	I
14			DRTQDAEFL	I
15	TGIF1	p.M226L	RLLPDLLRK	I
16	CDCA8	p.L202V	KTPGVRTPA	I
17	ZNF562	p.F268C	CTNFSQLSA	I
18			KTKNCGKSC	I
19	PARP4	p.T1170I	LSKENSLII	I
20	TUT1	p.R36T	RYRTVAMAA	I
21	ARHGAP39	p.N837S	HMDPVSDTK	I
22	CARD11	p.M369I	KHRMNTVIL	I
23	UNKL	p.P250H	YRSTPCHSV	I
24			RSTPCHSVK	I
25			QYRSTPCHS	I
26	AP4B1	p.T89R	LLAINRLCK	I

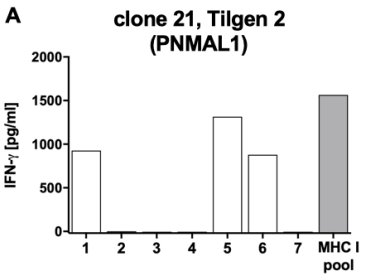
No.	Gene	Mutation	Sequence	HLA class
27	DCUN1D2	p.D150A	LKATAKFKDFYQFTFTF	II
28	SLC30A5	p.M748I	SGLSTGFHDVLAITKQMESMK	II
29	IFT172	p.P1340H	PQRNMEVVLAVGHQLIGIGKHSAAAEL	II
30	HEBP2	p.F142S	FIEDRAEMTVFVRSSDGFSSAQ	II
31	PUS7	p.E223K	TKDREGKKYIVAYHAA	II
32	BHLHE40	p.T163S	AKHENSRDLKSSQLVTHLHR	II
33	EMILIN2	p.K182R	KEGPQELQERKIQVLEEKVLRLTR	II
34	GIT2	p.T309S	DAVWLATQNHSALVSETTVVPFLPVNPE	II
35	CARS2	p.Q171H	LPPTVYLRVTENIPHIISFIEGIIAR	II
36	CDCA8	p.L202V	PRFDSRVFKTPGVRTPAAGERIY	II
37	DDB2	p.V164L	IKGAAWHPRYNLIVLGRY	II
38		p.V353L	PIKAAWHPRYNLIVLG	II
39	MEGF8	p.G2723R	AGVATLLLQLPGRPHAP	II
40	XPO5	p.F920Y	EHYEALVSPILGPLYTYLHMRLSQKWQVI	II
41	KCNK5	p.R112C	GCLFCVFYGLFGVPLC	II
42	KIF26B	p.S553Y	DGCVFCFGHAKLGKYYTMI	II
43	NUP160	p.L308V	EHDAFIFALCQDHKVRMWSYKEQMCLMVA	II
44	ECI1	p.Y136D	AGYWKAVQELWLRLDQSNLVLVSAINGAC	II
45	PNMAL1	p.P100R	RTQDAEFLKNLNEFL	II
46	CARD11	p.M369I	GKDCEMYKHRMNTVILQLEEV	II
47	TUT1	p.R36T	RWWQRCLCFCRYRTVAMAAVDSDVE	II
48	PCBP1	p.G52W	RIREESGARINISEW	II
49	INTS1	p.R1657G	FGPYLLTLFTHQSSWP	II
50	MTMR12	p.R642H	GPEIKVWAQHYLRWIPEAQILGGG	II
51	USP9X	p.G2131R	AKLIVFIAHFSLQDRPCPSP	II
52	ARHGAP39	p.N837S	SYLEGYIYRHMDPVSDTKGVAISTYAKY	II
53	ARHGAP39	p.N837S	GYIYRHMDPVSDTKVTQHIKELLE	II
54	PCNXL2	p.G2111A	LHDRCLAEAVADTLAVV	II
55	TBC1D9B	p.L714V	LQVALAVLDANMEQV	II
56	ZNF562	p.F268C	GKSCTNFSQLSAHAKTHK	II
57	RAB11FIP3	p.V298L	PDEFDDFLTYEANEVTD	II
58	AP4B1	p.T89R	APLKPDLALLAINRLCKD	II
59	TGIF1	p.M226L	CNWFINARRRLLPDLLRK	II
60	GALNS	p.C507S	GCEKLGKSLTPPESIPKKCLW	II
61	PTPN7	p.Y239F	YDGKEKVFIATQGPMPNTVSDF	II
62	PARP4	p.T1170I	KSLIIKLSKENSLIIQFTSFVAVEKRDEN	II
63	UNKL	p.P250H	RRNPRRFQYRSTPCHSVKHGD	II
64	PPP1R13L	p.D633H	ARLNPLVLLLHAALTGELEVVQ	II
65	CYP2D6	p.V370I	IHEVQRFGDIIPLGVTHMTSRDIEV	II

Table S4. All oligonucleotides and plasmids used for cloning and amplification are shown.

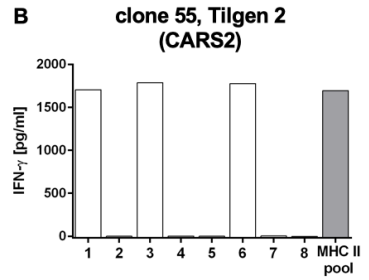
Construct	Cloning oligos	Sequence (5' → 3')	Plasmid	Marker gene
A1*1101	HLA 1101 For	CGCGGATCCACCATG GCCGTCATGGCGCC	pLZRS	NGFR
	HLA 1101Rev	CCGGAATTCTCACACTTTACAAGCTGTGAG		
DRB1* 1501	HLA DRB For	CGCGGATCCACCATGGTGTGTCTGAAGCTCC	MP71	NGFR
	HLA DRB Rev	CCGGAATTCTCAGCTCAGGAATCCTGTTGG		
DPB1* 0201	HLA DPA1 0103 For	CGCGGATCCACCATGCGCCCTGAAGACAGAATG	pLZRS	NGFR
	HLA DPA1 0103 Rev	CTCCACGTCACCGCATGTTAGAAGACTTCCTCTGCCCTCCAGGGTCCCCT GGGCCC		
	HLA DPB1 0201 For	TCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCTATGATGGTTCTG CAGGTTTCT		
	HLA DPB1 0201 Rev	CCGGAATTCTTATGCAGATCCTCGTTGAAC		
DPB1* 0401	HLA DPA1 0103 For	CGCGGATCCACCATGCGCCCTGAAGACAGA ATG	pLZRS	NGFR
	HLA DPA1 0103 Rev	CTCCACGTCACCGCATGTTAGAAGACTTCCTCTGCCCTCCAGGGTCCCCT GGGCCC		
	HLA DPB1 0401 For	TCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCTATGATGGTTCTG CAGGTTTCT		
	HLA DPB1 0401 Rev	CCGGAATTCTTATGCAGATCCTCGTTGAAC		
DRB1* 0701	HLA DRB1 0701 For	CGCGGATCCACCATG GTGTGTCTGAAGCTCC	pLZRS	NGFR
	HLA DRB1 0701 Rev	CCGGAATTC TCAGCTCAGGAATCCTGTTGG		
DQB1* 0202	HLA DQA1 0201 For	CGCAGATCTACCATGATCCTAAACAAAGCTCTG	pLZRS	NGFR
	HLA DQA1 0201 Rev	CTCCACGTCACCGCATGTTAGAAGACTTCCTCTGCCCTCCAAGGGCCCTT GGTGTCTG		
	HLA DQB1 0202 For	TCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCT ATGTCTTGGAAAAAGGCTT		
	HLA DQB1 0202 Rev	CCGGAATTCTCAGTGCAGGAG CCCTTTC		
RBMX wt/T55I	RBMX Start For	CGCGGATCCACCATGGTTGAAGCAGATCGCC	MP71	GFP
	RBMX Stop Rev	CCGGAATTCTAGTATCTGCTTCTGCCTCC		
RBMX T55I	RBMX mut For	GCTTTTGTCA**T**CTTTGAAAGCCCAGC	MP71	GFP
	RBMX mut Rev	GGGCTTTCAAAG**A**TGACAAAAGCAAATCC		
PNMAL wt/P100R	PNMAL Start For	CGCACGCGTCCACCATGTCCAAGACCATGGCGATG	MP71	GFP
	PNMAL Stop Rev	CGCCTCGAGTCAAACCTTTCTGGATTCATTGGT		
PNMAL P100R	PNMAL mut For	CTGTAGAGACC**G**TACCCAGGATGCTGAG	MP71	GFP
	PNMAL mut Rev	CATCCTGGGTA**C**GGTCTCTACAGACCAC		
CARS wt/Q171H	CARS2 Start For	CGCACGCGTCCACCATGTTGAGGACTAC	MP71	GFP
	CARS2 Stop Rev	CGCCTCGAGTCAGCCCGCTGATTTTTGG		
CARS2 Q171H	CARS2 mut For	GAAAATATTCCTCA**C**ATAATTTCTTTCATTG	MP71	GFP
	CARS2 mut Rev	GAAAGAAATTAT**G**TGAGGAATATTTTCGGTTAC		

Figures and legends

Figure S1



MHC I PP (pat. 2)	
Subpool	Peptides
1	1,2,3,4,5,6,7,8,9,10,11,12,**13**,14,15
2	1,2,3,4,5,16,17,18,19,20,21,22,23,24,25
3	1,6,7,8,9,16,17,18,19,26
4	2,6,10,11,12,16,20,21,22,26
5	3,7,10,**13**,14,17,20,23,24,26
6	4,8,11,**13**,15,18,21,23,25
7	5,9,12,14,15,19,22,24,25



MHC II PP (pat. 2)	
Subpool	Peptides
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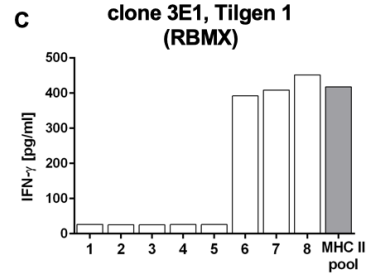
2	27,28,29,30,31,32,48,49,50,51,52,53,54,55, 56,57,58,59,60,61,62
3	27,33,34,**35**,36,37,48,49,50,51,52,63,64,65
4	28,33,38,39,40,41,48,53,54,55,56,63,64,65
5	29,34,38,42,43,44,49,53,57,58,59,63
6	30,**35**,39,42,45,46,50,54,57,60,61,64
7	31,36,40,43,45,47,51,55,58,60,62,65
8	32,37,41,44,46,47,52,56,59,61,62



MHC II PP (pat. 1)	
Subpool	Peptides
1	21,22,23
2	22,23,24
3	23,24,25
4	24,25,26
5	25,26,27
6	26,27,**28**
7	27,**28**,21
8	**28**,21,22

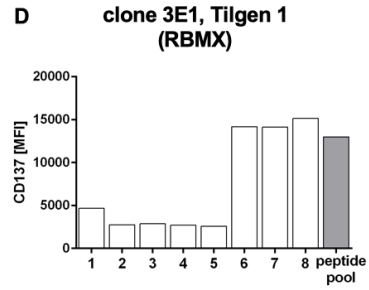


Fig. S1. *Peptides of the respective MHC class I or II pool were subpooled and tested for recognition by the T-cell clones in an IFN-γ ELISA*. The IFN-γ assay results are shown for (A) PNMAL1, (B) CARS2, and (C) RBMX. (D) Upregulation of CD137 as measured by flow cytometry upon peptide stimulation is shown for RBMX.

Figure S2

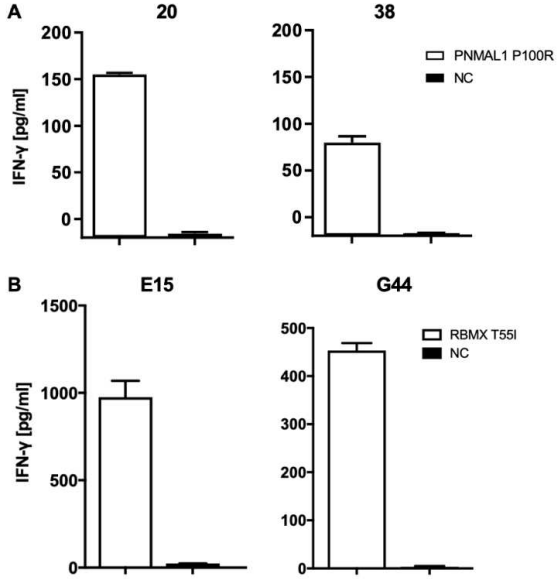


Fig. S2. Additional peptide pool reactive CD8+ T-cell clones from patient 2 (n=30) and CD4+ T-cell clones from patient 1 (n=2) were tested against PNMAL1 P100R (A) and RBMX T55I (B) peptides in an IFN-γ ELISA. For patient 2, two exemplary clones are depicted. NC (negative control): autologous EBV-LCL.

Figure S3

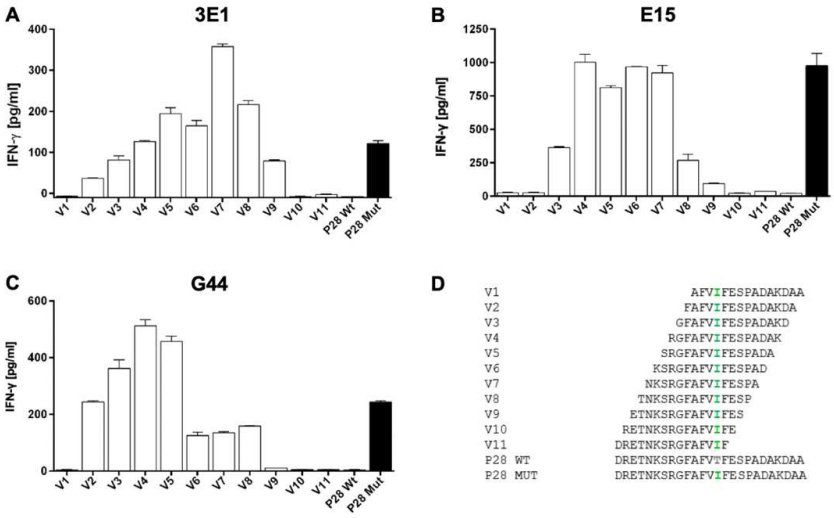


Fig. S3 The core epitope of RBMX T55I was identified for all three T-cell clones (A) 3E1, (B) E15, and (C) G44 by testing recognition of truncated peptides (D) loaded onto autologous EBV-LCL as measured in an IFN-γ ELISA. The means and SEM of duplicates are shown.

Figure S4

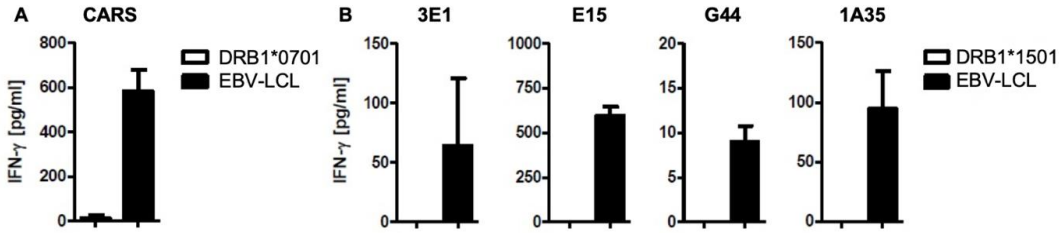


Fig. S4. T-cell recognition of HeLa cells retrovirally transduced with the predicted HLA-DRB1*0701/DRA1*0102 (A) and HLA-DRB1*1501/HLA-DRA*0102 (B). Depicted are the mean and SEM of triplicates (A) or duplicates (B) as measured after loading with CARS2 Q171H (A) and RBMX T55I (B) peptide. HLA compatible peptide-loaded EBV-LCLs were used as controls.

Figure S5

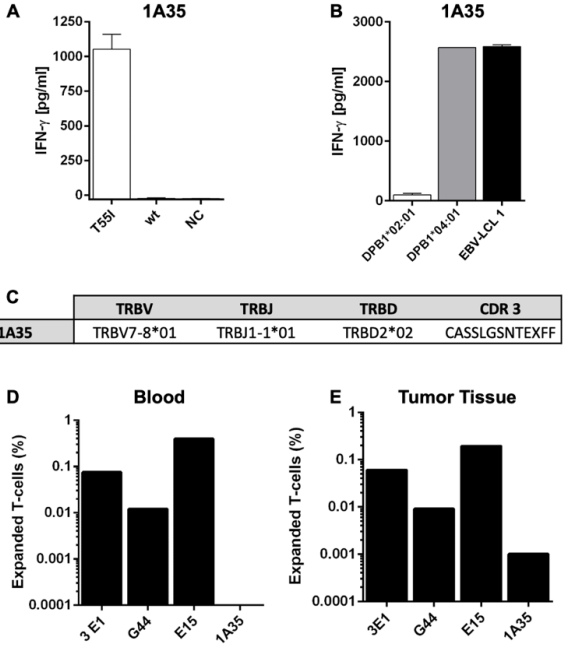


Fig. S5. *RBMX T55I specific T-cell responses persist after neoadjuvant chemotherapy*. (A) CD4+ T-cell clone 1A35 isolated from resected tumor tissue after neoadjuvant chemotherapy specifically recognizes RBMX T55I but not wt RBMX as measured in an IFN-γ ELISA against peptide-loaded autologous EBV-LCL from patient 1. Depicted are the mean and SEM of triplicates. NC: negative control (unloaded autologous EBV-LCL). (B) 1A35 recognizes peptide loaded HeLa cells retrovirally transduced with HLA-DPB1*0401, but not HLA-DPB1*0201 in the IFN-γ ELISA. Depicted are the mean and SEM of duplicates. (C) TCR sequencing of T-cell clone 1A35 reveals a differential variable beta chain and a unique CDR3 region. Clonotypic qPCR of all four RBMX T55I specific T-cell clones in blood (D) and tumor tissue (E) before and after neoadjuvant chemotherapy.

Figure S6

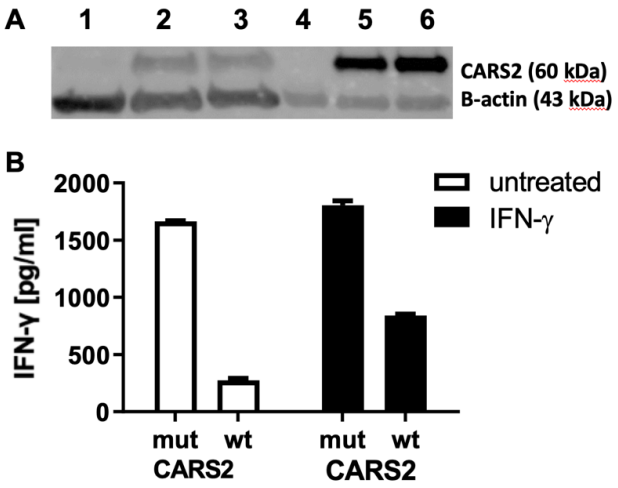


Fig. S6. (A) Western blot analysis of whole cell lysates of CARS2 mut/wt transduced and untransduced cells as control: 1- EBV-LCL 050; 2 - EBV-LCL 050 CARS2 mut; 3 - EBV-LCL CARS2 wt; 4 - MCF-7 wt; 5 - MCF-7 CARS2 mut; and 6 - MCF-7 CARS2 wt. Beta-actin serves as loading control. (B) T-cell recognition of CARS2 specific T-cell clone 55 of MCF-7 cell transduced with CARS2 Q171H and the HLA-DQ restriction molecule and additionally pulsed with the indicated peptide variants of CARS2 (wt or mut) as measured by IFN-γ ELISA.

References

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