SUPPLEMENTAL MATERIAL

Transcriptional and functional analyses of neoantigen-specific CD4 T cells during a profound response to anti-PD-L1 in metastatic Merkel cell carcinoma

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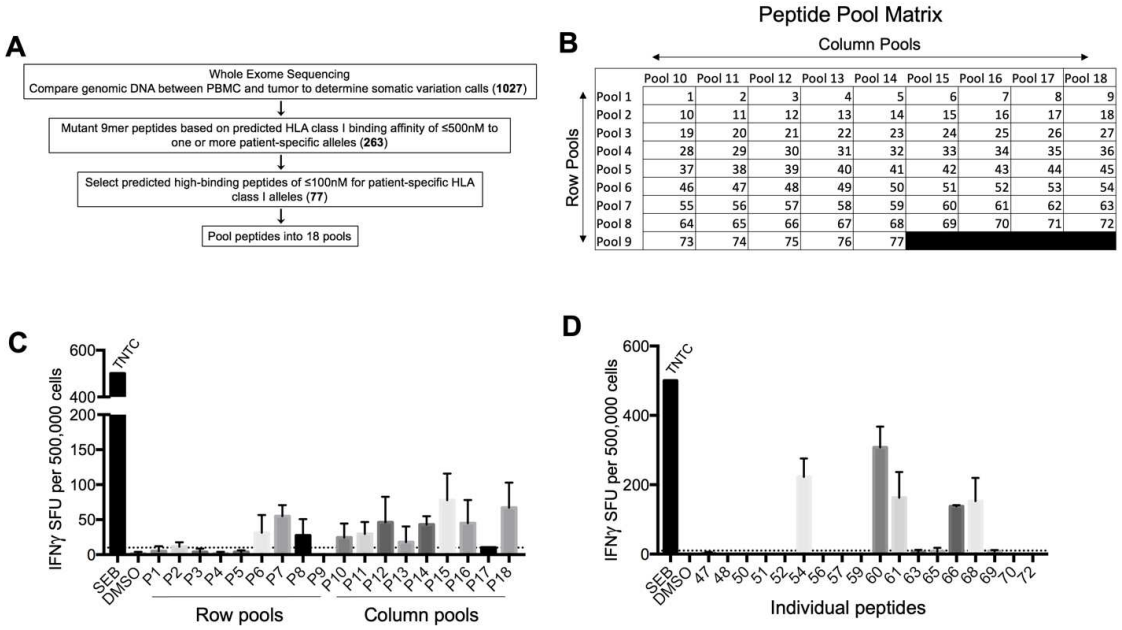
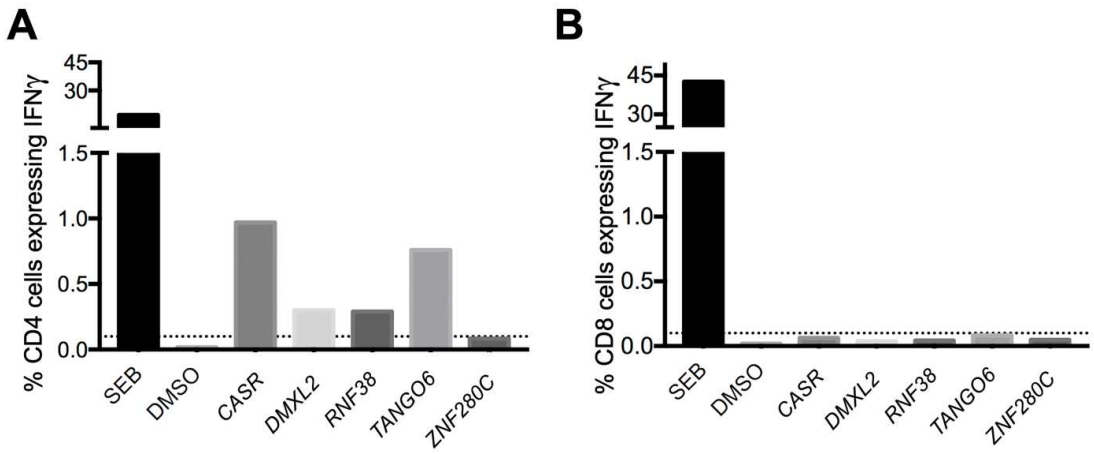
Figure S1: Selection and design of peptide pools and T cell responses



A) Overview of pipeline to identify tumor specific mutations, HLA class I restricted neoantigens and **B)** peptide pool design. **C)** IFNγ secretion in response to neoantigens. Pools of neoantigen peptides were used to probe PBMC directly ex vivo in an IFNγ ELISpot assay. 2x spot forming units (SFU) above DMSO control or >10 SFU and above were considered positive. n=4 technical replicates, the mean is graphed and error bars indicate standard deviation . **D)** Positive peptide pools were broken down to individual neoantigen peptides. n=2 technical replicates, the mean is graphed and error bars indicate standard deviation.

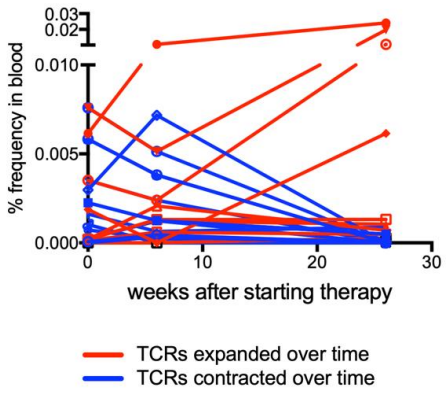
Figure S2: Flow cytometry assessment of IFNγ shows CD4 T cell reactivity to neoantigens



PBMC were cultured with each neoantigen peptide and supporting cytokines. After 14 days, T cell cultures were re-challenged with appropriate peptides. IFNγ expression by CD4 **(A)** and CD8 **(B)** T cells were assessed by flow cytometry. Cutoff for positivity set at 0.1% (dotted line). n=1 flow cytometry experiment.

Figure S3: Characterization of tumor-associated T cell receptor clones

A

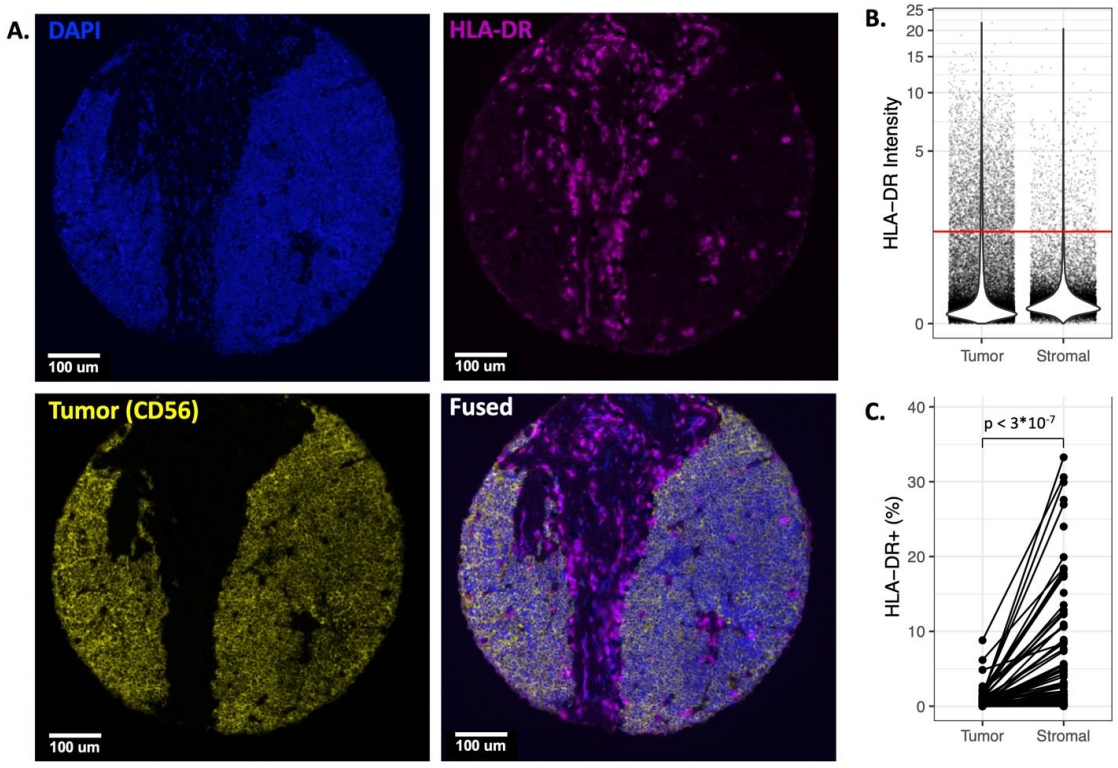


B TCR beta chain sequences of neoantigen-specific T cells sorted following activation induced markers from blood

Neoantigen	TCRB CDR3 amino acid	V beta family	% in tumor
CASR	CAISEADGGYTF	TRBV10-3*00	0
	CASSIQGYGASGANVLTF	TRBV19*00	0
DMXL2	CASSPDPDWGGNEQFF	TRBV28*00	0
	CSGVSSSYEQYF	TRBV20-1*00	0
	CASSEARAQHNEQFF	TRBV2*00	0
	CASSLAEGAYEQYF	TRBV7-2*00	0
	CASSLGRGDTEAFF	TRBV11-3*00	0
RNF38	CASSIRGLNEKLFF	TRBV12-4*00,TRBV12-3*00	0
	CASTGLPGNEKLFF	TRBV2*00	0
	CASSLGQGANTGELFF	TRBV7-6*00,TRBV7-7*00	0
	CATSRTRDRGTEAFF	TRBV15*00	0
TANGO6	CASSIGTSGLEQFF	TRBV6-5*00,TRBV06-08*01	0.21%
	CSARGQREAFF	TRBV20-1*00	0
	CASSQGITYEQYF	TRBV3-1*00	0
	CATSDTRGTGGEKLFF	TRBV24-1*00	0
	CAWRGASVSYEQYF	TRBV30*00	0
ZNF280C	CSVPDSIEQFF	TRBV29-1*00	0
	CASTSDRGRVQPQHF	TRBV12-4*00,TRBV12-3*00	0
	CSASRTSGRGGYF	TRBV20-1*00	0
	RASSLEREGPHEQFF	TRBV7-3*00	0

A) Tumor specific clonotypes were identified (TCR beta CDR3) in biopsy material and were then used as "barcodes" to track circulating T cell clones in the peripheral blood over time. Differentially expressed clonotypes (as determined by Fisher's exact test) are shown in red for upregulated and blue for downregulated TCRs. **B**) TCR beta amino acid sequences of neoantigen-specific CD4 T cells sorted from peripheral blood based on upregulation of CD154, CD137 and CD69 after stimulation with indicated neoantigens. One CD4 T cell clone isolated based on activation markers from PBMC was also found in the tumor biopsy.

Figure S4: Merkel cell carcinomas do not express HLA-DR



A) HLA-DR expression within the tumor and stroma revealed by mIHC staining of a representative tumor within an MCC tissue microarray (TMA). **B)** Intensity of HLA-DR expression among ~500,000 cells within the tumor or stroma across 76 unique MCC patient tumors. **C)** Paired frequency within each TMA core of HLA-DR+ tumor or stromal cells.

Table S1: List of 77 neoantigen-specific peptides used in immunoassays

Neoantigen peptides were selected based upon HLA class I predicted binding affinity using NetMHCv3.4 (https://services.healthtech.dtu.dk/?NetMHC-3.4), with a cut-off of ≤100nM (n=77 peptides). HLA class I and binding affinities (IC50 in nM) are shown.

Gene Name	Wild type sequence	Mutant sequence	Amino acid change	HLA allele prediction	IC50 (nM) of wild sequence	IC50 (nM) of mutant (neoantigen) sequence
XKR3	msftisfIi	msftisfNi	I74N	A6802	6	3
NAIF1	sAtaaaatv	sTtaaaatv	A159T	A6802	61	11
USP22	ittyvsfPl	ittyvsfSl	P429S	A6802	6	17
FANCD2	qmllcpfPf	qmllcpfLf	P1468L	A2402	75	26
ABHD8	mltgvTdgi	mltgvldgi	T6I	A6802	25	38
UBAP2	stavnscsP	stavnscsL	P82L	A6802	13273	53
VPS13B	dafPwtisl	dafSwtisl	P1153S	A6802	37	64
TSHZ1	Slakaaspi	Flakaaspi	S620F	C0802	2953	77
DRD3	fviySsvvs	fviyFsvvs	S192F	A6802	213	94
MAD2L1BP	etsStqepl	etsFtqepl	S38F	A6802	6	4
GCFC2	dtsiSfppv	dtsiLfppv	S93L	A6802	18	11
WWP1	ltVvldglv	ltAvldglv	V137A	A6802	76	18
OXR1	Dtehstnev	Ntehstnev	D390N	A6802	54	28
CD53	evlGmsfal	evlRmsfal	G199R	A6802	17	40
CNBD1	fisqsfhsF	fisqsfhsI	F235I	A6802	5138	54
CLN6	vfplewfPl	vfplewfSl	P75S	A2402	81	66
VPS8	qvfEflirl	qvfKflirl	E757K	A6802	25	77
NLRP7	yPdcklqtl	yLdcklqtl	P873L	C0802	262	97
NIPSNAP3A	Gvfhteyga	Evfhteyga	G182E	A6802	1801	7
CHD5	eaidrfnaP	eaidrfnaL	P1089L	A6802	7457	12
GPR52	mvlfriTsv	mvlfriIsv	T271I	A6802	19	18
KCNJ11	tlasarGpl	tlasarEpl	G366E	A6802	64	29
MAGEA1	vsarvRfff	vsarvCfff	R291C	A2402	160	41
NLRP5	lwmrDktli	lwmrNktli	D762N	A2402	124	54

VIT	sysngvqSl	sysngvqLl	S123L	A2402	285	68
TANGO6	yRtefgavv	yStefgavv	R172S	C0802	762	80
FBXO33	tavelErfv	tavelKrfv	E303K	A6802	237	99
EPB41	etepTvhhl	etepIvhhl	T618I	A6802	38	7
SIDT1	Gnmvashpi	Enmvashpi	G351E	A6802	3747	13
SLC38A10	ttfyvmvGf	ttfyvmvEf	G245E	A6802	19	20
USP44	Gntcymnsv	Entcymnsv	G279E	A6802	7309	30
ZFAND5	stSeksrnv	stFeksrnv	S84F	A6802	214	45
CACNA1F	dtfpqalIT	dtfpqalII	T692I	A6802	1939	55
NAPA	esvkeydSi	esvkeydFi	S268F	A6802	194	68
OPLAH	iciSvgaev	iciFvgaev	S720F	A6802	247	81
SEMA4A	tengfSypv	tengfLypv	S500L	A6802	60	99
MYO18B	Ssppplfsv	Fsppplfsv	S24F	A6802	15	7
CACNA1G	fGnyvlfnl	fSnyvlfnl	G951S	A6802	173	13
ORC1	itaiknsSv	itaiknsFv	S771F	A6802	56	22
IMMT	taaipPesl	taaipLesl	P617L	A6802	48	31
PTPRJ	Smasfdcev	Fmasfdcev	S721F	A6802	128	45
DSEL	fvsfksGkl	fvsfksEkl	G448E	A6802	138	55
KIAA1033	fvPdlediv	fvSdlediv	P946S	A6802	91	68
ZNF280C	fyygRhegv	fyygMhegv	R301M	A6802	5983	84
DUOX2	eisvvkaEl	eisvvkaKl	E1278K	A6802	32	100
ZSCAN9	etpgpReal	etpgpKeal	R67K	A6802	6	9
COPB1	maanviPvl	maanviSvl	P403S	A6802	79	13
PROM1	niiPvldei	niiSvldei	P242S	A6802	14	24
CPXM1	qDadpwfqv	qNadpwfqv	D169N	A6802	125	33
MAGI3	maytdtGmi	maytdtEmi	G307E	A6802	119	45
C1orf228	evggVltll	evggFltll	V105F	A6802	34	57
RNF38	sahpptlpP	sahpptlpL	P287L	A6802	12773	69
IPO11	liptLiesv	liptFiesv	L172F	A6802	146	85
ASTN1	tvmEdavev	tvmKdavev	E573K	A6802	5	9

DSCAM	esisiswSt	esisiswFt	S1110F	A6802	30	15
NOS3	Glgplhygv	Elgplhygv	G967E	A6802	6089	25
CPT1B	kylGvsspf	kylEvsspf	G634E	A2402	22	33
OSGIN2	tyitSvsrl	tyitFvsrl	S244F	A2402	72	49
ASCC3	nHhvaslsf	nYhvaslsf	H2139Y	A2402	3333	58
CASR	fPihfgvaa	fSihfgvaa	P39S	A6802	2430	73
IPO7	eaiihsPel	eaiihsSel	P103S	A6802	778	89
COL12A1	dtlySvnlv	dtlyFvnlv	S837F	A6802	18	10
AMBN	msfavpffP	msfavpffS	P31S	A6802	620	16
CDC45	atmslmesP	atmslmesL	P341L	A6802	12614	26
HSPA12B	lrffrEhal	lrffrKhal	E182K	B1402	37	34
PARP15	gvagvtSra	gvagvtFra	S31F	A6802	225	50
UBE3C	Sfarhyyfl	Ffarhyyfl	S813F	A2402	32	58
IPO5	liPyldnlv	liSyldnlv	P498S	A6802	94	73
MARCH7	nvpsasevP	nvpsasevL	P281L	A6802	16953	93
CCDC67	stmpplppS	stmpplppL	S526L	A6802	223	11
TNPO2	evRqssfal	evWqssfal	R681W	A6802	305	16
C12orf29	mPcvfvtev	mScvfvtev	P12S	A6802	703	26
PIGB	cqlcSwftw	cqlcFwftw	S181F	A2402	78	36
PDZRN3	tPyglyyps	tSyglyyps	P661S	A6802	1713	51
DMXL2	eidntvPpv	eidntvSpv	P154S	A6802	537	62
MAP3K1	svssSthft	svssFthft	S846F	A6802	237	74
COL15A1	frdfaisvV	frdfaisvM	V96M	C0802	45	93
HIV nef	rypltfgwcf	n/a	n/a	A2402	8	n/a

TABLE S2: Patient-specific neoantigen peptides and predicted HLA class II binding

Wild type and mutant 17mer peptides are listed. *Predicted CD8 epitopes used in immunoassays are bolded, mutant residues are underlined. Class II core peptide predictions are shown and binding affinities (via Net MHCv3.4) are listed.

HLA Restriction	Gene	Wild type 17mer	Mutant 17mer*	Class II core peptide	IC50 (nM) Wild Type	IC50 (nM) Mutant
DRB1*04:01	*CASR*	DIILGGLFPIHFGVAAK	DIILGGL**F<u>S</u>IHFGVAA**K	LGGLFSIHF	1055	294
DRB1*04:01	*DMXL2*	EEEIDNTVPPVLNDWKC	EE**EIDNTV<u>S</u>PV**LNDWKC	IDNTVSPVL	4764	1367
DRB1*04:01	*RNF38*	SAHPPTLPPSAPLQFLT	**SAHPPTLPL**<u></u>SAPLQFLT	TLPLSAPLQ	4175	1852
DRB1*04:01	*TANGO6*	GVGVPLRYRTEFGAVVQ	GVGVPLR**Y<u>S</u>TEFGAVV**Q	LRYSTEFGA	281	208
DRB1*04:01	*ZNF280C*	LVNEFYYGRHEGVTEKE	**LVNEFYYG<u>M</u>**HEGVTEKE	FYYGMHEGV	1219	492
DRB1*11:01	*CASR*	DIILGGLFPIHFGVAAK	DIILGGL**F<u>S</u>IHFGVAA**K	LGGLFSIHF	1892	1047
DRB1*11:01	*DMXL2*	EEEIDNTVPPVLNDWKC	EE**EIDNTV<u>S</u>PV**LNDWKC	IDNTVSPVL	8915	10377
DRB1*11:01	*RNF38*	SAHPPTLPPSAPLQFLT	**SAHPPTLPL**<u></u>SAPLQFLT	TLPLSAPLQ	14494	867
DRB1*11:01	*TANGO6*	GVGVPLRYRTEFGAVVQ	GVGVPLR**Y<u>S</u>TEFGAVV**Q	LRYSTEFGA	287	529
DRB1*11:01	*ZNF280C*	LVNEFYYGRHEGVTEKE	**LVNEFYYG<u>M</u>**HEGVTEKE	FYYGMHEGV	90	44

Materials and Methods

IFNγ detection via flow cytometry

T cell lines were co-cultured with Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) labeled, peptide loaded (5µg/ml), patient derived LCLs in the presence of anti-CD28 and anti-CD49d mAbs (BD Biosciences), and brefeldin A for 12–16 hours[1 2]. Overnight cultured cells were stained with anti-CD3, -CD4, -CD8, -CD14 and -CD19. After a cytofix/cytoperm step (BD Biosciences), cells were intracellularly stained with anti-IFNγ (BD Biosciences), washed and data collected on a FACSCanto II (BD Biosciences).

T cell receptor sequencing

Neoantigen-reactive CD4 T cells were sorted and cDNAs generated from single cells (as described above). cDNAs were used for paired TCR alpha and beta sequencing of the complementarity determining region 3 (CDR3) for each gene as previously described.[3] TCR alpha and beta CDR3 sequences were analyzed with the MiXCR program with the IMGT databased used as a reference.[4 5] TCR Immunoseq™: A DNeasy Kit (Qiagen) was used to isolate DNA from formalin fixed paraffin-embedded (FFPE) tissue and from peripheral blood and were submitted to Adaptive Biotechnology (Seattle, WA) for *TCB* CDR3 sequencing and data processing.

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