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T cell receptors for clinical therapy: *in vitro* assessment of toxicity risk

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Running title:

In vitro selection of safe T cell receptors

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Abstract

Adoptive therapy with T cell receptor (TCR)-engineered T cells has shown promising results in the treatment of patients with tumors, and the number of TCRs amenable for clinical testing is expanding rapidly. Notably, adoptive therapy with T cells is challenged by treatment-related side effects, which calls for cautious selection of target antigens and TCRs that goes beyond their mere ability to induce high T cell reactivity. Here, we propose a sequence of in vitro assays to improve selection of TCRs, and exemplify risk assessments of on-target as well as off-target toxicities using TCRs directed against Cancer Germline Antigens. The proposed panel of assays covers parameters considered key to safety, such as expression of target antigen in healthy tissues, determination of a TCR's recognition motif towards its cognate peptide, and TCR's cross-reactivity towards non-cognate peptides.

Introduction

Therapeutic use of anti-tumor T cells has proven feasible in a multitude of trials over the last decade. Alongside the demonstration of clinical benefit and enthusiasm about the therapeutic efficacy, the occurrence of toxicities has stimulated awareness of safety pitfalls. While initial studies demonstrated recognition, and sometimes destruction of healthy tissues (1-3), later studies demonstrated lethal adverse effects in individual patients (4,5). These studies highlight the two main challenges facing safety of T cell receptor (TCR) gene therapy: on- and off-target toxicities. With a quickly expanding panel of TCRs that have generally been selected for their ability to provide T cells with high avidities towards tumor antigens, there is an urgent need for streamlining the safety assessment of these TCRs prior to clinical usage.

Selecting target antigens

An ideal target antigen for adoptive T cell therapy (AT) displays two important features: it is immunogenic and shows selective and homogenous expression in tumor tissue. Immunogenicity is best explained as an antigen's ability to be recognized by and sufficiently activate T cells, a feature that is generally well addressed when selecting a target antigen and its corresponding TCR (reviewed in (6)). In example, characteristics related to the immunogenicity of two MAGE-C2 (MC2) antigen epitopes are summarized in Table I. Selective expression in tumor tissue, and hence its absence in healthy tissues, would reduce the risk for on-target toxicities. Differentiation, over-expressed or onco-fetal antigens are not absent from healthy tissues and the targeting of these antigens, e.g. MART-1 or carcinoembryonic antigen (CEA), by TCR-transduced T cells has resulted in severe destruction of melanocytes in the eyes, ears and skin (1) or inflammation of the colon (2,3). Candidate antigens that are exclusively expressed by tumor

tissues include neo-antigens and oncoviral antigens. Neo-antigens are derived from somatic DNA alterations and their identification requires analyses of mutations, gene expression, and algorithms that predict antigen processing and presentation by major histocompatibility complex (MHC) (7-9). AT studies with tumor infiltrating lymphocytes (TILs) in patients with melanoma and cholangiocarcinoma showed that clinical benefit was associated with T cell responses against neo-antigens (9-11). Current exploitation of neo-antigens in AT, however, is challenged by the uncertainty of current algorithms accurately predicting immunogenicity, and the fact that neo-antigens are usually specific per patient (12-14). Viral antigens are present in more than 10% of human cancers and are often the result of viral insertion into the genome and subsequent reactivation in tumors. AT studies using TILs reactive against either HPV or EBV have shown clinical successes in patients with cervical cancer or nasopharyngeal carcinoma, respectively (15,16). In addition to neo-antigens and onco-viral antigens, also certain cancer germline antigens (CGAs) demonstrate tumor-selective expression. In fact, CGAs are expressed in gonadal tissues and some in thymus (17), and certain CGAs are considered to be selectively de-repressed in tumor tissuess (for detailed reviews, see (6,18,19)). MAGE-A3 and NY-ESO1 are examples of CGAs that have already been targeted by TCR-engineered T cells in patients with metastatic melanoma, metastatic synovial sarcoma or multiple myeloma (4,20). Although off-target toxicities were observed with the targeting of the former antigen (most likely an issue of the TCRs; see next section), the safe use of selected CGAs was suggested by the targeting of NY-ESO1 demonstrating clinical benefit without toxicities (20,21).

We recommend for any antigen, with the exception of neo-antigens, to test the antigen's absence from a large panel of healthy organs. Online databases such as the protein atlas (http://www.proteinatlas.org/) or the CGA database (www.cta.lncc.br) combine extensive data

from transcriptomic analyses and antibody stainings from numerous normal, non-cancerous cell lines and tissues. When applying these tools to assess the expression of MC2, we observed that mRNA expression is restricted to cells from cancers and testes, the latter considered to possess an immune privileged status (no MHC expression; thus no detection by T cells). The use of commercially available cDNA libraries of a large series of healthy tissues enable researchers to extend online analyses and quantify antigen expression with a laboratory assay. When performing qPCR using such a cDNA library, we demonstrated absence of MC2 mRNA in healthy tissues as illustrated in figure 1A. In case specific antibodies are available, we would recommend to follow-up qPCR with immune histochemistry. Using an MC2-specific antibody, we confirmed the presence of MC2 protein in testis and melanoma as well as its absence in multiple healthy tissues, such as brain, heart, intestine and lung (see figure 1B). However, both qPCR and immune histochemistry cannot formally exclude the presence of rare antigen-positive cells within a tissue, e.g. stem cells. For example, mRNA of certain CGAs has been detected only in medullary thymic epithelial cells but not total thymus (17). As an additional means to exclude target antigen expression, sophisticated in vitro cell cultures have been developed to closely mimic complete tissues or organs (commented in next section) (5,22).

Selecting therapeutic TCRs

Once the safety of the target antigen has been assessed, one can start selecting TCRs. Procedures to obtain tumor-reactive T cells and hence TCRs can generally be divided into those that rely on tolerant and those that rely on non-tolerant repertoires of T cells. Tolerant repertoires, where deletion of T cells with an avidity outside the thymic selection window has occurred, have been used to obtain T cell clones from patients following successful TIL therapy, peptide vaccination,

or using *in vitro* pulsing of autologous dendritic cells (23,24). Notably, the thymic selection of T cells is most likely a trade-off between producing a self-tolerant yet sufficiently diverse and responsive TCR repertoire, and escape of self-reactive TCRs cannot be negated. Indeed, TCRs specific for over-expressed antigens and obtained from the native repertoire have been shown to initiate autoimmune side effects (1). The use of non-tolerant repertoires, with the rationale of allowing the generation of high-avidity T cells, has been applied in allogeneic *in vitro* as well as *in vivo* systems. For example, HLA-mismatched antigen-presenting cells (25) or artificial antigen-presenting cells pulsed with peptides of interest facilitate the *in vitro* generation of tumor-reactive T cells (26). *In vivo*, mice transgenic for human HLA as well as mice transgenic for human TCR and HLA-A2 genes (27) have been immunized and used as a source of TCRs. After having obtained antigen-reactive T cells, sequences of the TCRα and β chains can be determined by molecular techniques such as 5′ RACE (23), enhanced PCR methods, capturing and indexing of genomic DNA-encoding TCR chains (28), or sequencing and pairing of TCR chains based on combinatorial algorithms (29).

It is important to note that TCRs derived from above-mentioned repertoires, in particular the non-tolerant repertoir, have not been selected in the presence of all patient MHC alleles and in the case of mice neither against human peptides present in the thymus, and may show allo- and non-cognate reactivity. Moreover, TCRs, even though obtained from highly tumor-reactive T cells, have an inherent degeneracy for peptide recognition and are able to recognize more than a single peptide. This dynamic flexibility in antigen recognition is in part accredited to the bending ability of the TCR-CDR domains (30,31), but also to the dominant interaction of the TCR with a restricted number and order of amino acids present in the MHC-presented peptide. To minimize the risk of selecting a TCR that recognizes non-cognate self-peptides, we recommend a series of

assays that assess the risk of this so-called off-target toxicity. These assays are illustrated with two patient-derived MC2-specific TCRs 6 and 16 with details and evaluation of anti-tumor T cell responses mediated by TCR6 and TCR16 summarized in Table I.

Recognition of random epitopes

Initial assessment of a TCR's self-reactivity can be done by testing the responsiveness of TCR-transduced T cells towards random peptides known to be presented by the respective HLA restriction allele. Mathematical projections indicate that amongst a pool of ~10^{12} peptides, a single TCR may react with >10^6 peptides, supporting the notion of TCR degeneracy for peptide recognition, which potentially contributes to a more diverse TCR repertoire (32). We co-cultured TCR6 and TCR16 T cells with antigen-presenting cells loaded with saturating concentrations of >100 common, HLA-A2-eluted self-peptides (4). As depicted in figure 2, both T cell populations mediated a T cell response to their respective cognate peptides, but to none of the other peptides. Importantly, these data hint to lack of cross-reactivity of these two TCRs, but we cannot fully exclude recognition to random peptides. Another assessment of self-reactivity can be conducted by testing TCR-transduced T cells towards allogenic HLA molecules. In order to exclude activation of T cells upon recognition of foreign HLA molecules, panels of lymphoblastoid B cell lines with various HLA allotypes have proven valuable (33,34).

Recognition of cognate epitope via critical amino acids

Further assessment of a TCR's self-reactivity, and a key assay in this communication, is the testing of a TCR's intrinsic capacity to recognize peptides highly homologous to its cognate

epitope (35,36). To this end, one can determine the recognition motif, i.e. the position and sequence of amino acids within the cognate epitope that are crucial for binding to the TCR. This motif is unique per TCR and can be considered a surrogate measure for the extent of cross-reactivity of TCRs (22,33). The importance to assess such motifs became apparent from two recent clinical trials using AT with TCR-engineered T cells. The first trial targeting MAGE-A3 and A9 (MA3/9) in the context of HLA-A2 utilized the TCR 9W11 and reported neurological toxicities in two patients with metastatic melanoma (4). The second trial targeting MA3 in the context of HLA-A1 utilized the TCR a3a and reported cardiac toxicities in one patient with metastatic melanoma and one patient with multiple myeloma (5). Both TCRs were affinity enhanced in vitro and mediated toxicity by recognizing peptides highly similar to the cognate peptide, namely peptides derived from MAGE-A12 and Titin present in brain and heart tissue, respectively. These studies clearly underline the need to assess recognition motifs, and the search for T cell reactivities against homologous self-peptides, prior to clinical application. The importance of recognition motifs is timely and its assessment has only occurred for a limited number of TCRs to date, which is summarized in Table II.

Using a set of altered peptide ligands (APLs), peptides containing individual alanines replacements at every single position in the cognate peptide (in case of an endogenous alanine→glycine), we conducted stimulation assays with TCR6 and 16 T cells. Critical amino acids are defined as those that, when testing the respective APLs, result in a drop of the T cell response (generally using IFNγ production as a readout) of > 50% when compared to the response toward the cognate peptide. Following the determination of these motifs, as exemplified in figure 3A, we used ScanProSite (http://prosite.expasy.org/scanprosite/) (37) and identified target antigens, listed in figure 3B, that harbor the recognition motif and represent potential

cross-reactive targets for TCRs 6 and 16. Subsequently, these self-peptides were tested for their ability to induce T cell responses towards HLA-A2-postive antigen presenting cells loaded with saturating concentrations (1μM) of peptide. These experiments yielded a short list of self-peptides that are actually recognized by the TCRs under study, defined as those that resulted in at least a T cell IFNγ response > 2.5% of the response to cognate peptide (figure 3B: underlined antigens).

Recognition of non-cognate epitopes that contain recognition motif

Once self-peptides that can be recognized by TCR T cells have been identified, we recommend to execute two additional tests to more stringently assess the risk for self-reactivity. These tests aim to provide measures for T cell avidity as well as efficiency of cellular processing and presentation. Towards the first test, one can titrate amounts of non-cognate peptides and determine the concentration of these peptides that elicits 50% of the maximal T cell response (EC_{50}). For both TCR6 and 16 T cells, we found that only a single self-peptide, namely a peptide from the antigen MAGE-B4 (MB4) or MAGE-C1 (MC1; see figure 3B for homology and predicted peptide-HLA binding), respectively, revealed detectable EC_{50} values that were only 2-5 fold lower than those of the cognate peptides (figure 4). Extent of homology and predicted peptide-MHC binding of peptides that induce T cell IFNγ are listed in figure 3B, which shows that loss of homology and peptide-MHC affinity was least affected for MB4 and MC1 peptides. All other self-peptides revealed no T cell reactivities at titrated doses, or at the very best 5 log scales lower compared to cognate peptide. These were considered not recognizable by the TCRs under study and excluded from further assays. Towards the second test, one can predict whether self-peptides are the result of antigen processing and presentation to enable T cell recognition *in*

vitro. NetCTLpan (http://www.cbs.dtu.dk/services/NetCTLpan/) (38) takes proteasomal C terminal cleavage, TAP transport efficiency, and peptide MHC class I binding of peptides into account, and can be employed to obtain an initial score for antigen processing and presentation. Analysis of the MB4 peptide, but not MC1 peptide, yielded a high score according to this web-based tool. Such predictions may not be fully accurate and should be verified using cells known to express the antigen or antigen presenting cells (e.g. dentritic cells) transfected with antigen-encoded RNA followed by co-cultivation with TCR-transduced T cells. To this end, we stimulated TCR T cells with the esophageal cancer cells line OEC-19, which natively expresses the MB4 and MC1 antigens, but is devoid of the MC2 antigen, and observed that both TCRs failed to initiate T cell activation against either MB4 or MC1 (see figure 4B). When using cell lines that natively express the MC2 antigen, as a control for the processing and presentation of MAGE antigens, we observed that both TCRs did initiate T cell activation. It is noteworthy that standard tissue culture systems may not always accurately reflect antigen processing and presentation. This was evidenced by the recognition of Titin by the TCR a3a that could only be observed in more elaborate tissue culture systems, such as 3D cultures of beating cardiomyocytes derived from induced pluripotent stem cells (22). In case the above two assays do not exclude self-reactivity of TCR T cells, one could pursuit assessment of the tumor-selective expression of such new antigens. In case expression of new antigens is not selective for tumors, the corresponding TCR should be excluded. Using online tools (39) as well as qPCR (figure 1A), MB4 showed expression within epididymis and vagina, whereas MC1 showed no expression in any of the healthy non-gonadal tissues. These data highlight the stringent safety profile of TCR16, the TCR selected for a clinical trial to treat melanoma and head-nand-neck carcinoma, currently prepared at Erasmus MC.

Future perspective of in vitro assays assessing risk of TCR-mediated toxicities

The proposed collection and sequence of *in vitro* assays to assess risks for toxicities are presented in figure 5. We advocate this testing for TCRs with clinical intent, in particular those TCRs reactive against a self-peptide and derived from a non-tolerant repertoire and/or following gene-enhancement. In extension to gene-enhancement, introduction of TCR-CDR mutations has been a commonly used tool to generate high-affinity TCRs (2,4,5,20). While such gene-enhanced TCRs recognize target peptides at increased affinities when compared to the corresponding wild-type TCRs, consequently such TCRs are also at risk to recognize non-cognate peptides. To test whether affinity enhancement led to an increase in degeneracy for peptide recognition, we made use of a panel of 8 TCRs specific for the same cognate peptide $gp100_{181\text{-}188}$:HLA-A2) but harboring 2-3 mutated amino acids in either their CDR2β, CDR3α or CDR3β domains (Govers, Ms submitted). Upon assessment of the recognition motifs and search for motif-harboring self-peptides, it became apparent that enhanced affinity was accompanied by drastic increase in the TCR's ability to recognize self-peptides (Table III). These data extend earlier findings regarding a correlation between affinity enhancement and loss of TCR specificity (40), and warrant caution when trying to change the TCR-CDR structure as it compromises the stringet recognition of cognate peptide (31,41).

Taken together, here we propose a platform of *in vitro* assays that in combination with available online-databases and tools allows for optimal toxicity risk-assessment for target antigens and TCRs currently under consideration for clinical trials.

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Figure Legends

Figure 1. Target antigen is absent or shows restricted expression in healthy tissues.

(A) MC2, MC1 and MB4 qPCRs were performed on a cDNA library of 48 healthy, human tissue samples (OriGene Technologies). Patient-derived melanoma cell line EB81-MEL and patient-derived esophageal cancer cell line OEC-19 served as positive controls. Spiking experiments were conducted separately to determine optimal PCR conditions for maximum sensitivity (detection limit: 1:1,000-10,000 $MC2^+$ cell: $MC2^-$ cells; data not shown). Relative mRNA levels are corrected for GAPDH and expressed as fold increase compared to antigen expression in the testis. Part of the displayed data is derived from (23); Copyright © 2016 The American Association of Immunologists, Inc. (**B**) Immune stainings of melanoma and healthy tissues were conducted using a monoclonal antibody specific for MC2 (42) on tissue micro arrays (TMA). EnVision System (Agilent Technologies) was used for signal visualization, TMAs were scanned via Nano zoomer (Hamatsu) and manually scored using Distiller (SlidePath). Exemplary tissue sections of patient-derived melanoma tissue as well as various healthy tissues obtained from the Erasmus MC pathology department are displayed with 20x magnification. MC2 positivity is scored as brown colored nuclear staining, with 3 smaller TMAs exemplifying different staining intensities.

Figure 2. TCRs do not recognize random peptides eluted from the restricting MHC allele.

TCR6 or TCR16 T cells were co-cultured with T2 cells loaded with 1µM of 114 different HLA-A2-eluted peptides (4) for 24h. Cognate MC2 peptides (TCR6: LLFGLALIEV; TCR16: ALKDVEERV) served as positive controls. IFNγ levels in 24h culture supernatants were measured by ELISA and are displayed as mean ± SEM (n=3). Left-hand panel is adapted from (23); Copyright © 2016 The American Association of Immunologists, Inc.

Figure 3. TCRs recognize cognate peptide via unique or restricted motif.

(**A**) TCR6 or TCR16 T cells were co-cultured with T2 cells loaded with 1µM of cognate peptides or peptides with a single alanine replacement (Altered Peptide Ligands (APL), in case of alanine in original peptide: glycine). IFNγ levels in 24h culture supernatants were measured by ELISA. IFNγ response to APLs is displayed as mean % relative to response to cognate peptides ± SEM (n=4). Responses < 50% (dashed line) were indicative of amino acids critical for TCR recognition (recognition motif: underlined amino acids). (**B**) Homologous motifs from (A) were queried against a human protein database using ScanProSite. This yielded 27 and 1 non-cognate matches for TCR6 and TCR16, respectively. Subsequently, TCR6 or TCR16 T cells were co-cultured with T2 cells loaded with 1µM of these 27 and 1 peptide(s), and IFNγ levels were measured in 24h culture supernatants by ELISA (n=4). Underlined peptides induced a T cell

IFNγ response > 2.5% of the response to cognate peptide. Homology to cognate peptide (diverging amino acids underlined) as well as peptide-MHC affinity (IC_{50} calculations according to NetMHCpan; http://www.cbs.dtu.dk/services/NetMHCpan/) are indicated for those peptides with a detectable IFNγ response.

Figure 4. TCRs mediate negligible T cell avidity nor recognize natively presented non-cognate peptides that harbor recognition motif.

(**A**) Non-cognate peptides underlined in figure 3B were titrated from 10μM (10^{-5}M) to 1pM (10^{-12} M) and tested for T cell IFNγ response as described in previous legends and displayed as mean ± SEM (n=4). EC_{50} values for cognate and selected non-cognate peptides were calculated in GraphPad, using non-linear regression; n.d. = not detectable. (**B**) TCR6 or TCR16 T cells were co-cultured with tumor cell lines or T2 cells (as a negative control). Expression status of MC2, MC1, MB4 and HLA-A2 for these cells was assessed via qPCR and indicated below plots with plus or minus. IFNγ levels in 24h culture supernatants were measured by ELISA and are displayed as mean ± SEM (n=3). Left-hand plots are adapted from (23);

Figure 5. Platform of *in vitro* assays to select antigens and TCRs with limited risk for *in vivo* toxicity.

Flowchart proposes a series and sequence of *in vitro* techniques, exemplified in figures 1 to 4 and explained in detail in text. Such a sequence of assays would facilitate selections of potentially safe target antigens and TCRs prior to their use in clinical trials.

Table I Properties of cognate MAGE-C2 epitopes and corresponding TCRs that relate to T cell reactivity

Epitopes	**LLFGLALIEV** (MAGE-C2$_{191-200}$)	**ALKDVEERV** (MAGE-C2$_{336-344}$)
HLA-A2 binding	predicted affinity[a]: 9.9nM measured affinity[b]: 2.5µM	predicted affinity[a]: 342.2nM measured affinity[b]: 20.0µM

TCR	**TCR 6**		**TCR 16**	
TCR genes[c]	α-chain: Vα12-2*01 / Jα23*01 / Cα β- chain: Vβ15*02 / Jβ2-3*01 / Cβ2		α-chain: Vα3*01 / Jα3*01 / Cα β- chain: Vβ28*01 / Jβ2-5*01 / Cβ2	
pMHC binding[d]	EC_{50} $CD8^+$ T cells: 348nM EC_{50} $CD4^+$ T cells: 501nM		EC_{50} $CD8^+$ T cells: 663nM EC_{50} $CD4^+$ T cells: n.d.	
functional T cell avidity[e]	EC_{50}: 3.19nM		EC_{50}: 73.8nM	
tumor cell recognition[f]	EB81-MEL:	1090pg/ml	EB81-MEL:	1738pg/ml
	518-A2:	605pg/ml	518-A2:	461pg/ml
	607-B:	835pg/ml	607-B:	626pg/ml
	SCC-9:	311pg/ml	SCC-9:	207pg/ml
	93-VU-120:	292pg/ml	93-VU-120:	26pg/ml
	SUM-195-PT:	217pg/ml	SUM-195-PT:	466pg/ml

[a] according to http://www.cbs.dtu.dk/services/NetCTLpan/.

[b] T2 cells were pulsed with titrated amounts of ALK or LLF peptide and formation of pMHC class I complexes on the cell surface was quantified via flow cytometry using PE-labeled HLA-A2 mAb (23).

[c] cDNAs derived from patient-derived T cell clones were PCR amplified using either a set of TCR-Vα or Vβ sense primers and a corresponding TCR-Cα or Cβ antisense primer or 5'RACE (rapid amplification of cDNA ends). Following nested PCRs and cloning, TCRα and β sequences were identified using www.imgt.org and classified according to the Lefranc nomenclature (see (23) for details).

[d] TCR T cells were pulsed with titrated amounts of PE-labeled pMHC multimer, and binding was quantified via flow cytometry.

[e] TCR T cells were antigen-presenting cells that were pulsed with titrated amounts of cognate peptide, and functional T cell avidity was quantified via ELISA measurements of IFNγ production.

[f] TCR-transduced T cells were co-cultured with 3 melanoma, 2 head-and-neck carcinoma and 1 triple-negative breast cancer cell lines all positive for HLA-A2 and MC2 (determined by qPCR) at an E:T ratio of 3:1 for 24h. Cell lines were treated with epigenetic drugs Azacytidine and Valproate as well as IFNγ prior to co-culture (see (23) for details). IFNγ levels in 24h culture supernatants were measured by ELISA; displayed values are means of five experiments. Note that epigenetic drugs induce enhanced and tumor-selective expression of MC2 *in vitro (23)* as well as enhanced tumor immunogenicity *in vivo* (18).

n.d. = not determined.

Table II Recognition motifs of TCRs utilized in clinical and pre-clinical research

TCR name	target antigen	HLA restriction	cognate epitope	recognition motif[a]	number of antigens with motif[b]	number of epitopes with high MHC affinity (<10μM)[c]	reference
a3a	MAGE-A3	A1	EVDPIGHLY	**E**x**DPI**xxx**Y**	5	0	(22)
9W11	MAGE-A3	A2	KVAELVHFL	-xx**E**-x**H**--[d]	-	-	(43)
T1367	MAGE-A1	A2	KVLEYVIKV	xxx**EY**x**IK**x	62	6	(33)
s24-TCR	survivin	A2	ELTLGEFLKL	x**LT**x**GEFLK**x	1	1	(44)
gp100 wt	gp100	A2	YLEPGPVTA	x**LEPGP**xx**A**	4	4	Govers, Ms. submitted
fl-MPD	gp100	A2	YLEPGPVTA	**YxEPxxxxx**	>1000	>500	(45)
fl-296	gp100	A2	YLEPGPVTA	**YxExxxxxx**	>1000	>500	(45)
TCR 4	MAGE-C2	A2	LLFGLALIEV	xx**FGL**x**L**xxx	260	122	(23)
TCR 6	MAGE-C2	A2	LLFGLALIEV	**L**x**F**x**L**x**L**x**E**x	28	9	(23)
TCR 11	MAGE-C2	A2	LLFGLALIEV	xx**FGL**x**L**x**E**x	21	17	(23)
TCR 16	MAGE-C2	A2	ALKDVEERV	x**LKDVEER**x	2	2	(23)

[a]Recogniton motifs are defined through T cell IFNγ production in response to alanine scanned cognate epitopes.

[b]number of human proteins containing matching recognition motif according to ScanProSite (http://prosite.expasy.org/scanprosite/).

[c]number of proteins containing matching recognition motif (according to [b]) and a predicted affinity value <10μM for binding of the peptide to its respective MHC; affinity calculations according to NetMHCpan (http://www.cbs.dtu.dk/services/NetMHCpan/).

[d]recognition motif incomplete, amino acids on positions 2-4 and 6-7 are based on stimulation assays conducted by Chinnasamy et al. with a panel of MAGE-peptides with highly similar sequences (43); '-' indicates amino acids with unknown relevance to the recognition motif.

Table III TCR affinity enhancement and its effect on off-target recognition[a]

TCR name	K_D [μM]	target antigen	cognate epitope	recognition motif[a]	number of antigens with motif[b]	number of epitopes with high MHC affinity (<10μM)[c]
gp100 wt	18.5	gp100/HLA-A2	YLEPGPVTA	x**LEPGP**xx**A**	4	4
gp100 TCR 1	7.9	gp100/HLA-A2	YLEPGPVTA	x**LE**x**GP**xx**A**	23	13
gp100 TCR 2	4.0	gp100/HLA-A2	YLEPGPVTA	x**LE**x**GP**xxx	240	97
gp100 TCR 5	1.1	gp100/HLA-A2	YLEPGPVTA	x**LE**x**GP**xxx	240	97
gp100 TCR 8	0.026	gp100/HLA-A2	YLEPGPVTA	x**Lx**xxxxxx	>10000	>10000

[a]Govers, Ms, submitted.

[b]number of human proteins containing matching recognition motif according to ScanProSite (http://prosite.expasy.org/scanprosite/).

[c]number of human proteins containing matching recognition motif (according to [b]) and a predicted affinity value of <10μM for binding of the peptide to its respective MHC; affinity calculations according to NetMHCpan (http://www.cbs.dtu.dk/services/NetMHCpan/).

Figure 1:

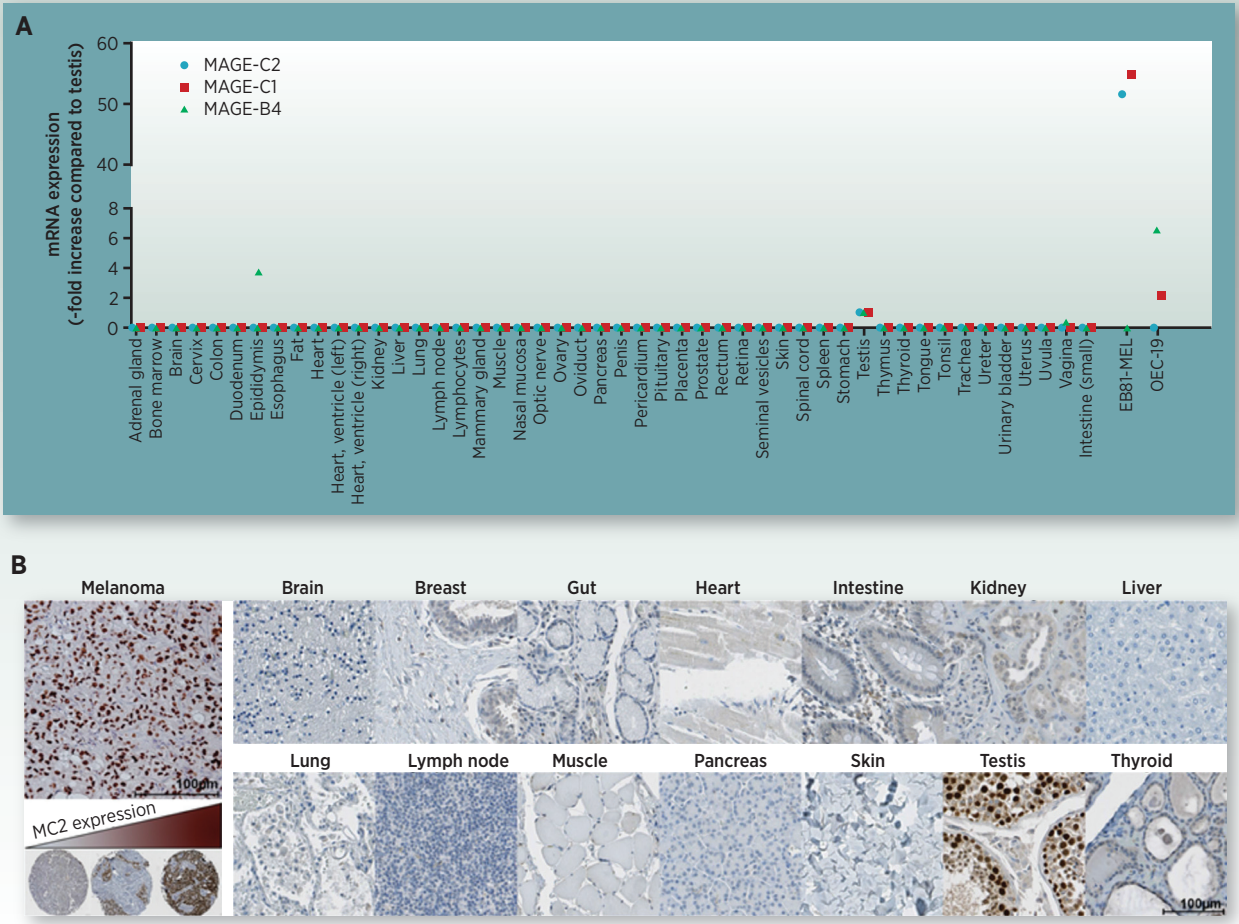

A
mRNA expression
(-fold increase compared to testis)
60
50
40
8
6
4
2
0
MAGE-C2
MAGE-C1
MAGE-B4
Adrenal gland
Bone marrow
Brain
Cervix
Colon
Duodenum
Epididymis
Esophagus
Fat
Heart
Heart, ventricle (left)
Heart, ventricle (right)
Kidney
Liver
Lung
Lymph node
Lymphocytes
Mammary gland
Muscle
Nasal mucosa
Optic nerve
Ovary
Oviduct
Pancreas
Penis
Pericardium
Pituitary
Placenta
Prostate
Rectum
Retina
Seminal vesicles
Skin
Spinal cord
Spleen
Stomach
Testis
Thymus
Thyroid
Tongue
Tonsil
Trachea
Ureter
Urinary bladder
Uterus
Uvula
Vagina
Intestine (small)
EB81-MEL
OEC-19
B
Melanoma
Brain
Breast
Gut
Heart
Intestine
Kidney
Liver
100µm
MC2 expression
Lung
Lymph node
Muscle
Pancreas
Skin
Testis
Thyroid
100µm

Figure 2:

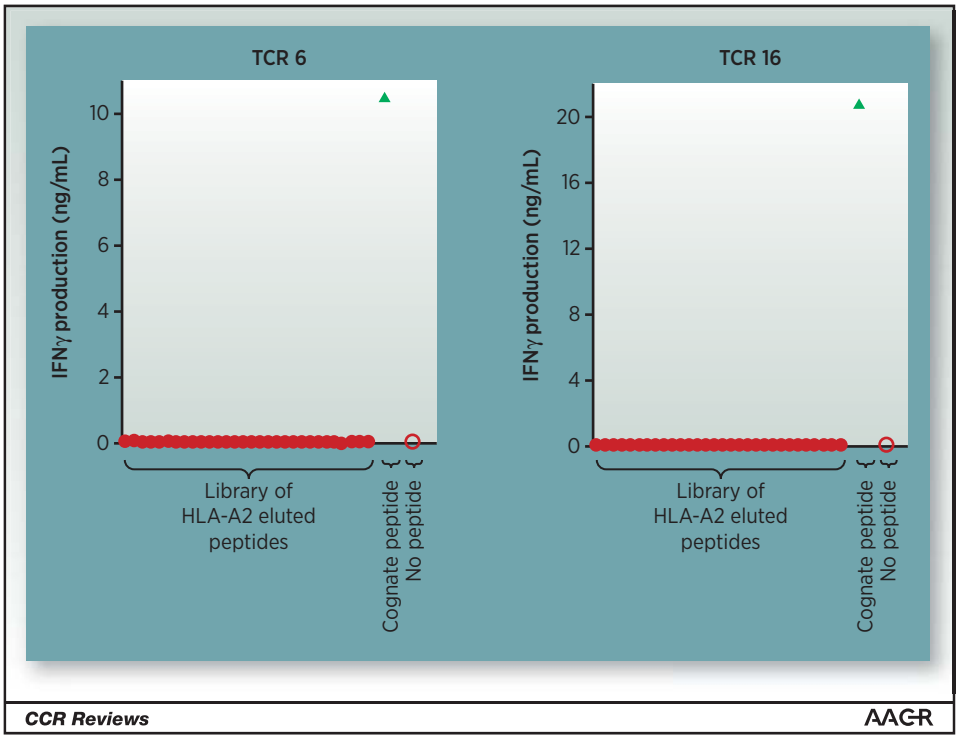
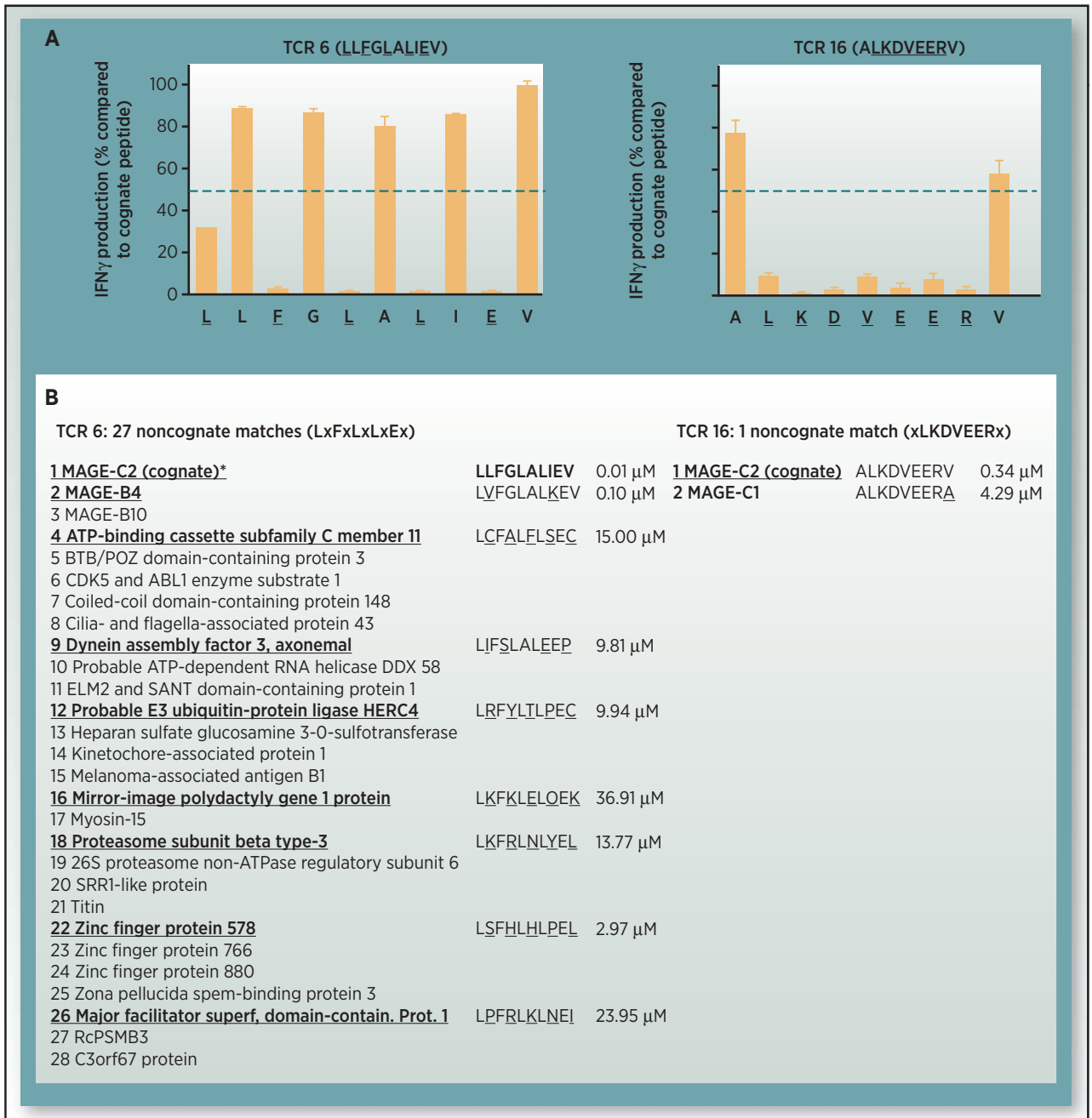

TCR 6
TCR 16
IFNγ production (ng/mL)
0
2
4
6
8
10
12
16
20
Library of HLA-A2 eluted peptides
Cognate peptide
No peptide
CCR Reviews
AACR

Figure 3:



B

TCR 6: 27 noncognate matches (LxFxLxLxEx)			TCR 16: 1 noncognate match (xLKDVEERx)		
1 MAGE-C2 (cognate)*	**LLFGLALIEV**	0.01 μM	1 MAGE-C2 (cognate)	ALKDVEERV	0.34 μM
2 MAGE-B4	LVFGLALKEV	0.10 μM	2 MAGE-C1	ALKDVEERA	4.29 μM
3 MAGE-B10					
4 ATP-binding cassette subfamily C member 11	LCFALFLSEC	15.00 μM			
5 BTB/POZ domain-containing protein 3					
6 CDK5 and ABL1 enzyme substrate 1					
7 Coiled-coil domain-containing protein 148					
8 Cilia- and flagella-associated protein 43					
9 Dynein assembly factor 3, axonemal	LIFSLALEEP	9.81 μM			
10 Probable ATP-dependent RNA helicase DDX 58					
11 ELM2 and SANT domain-containing protein 1					
12 Probable E3 ubiquitin-protein ligase HERC4	LRFYLTLPEC	9.94 μM			
13 Heparan sulfate glucosamine 3-0-sulfotransferase					
14 Kinetochore-associated protein 1					
15 Melanoma-associated antigen B1					
16 Mirror-image polydactyly gene 1 protein	LKFKLELQEK	36.91 μM			
17 Myosin-15					
18 Proteasome subunit beta type-3	LKFRLNLYEL	13.77 μM			
19 26S proteasome non-ATPase regulatory subunit 6					
20 SRR1-like protein					
21 Titin					
22 Zinc finger protein 578	LSFHLHLPEL	2.97 μM			
23 Zinc finger protein 766					
24 Zinc finger protein 880					
25 Zona pellucida spem-binding protein 3					
26 Major facilitator superf, domain-contain. Prot. 1	LPFRLKLNEI	23.95 μM			
27 RcPSMB3					
28 C3orf67 protein					

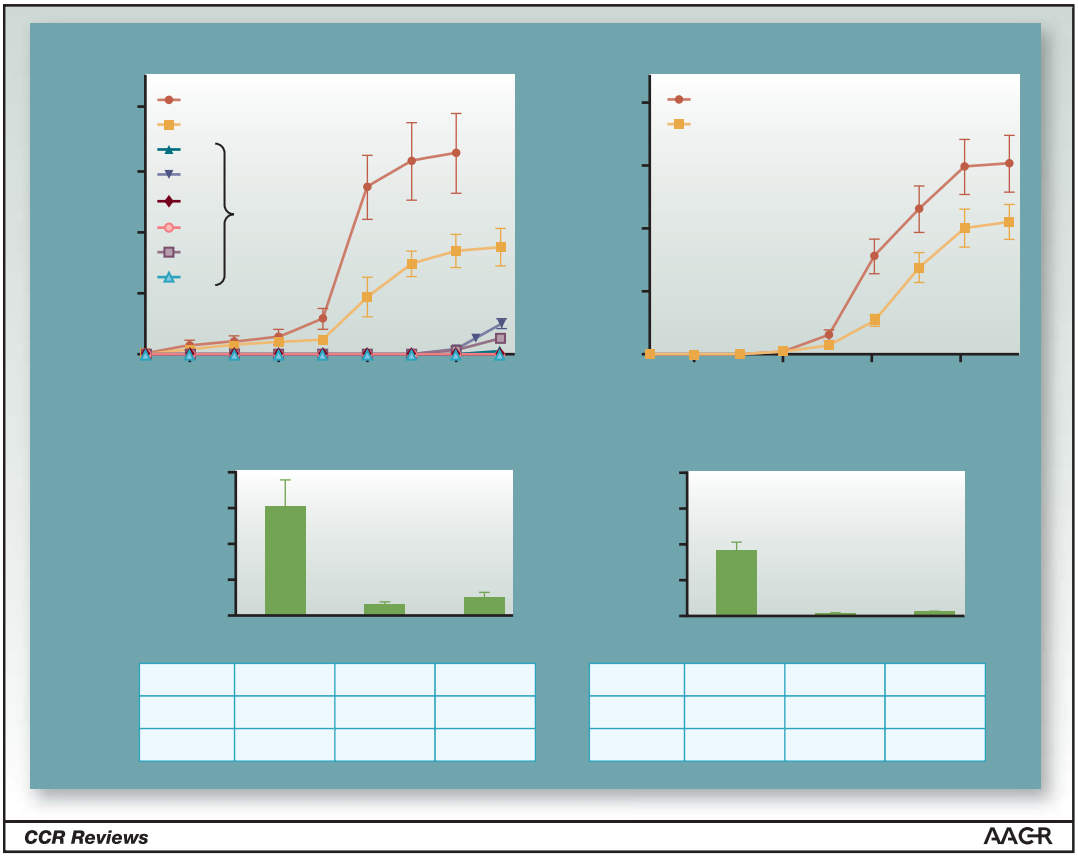


Figure 5: