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ORIGINAL ARTICLE

Targeting the epidermal growth factor receptor (HER) family by T cell receptor gene-modified T lymphocytes

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Abstract Human epidermal growth factor receptor 2 (HER2) has been successfully targeted as a breast cancer-associated antigen by various strategies. HER2 is also overexpressed in other solid tumors such as stomach cancer, as well as in hematological malignancies such as acute lymphoblastic leukemia. HER2-targeted therapies are currently under clinical investigation for a panel of malignancies. In this study, we isolated the T cell receptor (TCR) genes of a HER2-reactive allo-human leukocyte antigen-A2-restricted CTL clone and introduced the TCRα- and β-chain genes into the retrovirus vector MP71. Murinization and codon optimization of the HER2-reactive TCR was required for efficient TCR expression in primary human T cells. The tumor recognition efficiency of HER2-TCR gene-modified T cells was similar to the parental CTL clone from which the TCR genes were isolated. The known cross-reactivity of the HER2-reactive TCR with HER3 and HER4 was retained when the TCR was transduced into primary T cells. Our results could contribute to the development of a TCR-based approach for the treatment of HER2-positive breast cancer, as well as of other malignancies expressing HER2, HER3, and/or HER4.

Keywords Adoptive T cell therapy · TCR gene transfer · HER2-TCR · Retroviral vector · ErbB2 · HER2

Introduction

The human epidermal growth factor receptor 2 (HER2) has been an important target for novel strategies for breast cancer as well as for other HER2-expressing malignancies,

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including acute B lymphoblastic leukemia (B-ALL) where HER2 is overexpressed in one third of adult patients [1–3]. These strategies include monoclonal antibodies (mAb), kinase inhibitors, and cancer vaccines. The mAb trastuzumab (Herceptin) and the kinase inhibitor lapatinib, both directed against HER2, led to objective remissions of breast cancer and belong to the standard treatment for patients with HER2-expressing breast cancer. There is an ongoing study where tolerance and efficacy of trastuzumab is being investigated in patients with relapsed/refractory B-ALL [4]. In contrast, tumor regression following immunization with HER2-directed vaccines is rare, even though HER2-specific T cells can be induced [5–8]. The expansion of T cells with only poor function following vaccination can be explained by self tolerance, since HER2 is a self antigen. In order to overcome tolerance, adoptive T cell transfer strategies have been developed to activate a patient's T cells ex vivo and then transfer them back into the patient [9]. We have shown that the transfer of HER2-reactive human CTL clones can lead to the elimination of disseminated tumor cells in a patient with HER2-expressing breast cancer [10]; however, the broad clinical application of adoptive T cell transfer is limited because the isolation and characterization of HER2-reactive T cells is laborious. Therefore, we established a panel of allo-restricted T cell clones by stimulation of $CD8^+$ T cells from a human leukocyte antigen (HLA)-$A2^-$ donor with HER2 peptide-loaded HLA-$A2^+$ dendritic cells as a source for the isolation of HER2-reactive T cell receptor (TCR) genes, in order to redirect T cell antigen specificity [11]. Of note, these CTL clones were directed towards the immunodominant $A2/HER2_{369–377}$ peptide and displayed efficient tumor recognition — a characteristic seen only intermittently for $HER2_{369–377}$-specific T cells [5, 12, 13]. HER2 shares a high homology with the other members of the HER family, and some of the established HER2-reactive CTL clones specifically cross-reacted with the analogous nonamer epitopes derived from HER3 and HER4, but not from HER1 [11].

The transfer of TCRαβ genes into T cells has been developed over the past years as a strategy to endow T cells with a defined antigen specificity [14]. The potential value of T cell therapy using TCR gene-modified cells was shown in murine tumor- and virus-disease models. The feasibility of infusion of TCR gene-modified antigen-redirected autologous T cells was demonstrated in clinical trials [15, 16]; however, the generation of effective TCR-redirected T cells is often hampered by low cell surface expression of the transferred TCR, which subsequently leads to a low functional avidity of the gene-modified cells. Therefore, modifications of TCR genes were employed to increase the avidity of TCR-engineered cells [17]. Among these modifications, the replacement of the human TCR constant (C) regions by mouse counterparts [18–20], the codon optimization of TCRαβ genes [21], and the introduction of an additional cysteine bond in the C-region of the transgenic TCR [22] resulted in more efficient expression of TCR genes and in higher avidity of TCR gene-modified T cells.

Material and methods

Cell lines

293T (American Type Culture Collection (ATCC), Manassas, VA, USA: CRL-11268) A-498 (ATCC: HTB-44), BT-549 (ATCC: HTB-122), MCF7 (ATCC: HB-22), MDA-MB-231 (ATCC: HTB-23), MDA-MB-468 (ATCC: HTB-132), RCC-26 (provided by D. Schendel, Helmholtz Center Munich, Germany), SK-OV3 (ATCC: HTB-77), SK-OV3tA2 (supplied by M. L. Disis; University of Washington, Seattle, WA, USA), SK-MEL-29 and SK-MEL-37 (provided by L. Old, Memorial Sloan-Kettering Cancer Institute, New York, NY, USA), SW-620 (ATCC: CCL-227), SW-1116 (ATCC: CCL-233), and T 47 DD1-3 (ATCC: HTB-133) were cultured in Dulbecco's modified Eagle's medium (PAA, Pasching, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 IU/ml of penicillin/streptomycin (PAA). Me-324, LT-M1, LSW-B1 (provided by Andreas Moosmann, Helmholtz Center Munich, Germany), and T2 cells (ATCC: CRL-1992) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA), 10% FCS, 2 mM glutamine, and 100 IU/ml of penicillin/streptomycin. K562tA2 cells (provided by T. Wölfel, Mainz, Germany) transduced with either MigR1 (K562-A2; as mock control) or MigR1-HER2 (K562-A2-HER2) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml of penicillin/streptomycin, and 1 mg/ml of G418 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

$CD8^+$ T cells were cultured in RPMI 1640 medium supplemented with human AB serum (Milan Analytica AG, Magden, Switzerland), 2 mM glutamine, and 100 IU/ml of penicillin/streptomycin.

Isolation and amplification of HER2-specific TCR genes

TCRα- and β-chain sequences were derived from the $HER2_{369–377}$-reactive CTL clone KU1 [11]. To identify the variable regions of the TCR, a 5′-RACE-PCR (GeneRacer, Invitrogen, Karlsruhe, Germany) was performed using the following primers (MWG Biotech, Martinsried, Germany): TCRα-chain (5′-ggt aca cgg cag ggt cag ggt tct-3′) and TCRβ-chain (5′-gtg gcc ttt tgg gtg tgg gag at-3′). By using gene-specific primers, TCR chain genes were amplified by PCR, introducing a NotI restriction site (underlined) and a Kozak sequence in the sense primers and an EcoRI site

(underlined) in the antisense primers (TCRα-chain, sense: 5′-agg cgg ccg cca cca tgg cat gcc ctg gct tc-3′, antisense: 5′-tgg aat tct cag ctg gac cac agc cgc agc-3′; TCRβ-chain, sense: 5′-ggg cgg ccg cca tgg gca cca ggc tcc tc-3′, antisense: 5′-tgg aat tcc tag cct ctg gaa tcc ttt ctc-3′).

Generation of HER2-TCR retroviruses and transduction of T cells

The TCRα- and β-chain genes were either cloned separately (Fig. 1a) or linked by a P2A element in the orientation β-P2A-α [23] and inserted as a single transgene cassette into the retroviral vector MP71-PRE [24]. Additionally, the TCR cassette was optimized by murinization of the TCR C-regions [19] and codon optimization (GENEART, Regensburg, Germany) [21], and cloned into MP71-PRE (Fig. 1b). HER2-TCR retroviruses were generated by transfection of 293T cells using plasmids encoding MoMLV-*gag-pol* (pcDNA3.1MLVg/p, provided by C. Baum, Hannover, Germany), MLV-10A1-*env* [25], and MP71-HER2-TCR, as described [23]. Peripheral blood lymphocytes were isolated from healthy donors after informed consent by Ficoll gradient centrifugation [25]. $CD8^+$ T cells were enriched ($CD8^+$ T cell isolation kit II, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and transduced [23]. The experiments were in accordance with the stipulations of the latest Helsinki Declaration and approved by the local Ethics Committee.

Flow cytometry

T cells were stained using an anti-CD8 FITC-labeled mAb (Caltag Laboratories, Burlingame, CA, USA) and PE-labeled multimers specific for HLA-A2/HER2, HLA-A2/HER3, and HLA-A2/HER4 (manufactured by D. H. Busch), respectively, and were measured using a Coulter Epics XL flow cytometer (Beckmann-Coulter, Hialeah, IL, USA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

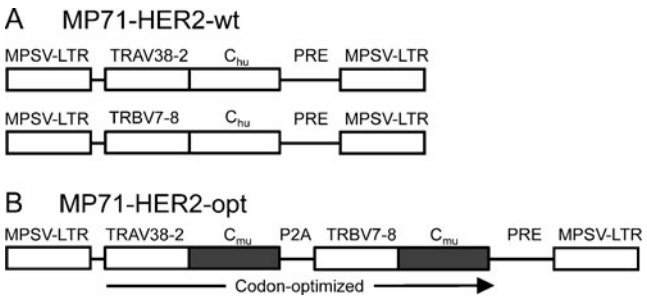

Fig. 1 Schematic depiction of wild-type (**a**) and optimized (**b**) MP71-HER2-TCR retroviral vector. C_{mu}: constant murine TCR-region, C_{hu}: constant human TCR-region, *MPSV-LTR*: long terminal repeat of the myeloproliferative sarcoma virus, *PRE*: post regulatory element, *P2A*: 2A element of porcine teschovirus

Functional analysis of T cells

4×10^4 T cells were incubated with 2×10^4 target cells in 96-well round bottom plates (TPP, Trasadingen, Switzerland) in a volume of 200 μl RPMI 1640 medium. The supernatants were analyzed for interferon (IFN)-γ content by enzyme-linked immunosorbent assay (ELISA; BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. To test the functional avidity, the same E:T ratio was used for peptide-pulsed ($HER2_{369–377}$: KIFGSLAFL, $HER3_{356–364}$: KILGNLDFL, $HER4_{361–369}$: KINGNLIFL, $gp100_{209–217}$: ITDQVPFSV, and $WT\text{-}1_{126–134}$: RMFPNAPYL, provided by Biosyntan, Berlin, Germany) T2 target cells. The peptide concentration was titrated from 10^{-5} to 10^{-14} M. To analyze the epitope specificity of the modified HER2-TCR, the same protocol was used, and the cells were loaded with 10^{-5} M of the respective peptide. $HER2_{369–377}$ was used as template for generation of peptide analogs, and each amino acid was sequentially substituted by alanine (Biosyntan). Cytolytic activity was analyzed in a standard 4 h ^{51}chromium release assay [11].

Results

Human T cells transduced with an optimized HER2-TCR show similar antigen recognition compared to the parental HER2-reactive CTL clone

Previously, the CTL clone KU1 was generated by stimulation of $HLA\text{-}A2^-$ $CD8^+$ T cells with allogeneic $HLA\text{-}A2^+$ (A2) DC pulsed with the HLA-A*0201-restricted epitope $HER2_{369–377,}$ followed by sorting and cloning of A2/HER2 $multimer^+$ T cells. This CTL clone showed specific lytic activity and IFN-γ secretion towards HER2-, as well as HER3- and HER4-overexpressing tumor cells [11]. Therefore, clone KU1 was selected for isolation of the TCR genes. Sequence analysis of the PCR products revealed the following TCR gene identities: TCRα (TRAV38-2J3701, CDR3: CAYRNDNTGKLIF) and TCRβ: (TRBV7-801J2-201D101, CDR3: CASSFSRQLNTGELFF).

The recognition of HER2 peptide/MHC complexes of the CTL clone KU1 was documented by A2/multimer staining. As $HER2_{369–377}$ has 66% homology with the other members of the HER family, the epitopes $HER3_{356–364}$ and $HER4_{361–369}$ were also recognized by the HER2 clone KU1 and exhibited staining with A2/HER2, A2/HER3, and A2/HER4 multimers (Fig. 2, left panel). A2 multimers containing an irrelevant peptide ($gp100_{209–217}$) did not bind to clone KU1. HER2-TCR-wt-transduced primary human $CD8^+$ T cells displayed only marginal staining with A2/HER2 multimer and no staining above background with A2/HER3 and A2/HER4 multimers, indicating the low expression level of the non-

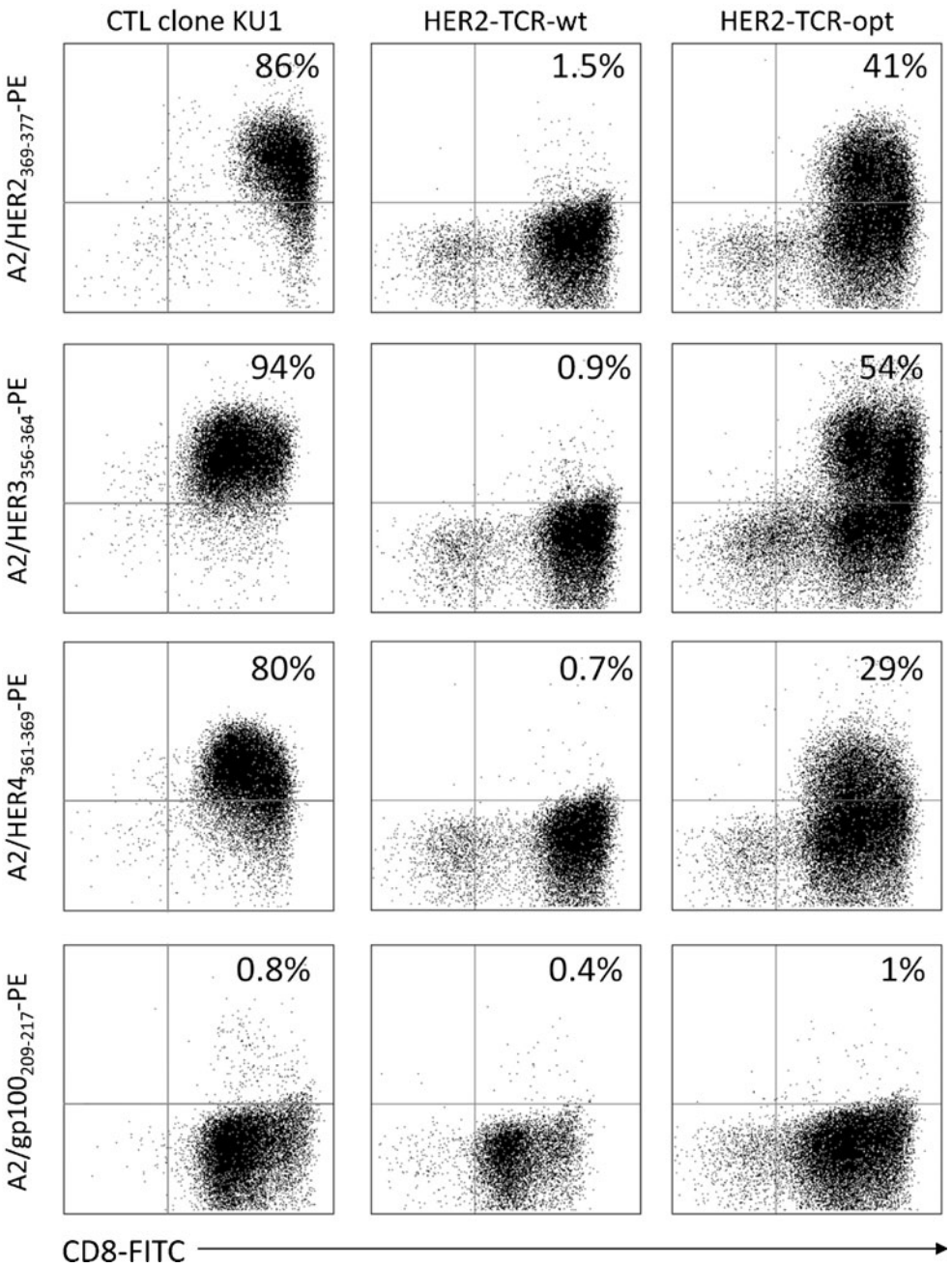

Fig. 2 HLA-A2/HER multimer staining of the parental CTL clone KU1 and TCR-transduced T cells. HLA-A2-restricted $HER2_{369–377}$-reactive CTL clone KU1 and $CD8^+$ T cells transduced with either wild-type (HER2-TCR-wt) or optimized HER2-specific TCR (HER2-TCR-opt) were stained with indicated A2/peptide multimers. Numbers represent the percentage of multimer$^+$ cells

optimized TCR (Fig. 2, middle panel). Efficient cell surface expression of the TCR and improved functional avidity of the gene-modified T cells were achieved after murinization and codon optimization of the TCR genes, and application of the P2A linker to combine the TCRα- and β-chain genes (HER2-TCR-opt). Thus, the TCR expression in transduced $CD8^+$ T cells increased from 1.5% to 41%, as measured by A2/HER2 multimer staining (Fig. 2). The reactivity of clone KU1 with HER2, HER3, and HER4 was retained, as demonstrated by the positive staining with the respective HLA multimer (Fig. 2, left/right panel). The gp100 tetramer did not stain transduced T cells with either construct, indicating that there was no unspecific reactivity of the HER2-TCR gene-modified T cells.

Avidity and tumor recognition of transduced T cells are retained with HER2-TCR-opt

We analyzed whether the avidity toward the epitopes of HER2, HER3, and HER4 of TCR gene-modified $CD8^+$ T cells was comparable to that of the CTL clone KU1 which showed a half maximum IFN-γ secretion at 10^{-7} M toward HER2 and HER4 peptide-loaded T2 cells and at 10^{-9} M toward HER3 peptide-loaded cells (Fig. 3a, left). $CD8^+$

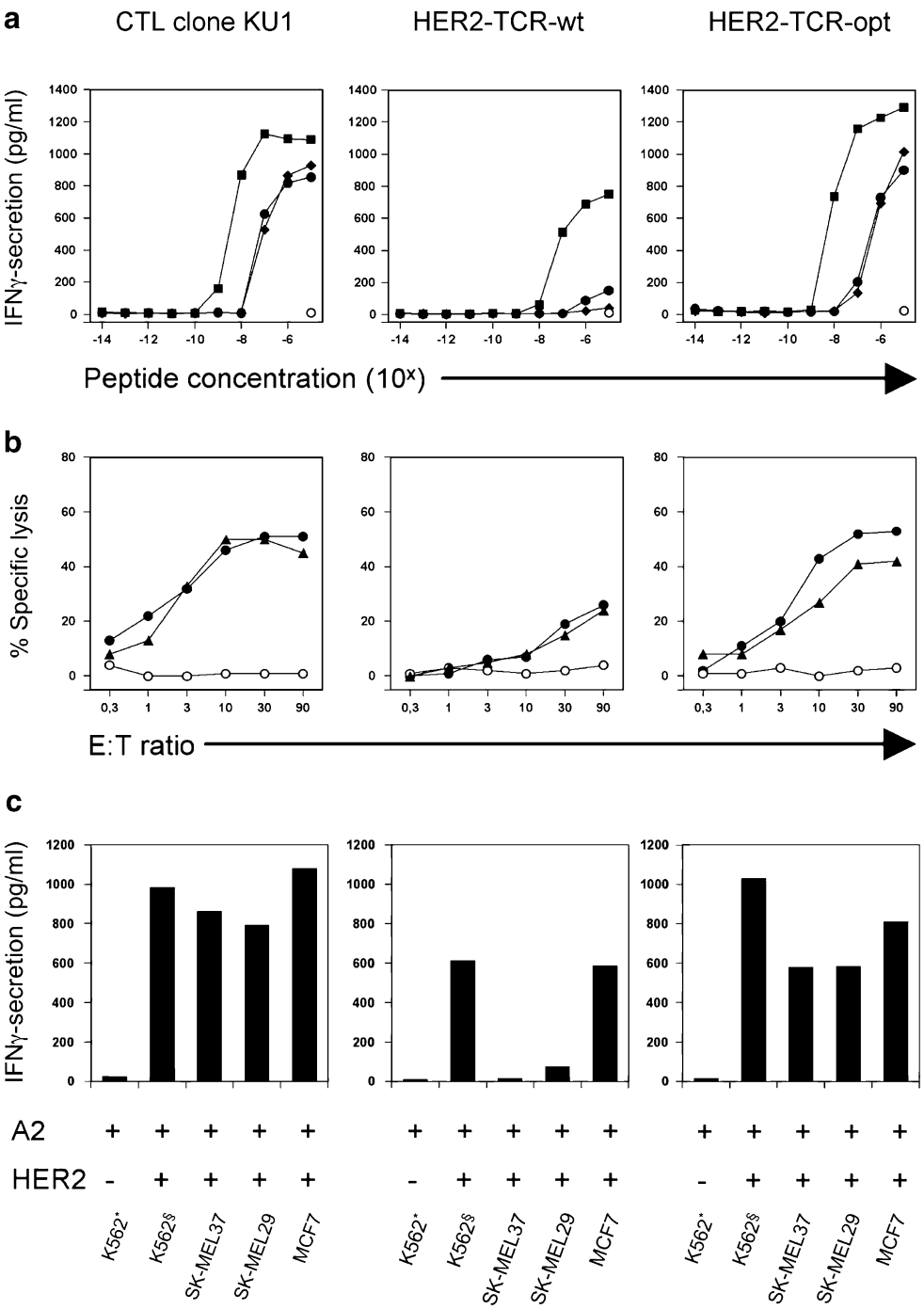

Fig. 3 Recognition of HER peptides and HER2/3/4-expressing cell lines by the CTL clone KU1 and TCR-transduced T cells. **a** Clone KU1 and $CD8^+$ T cells transduced with either wild-type (HER2-TCR-wt) or optimized HER2-TCR (HER2-TCR-opt) were tested for their avidity and cross-reactivity toward $HER1_{364–372}$ (*white circle*), $HER2_{369–377}$ (*black circle*), $HER3_{356–364}$ (*black square*), and $HER4_{361–369}$ (*black diamond*). Functional avidity was determined by IFN-γ secretion with T2 cells pulsed with graded amounts of peptides. **b** CTL recognition of endogenously processed epitope $HER2_{369–377}$ was evaluated by targeting K562-A2 cells retrovirally transduced with either HER2 (*black circle*) or mock vector transduced cells (*white circle*) or HER2-overexpressing MCF7 cells (*black triangle*) in a cytotoxicity assay using ^{51}Cr-labeled target cells and different E:T ratios of CTLs. **c** IFN-γ release by clone KU1 and TCR-transduced $CD8^+$ T cells was determined toward K562 cells transfected with A2 (*asterisk*) or A2 and HER2 (*section sign*) and toward the indicated $HER2/3/4^+$ tumor cell lines

T cells transduced with HER2-TCR-opt achieved the same avidity as clone KU1 toward these HER epitopes (Fig. 3a, right). In contrast, $CD8^+$ T cells transduced with HER2-TCR-wt only recognized HER3 with a half maximum IFN-γ secretion at 10^{-7} M, while no IFN-γ secretion was detected towards HER2 and HER4.

To answer the question whether TCR gene-modified T cells could recognize the endogenously processed HER2 peptide, HLA-A2-matched, HER2-transfected cells, and HER2-expressing tumor cells lines were investigated. HER2-TCR-opt-transduced $CD8^+$ T cells lysed HER2-expressing tumor cell lines as efficiently as the clone KU1 (Fig. 3b). Of note, T cells transgenic for HER2-TCR-opt exhibited similar lytic activity as the CTL clone, although only 41% of the transduced T cells showed A2/HER2 multimer binding, compared to 86% for the clone (Fig. 2). In contrast, HER2-TCR-wt-transduced $CD8^+$ T cells displayed a low TCR surface expression of 1.5% and little lytic activity (25%) even at a high E:T ratio of 90:1 (Fig. 3b, middle).

Next, the ability of HER2-TCR gene-modified T cells to secrete IFN-γ in response to $A2^+$/HER2/3/4$^+$ breast cancer (MCF7) and melanoma (SK-MEL-29, SK-MEL-37) tumor cells was analyzed. Following transduction with HER2-TCR-opt $CD8^+$ T cells secreted equal amounts of IFN-γ in comparison to the clone KU1 in the presence of all $A2^+$/HER2/3/4$^+$ tumor cells (Fig. 3c). HER2-TCR-wt-transduced T cells secreted IFN-γ only in the presence of MCF7 and K562-A2-HER2 but hardly any in the presence of the $A2^+$/HER2/3/4$^+$ melanoma cell lines.

Genetic modifications of the TCR do not alter its antigen specificity

Additional experiments were performed to analyze whether genetic modifications of the TCR led to cross-reactivity to epitopes other than HER2/3/4 and/or to HLA-A2 as an allo-antigen. A panel of 48 tumor cell lines was screened for its HER2/3/4 and HLA-A2 expressions. Figure 4a exemplarily shows the classification of $A2^{+/-}$ and HER2/3/4$^{+/-}$ cells. Cocultivation of HER2-TCR-opt-transduced T cells with $A2^+$/$HER2^+$ tumor cell lines induced IFN-γ secretion independent of coexpression of HER3 and HER4 (Fig. 4b). Unfortunately, among these tumor cell lines, we did not find cells which where HLA-$A2^+$ and exclusively $HER3^+$ or $HER4^+$; however, Fig. 4b shows that the HER2-TCR-opt-transduced T cells recognized the HER antigen(s) in an HLA-A2-dependent fashion, and there was no direct recognition of HLA-A2 as allo-antigen, independent of HER2. Upon cocultivation, HER2-TCR-opt-transduced T cells did not secrete IFN-γ when the target cells were HLA-$A2^+$ but negative for the three HER family members. Thus, the modification of the HER2-TCR did not change its antigen specificity.

An epitope scan was performed in order to further clarify whether the HER2-TCR shows cross-reactivity to epitopes other than HER2/3/4. Alanine was used to substitute each residue of the $HER2_{369–377}$ peptide (KIFGSLAFL) sequentially. Substitution of the amino acid residues resulted in a reduction in peptide reactivity, as measured by relative IFN-γ secretion of HER2-TCR-opt-transduced T cells upon cocultivation with peptide-loaded T2 cells. In most cases, IFN-γ secretion did not increase above background levels (Fig. 4c).

Discussion

The HER2 peptide sequence at position 369–377 was first identified as an immunodominant epitope for HLA-A2-restricted T lymphocytes that had been isolated from malignant ascites of patients with HER2-overexpressing ovarian cancer [12]. Since this discovery, the synthetic peptide $HER2_{369–377}$ has been widely investigated for the ex vivo and in vivo generation of HER2-specific CTL following in vitro stimulation [13, 26–31] and vaccination [5, 6, 8, 32, 33]. Results obtained from different groups led to the conclusion that $HER2_{369–377}$-specific T cells can potentially lyse HLA-A2-matched HER2-overexpressing tumor cells [13, 26–31] and may even be able to eliminate tumor cells in vivo [8, 10, 34]; however, several groups have shown that $HER2_{369–377}$-specific CTLs generated by in vitro or in vivo stimulation using the $HER2_{369–377}$ peptide do not necessarily recognize the endogenously processed peptide and, therefore, may fail to lyse HLA-$A2^+$ HER2-overexpressing tumor cell lines [5, 29]. We generated several CTL clones that reacted with the immunodominant epitope $HER2_{369–377}$ and displayed efficient in vitro tumor recognition [11].

In continuation to our earlier work, we show here that HER2 specificity can be transferred from a CTL clone to primary T cells by TCR gene transfer and that the TCR gene-modified T cells are able to recognize endogenously processed antigen on tumor cells.

The highest functional avidity of TCR gene-modified T cells was achieved when the HER2-TCR expression cassette was optimized by murinization and codon optimization. Most probably this is due to the improved ability of the HER2-TCR-opt in comparison with HER2-TCR-wt to compete with endogenous TCR for the indispensable CD3 complex needed for cell surface expression. The introduction of mouse C-regions into human TCR causes a reduction of mispairing between transferred and endogenous TCR chains and leads to an increased surface expression of the transferred TCR [18, 19]. Codon optimization increases the abundance of transferred TCR molecules in transduced T cells which stochastically outrun the endogenous TCR. The result for the HER2-TCR-opt is in line with reports for several other TCRs which were only efficiently expressed when the TCR genes were optimized [21].

Similar to the original CTL clone KU1, the TCR gene-modified T cells showed not only reactivity to HER2-, but also to HER3- and HER4-expressing cells; however, despite the cross-reactivity toward three members of the HER family, the HER2-TCR-opt showed no reactivity toward other epitopes expressed on a large variety of tumor cell lines. Moreover, no epitopes were found in an alanine scan, despite the homology of the synthesized peptides to the recognized $HER2_{369–377}$ epitope.

The reactivity of HER2-specific T cells towards HER3 and HER4 may enhance HER-directed T cell therapy because targeting several members of the HER family may inhibit the selective outgrowth of immune escape variants, which have been described following HER2-directed immunotherapy [35, 36].

On the other hand, there is a definite potential that this may also cause unwanted side effects through reactivity with normal tissues expressing HER molecules.

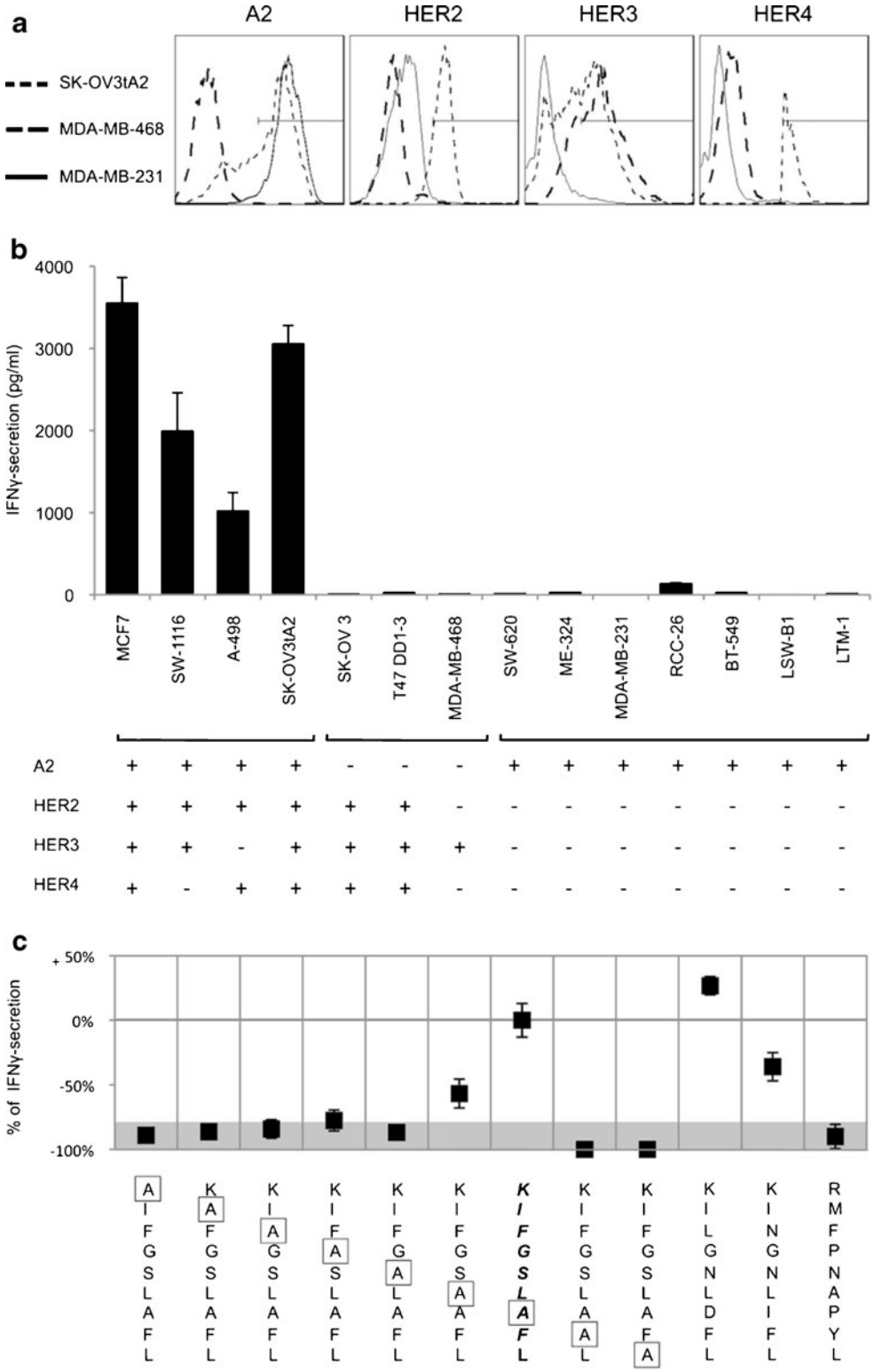

Fig. 4 HLA-A2- and HER2/3/4-restricted recognition of tumor cell lines and epitope specificity of the modified HER2-TCR. **a** Tumor cell lines were tested by FACS measurement for their HLA-A2 and HER2/3/4 expression. Exemplarily shown are SK-OV3tA2 (*dashed line*), MDA-MB-468 (*dotted line*), MDA-MB-231 (*solid line*). **b** IFN-γ release by TCR-HER2-opt-transduced T cells was determined toward indicated HER2/3/4- and HLA-A2-positive or negative tumor cell lines. **c** An alanine scan was performed and relative IFN-γ secretion of TCR-HER2-opt-transduced T cells was calculated based on their reactivity against T2 cells pulsed with indicated $HER2_{369–377}$ peptide analogons. The IFN-γ amount obtained with nonmodified $HER2_{369–377}$ (KIFGSLAFL), indicated in *bold/italic letters*, was used for data normalization. $HER3_{356–364}$ (KILGNLDFL), $HER4_{361–369}$ (KINGNLIFL) and $WT\text{-}1_{126–134}$(RMFPNAPYL) served as control peptides

Reactivity to non-malignant cells and the expression level of HER antigens in normal tissues has to be thoroughly analyzed in preclinical models before starting a HER-directed T cell therapy. The new strategy of "double or triple targeting" will broaden the group of cancer patients available for HER-directed T cell therapy, since the coexpression and subsequent heterodimerization of the HER family members may contribute to the malignant phenotype of solid tumors and hematological malignancies and, therefore, could be targeted more efficiently with the TCR described here.

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Disclosure statement P.M. and H.C. performed research, collected and analyzed data, and wrote the manuscript; L.S. and M.L. performed research; D.B. provided vital tools; and W.U. and H.B. designed and supervised research, reviewed the data, and wrote the manuscript. The authors declare no competing financial interests.

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