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Promiscuous behavior of HPV16E6 specific T cell receptor beta chains hampers functional expression in TCR transgenic T cells, which can be restored in part by genetic modification

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Abstract. *Background*: T cell receptor gene transfer is a promising strategy to treat patients suffering from HPV induced malignancies. Therefore we isolated the TCR$\alpha\beta$ open reading frames of an HPV16E6 specific CTL clone and generated TCR transgenic T cells. In general low level expression of the transgenic TCR in recipient human T cells is observed as well as the formation of mixed TCRs dimers. Here we addressed both issues employing three different expression platforms.

Methods: We isolated the HVP16E6 specific TCRα and TCRβ open reading frames and retrovirally transduced human T cells with either wild-type (wt), or codon-modified (cm) chains to achieve enhanced TCR expression levels, or used codon-modification in combination with cysteinization (cmCys) of TCRs to facilitate preferential pairing of the introduced TCRα and TCRβ chains.

Results: Careful analysis of recipient T cells carrying the HPV16E6 TCRβ and endogenous TCR chains revealed the transgenic TCRβ chain to behave very promiscuously. Further analysis showed that the percentage of tetramer positive T cells in codon-modified/cysteinized TCR transgenic T cells was four-fold higher compared to wild-type and two-fold higher compared to codon-modification only. Functional activity, as determined by IFN-γ production, was high in cmCysTCR transgenic T cells, where it was low in cm and wt TCR transgenic T cells. Recognition of endogenously processed HPV16E6 antigen by cmCysTCR transgenic T cells was confirmed in a cytotoxicity assay.

Conclusion: Promiscuous behavior of the HPV16E6 specific TCRβ chain can in part be forced back into specific action in TCR transgenic T cells by codon modification in combination with the inclusion of an extra cysteine in the TCR chains.

Keywords: Immunotherapy, cervical carcinoma, adoptive transfer, T cell receptors

Abbreviations

CxCa	cervical cancer;
CTL	cytotoxic T lymphocyte;
GFP	green fluorescence protein;
HPV	human papilloma virus;
IFN	interferon;
IRES	internal ribosome entry site;
NGFR	nerve growth factor receptor;
TCR	T cell receptor.

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1. Introduction

Human Papilloma viruses (HPV) are DNA containing viruses belonging to the family of papovaviridae.

To date, more than 120 HPV types have been identified. These can be divided into "low" risk and "high" risk HPV types [14]. Infection with "low" risk HPV types, including HPV6 and 11, may result in benign epithelial lesions like warts. In contrast, "high" risk or oncogenic HPV types, of which HPV16 and HPV18 are the most predominant, are associated with precancerous lesions [32,52]. In the early 1980s Zur Hausen et al. were the first to associate HPV infections with genital lesions and cervical cancer (CxCa) [60]. An association between infection with HPV and cancer has also been shown for a number of other anogenital carcinomas, including vulvar and penile carcinomas [11, 37], and for some head and neck squamous cell carcinomas [5,44].

Prophylactic vaccines designed to induce neutralizing antibodies are now in use for the protection of young females against HPV infection [33,39]. These vaccines however are of no use for the treatment of preexisting HPV lesions or cancers. Therefore there is still a need to develop therapeutics for this group of patients and for non-vaccinees at risk. Attractive candidates for therapeutic approaches are the early proteins E6 and E7, since these proteins are constitutively expressed and necessary to maintain a transformed phenotype of the tumor cells [15]. For the treatment of melanoma, adoptive transfer of tumor specific T cells has been effective in animal models [49], and more importantly also in patients [12,56]. Since effectiveness of HPV specific T cell treatment has been shown in animal models [48,61], adoptive transfer of HPV specific T cells could also be valuable for the treatment of HPV induced malignancies in patients [23]. Low frequencies of pre-existing HPV specific T cells however hampers the routine isolation of sufficient numbers of HPV specific T cells on a per patient basis [42,57].

Alternatively, large numbers of tumor specific T cells can be generated by T cell receptor transfer [6,17,31]. TCR transgenic T cells have indeed been shown to express the exogenously introduced TCRs and to recognize and kill tumor cells *in vitro* [4,36,38, 41]. Importantly, both $CD8^+$ and $CD4^+$ TCR transgenic T cells were functionally active and capable of delaying growth of established tumors in animal models [2,24]. In humans, the first clinical trial using TCR transgenic T cells has recently been conducted in melanoma patients [30]. Although promising results have been obtained in this clinical trial it should be emphasized that there are still some major improvements warranted in curing low level expression of transgenic TCRs and avoiding the formation of mixed TCR dimers. Formation of mixed TCR dimers could lead to TCRs with unknown and potential harmful specificities [3]. Molecular engineering approaches like codon-modification [22,40], the inclusion of murine constant domains [50], the introduction of an extra cysteine in the constant domains [8,27], referred to as cysteinization [46], and the "hole-into-knob" configuration [51] can contribute to enhanced TCR expression levels and the safety of TCR gene therapy.

Different retroviral constructs have been used to introduce the TCRα and TCRβ into recipient T cells. For clinical application the use of one retroviral construct encoding both TCR chains is preferred. In these cases the TCR ORFs are separated by an IRES sequence [16], or a picorna virus derived 2A sequence [40], or one TCR ORF is under the control of a long terminal repeat (LTR) promoter while the other TCR ORF is under the control of an internal promoter, such as an pgk promoter [6,31,35]. We used two retroviral vectors each containing a different marker gene. The TCRα ORF was followed by the marker gene GFP and the TCRβ ORF was followed by the marker gene NGFR [17,53,54], thus allowing detailed investigation of proper TCR pairing.

In the current study we describe the isolation of the $\alpha\beta$TCR ORFs encoding the TCR from CTL clone 7E7 [13], which is directed against HPV16E6$_{29–38}$, and detail the functional expression in recipient CD8 positive T cells. We performed comparative analyses employing three different expression platforms in one and the same retroviral vector. The expression level and functional activity was charted of TCR transgenic T cells in which the TCR was either encoded by wild-type (wt) or codon-modified (cm) ORFs or a combination of codon-modification with the addition of an extra cysteine (cmCys) in the constant domains of both TCR chains to facilitate improved TCR$\alpha\beta$ pairing.

2. Materials and methods

2.1. Cell lines and T cell culture

The HPV16-positive CxCa cell lines SiHA (American Type Culture Collection, ATCC, Manassas, VA, USA) and SiHA-A2 (SiHA transfected with HLA-A2.1), were cultured in keratinocyte-serum free medium (Life technologies, Paisley, UK) supplemented with 5% (v/v) foetal calf serum (FCS; Perbio, Helsingborg, Sweden), 5 μg/ml bovine pituitary extract (Life technologies), 0.5 ng/ml epidermal growth fac-

tor (Life technologies) and antibiotics (100 IE/ml penicillin and 100 µg/ml streptomycin, Life technologies). JurMA cells, which are devoid of endogenous TCRβ chains, were cultured in IMDM supplemented with 8% FCS and antibiotics. The EBV transformed B cell line JY was cultured in IMDM supplemented with 8% FCS and antibiotics. CTL were cultured in Yssel's medium [58] supplemented with 1% human serum (HS; ICN Biomedicals, Aurora, OH, USA) and antibiotics. Once a week the CTLs were stimulated with an irradiated (5,000 rad) feeder mixture containing 1×10^6 allogeneic PBMC, 0.1×10^6 JY cells, 20 U/ml IL2 (Chiron, Amsterdam, The Netherlands) and 100 ng/ml phytohemagglutinin (PHA; Murex Biotec, Dartford, UK) in Yssel's medium supplemented with 1% HS and antibiotics [20]. For the generation of a $CD8^+$ T cell population, healthy donor derived PBMC were isolated from an HLA-A2.1 positive buffycoat (Sanquin, Amsterdam, The Netherlands) by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). Subsequently, isolation of resting CD8β positive CTL precursors from total PBMC was performed by positive selection using a MACS column (MACS; Miltenyi Biotec, Bergisch Galdbach, Germany). For this purpose, total PBMC were stained with anti-CD8β mAb and microbead-conjugated anti-mouse IgG Abs (Miltenyi Biotec), followed by MACS sorting according to the manufacturer's protocols. All cells were mycoplasm free and were maintained at 37°C in humidified air containing 5% CO_2.

2.2. *Isolation of the HPV16E6 specific TCR alpha and beta open reading frames*

Total RNA was isolated from 2×10^6 CTL using RNAzol (Campro Scientific, Veenendaal, The Netherlands) according to the manufacturer's instructions. Copy DNA was synthesized from 2–5 µg of RNA using oligo(dT) primers and reverse transcriptase (Life Technologies) in a volume of 20 µl according to the manufacturer's instructions. PCR was performed using 12 mixtures of four to five primers (the primers used were a kind gift from Dr. T.N. Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands)) complementary to the variable TCRα chain or the variable TCRβ chain in combination with the downstream constant α or β primer, respectively. PCR was performed on 1 µl of cDNA in the presence of 2 mM $MgCl_2$, 15 µM of each primer, 200 µM dNTPs, and 2.5 U *Taq* polymerase (Roche, Almere, The Netherlands) in a total volume of 50 µl. When a band of the expected size was visible on an agarose gel stained with ethidium bromide, the PCR was repeated using each of the variable primers separately together with the constant TCR primer. PCR products were purified using a GeneClean III kit (Q-BIOgene, Amsterdam, The Netherlands) and ligated into the pCR2.1 vector (Invitrogen). Sequence analysis (BaseClear, Leiden, The Netherlands) was subsequently performed to confirm isolation of open reading frames encoding the TCRα and TCRβ chains and to identify Vα and Vβ TCR family usage of the original CTL clone.

2.3. *Molecular cloning of TCRα and TCRβ open reading frames and retroviral transduction*

Analysis of codon usage of the 7E7 TCRα and TCRβ chains showed several rare codons in the natural 7E7 TCRα and TCRβ genes (Suppl. Fig. 1A: www.qub.ac.uk/isco/JCO/). Further analysis of these TCR genes also showed the presence of procarya inhibitory motifs and cryptic splice donor sites which may negatively influence the expression of the TCR by retaining the mRNA within the nucleus (Suppl. Fig. 1B: www.qub.ac.uk/isco/JCO/). Codon-modified TCR open reading frames were synthesized by GeneArt (Regensburg, Germany). Codon-modified ORFs in combination with an extra cysteine in the constant domain of both the TCRα and TCRβ were obtained by mutagenesis. Residue 48 in the constant TCRα region has been altered from a threonine into a cysteine and residue 57 in the constant TCRβ region from a serine into a cysteine [27]. The TCRα and TCRβ ORFs were cloned into the Moloney Murine Leukaemia Virus based retroviral vector LZRS [26] containing an internal ribosome entry sequence (IRES) followed by the markers eGFP or the signaling defective truncated version of the nerve growth factor receptor (NGFR). The TCRα chain was cloned into LZRS-mcs-IRES-GFP and the TCRβ chain in LZRS-mcs-IRES-NGFR [17]. These constructs were transfected into the packaging cell line Phoenix-a using lipofectamine (Invitrogen). Two days after transfection 2 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA) was added to select for virus producing cells. Ten to 14 days after transfection of the Phoenix cells, puromycin free retroviral supernatant was harvested, aliquoted and stored at −80°C for future use. JurMA [1,4,41] and $CD8^+$ T cells were retrovirally transduced as described in detail previously [19]. Briefly, two days prior to retroviral trans-

duction, $CD8^+$ T cells were stimulated using a feeder mixture as described above. 5×10^5 JurMA or $CD8^+$ T cells were resuspended in 0.5 ml virus supernatant supplemented with 100 U/ml IL-2 and transferred to a fibronectin (RetroNectin; Takara, Otsu, Japan)-coated well of a non-tissue-culture-treated flat bottom 24-well plate (BD Biosciences, Mountain View, CA, USA). Plates were centrifuged for 90 min at 2000 rpm, followed by 4–5 h incubation at 37°C. The cells were subsequently harvested, washed and resuspended in Yssel's medium for T cells or complete medium for JurMA, and kept at 37°C overnight. After 18 hours, retroviral transduction was repeated as described. Expression of the TCR was determined by flow cytometric analysis after 48 h, and at later time points after the second transduction. PE labeled antibodies directed against human Nerve Growth Factor Receptor (NGFR; Chromoprobe, Aptos, CA, USA), TCRVβ1 and TCRVβ8 were used, and allophycocyanin (APC) labeled anti-NGFR (Chromoprobe, Aptos, CA, USA) antibody. PE- and APC-labeled HLA-A2.1 tetramers presenting the HPV16E6$_{29–38}$ epitope [42] were used. Tetramer and Ab staining of cells was performed in PBS supplemented with 0.1% BSA and 0.01% Azide (PBA) for 15 min at 37°C and 20 min on ice respectively, followed by washing with PBA. Stained cells were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) using CellQuest software (BD Biosciences). T cells expressing GFP and NGFR were isolated by NGFR/GFP directed flow sorting using a FACS Aria (BD, Biosciences). TCR transgenic T cells were stimulated weekly as described above.

2.4. Functional read-out assays

Peptides were obtained from Leiden University Medical Center (LUMC). Peptides were >90% pure as analyzed by reverse-phase HPLC, dissolved in DMSO and stored at −20°C for future use.

Activation of TCR transduced JurMA cells was measured by the production of luciferase [1]. To measure the activation of the TCR transduced JurMA cells by JY cells loaded with E7$_{11–20}$ peptide or E6$_{29–38}$, 1×10^5 JurMA cells were incubated overnight with 5×10^4 JY cells in a 96-well plate. After incubation with various stimuli, cells were washed in PBS and lysed in 20 μl cell-culture-lysis buffer (Promega, Madison, WI, USA). To 10 μl of cell extract 50 μl Luciferase assay reagent (Promega) was added. Luminescence was subsequently measured in a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Luciferase activity in stimulated JurMA cells was expressed in Relative Luminescence Units (RLU) related to the luciferase activity of nonstimulated JurMA cells, which was set at a value of one.

Production of interferon-γ by stimulated CTL was determined using intracellular staining of permeabilized CTL with a PE labeled IFN-γ specific antibody according to the manufacturer's instructions (CytoFix/CytoPerm kit with GolgiStop, BD Biosciences). Stimulations were performed for 4 h at 37°C in a round bottom 96-well plate (Nunc) containing 1×10^5 responder CTL and 5×10^4 target cells per well, followed by NGFR staining as described above, and intracellular IFN-γ staining. Samples were subsequently analyzed by flow cytometry in order to calculate the percentage of responding CTL.

Cytotoxic activity of retrovirally TCR transduced $CD8^+$ T cells was measured in a standard chromium release assay [42]. Briefly, 1×10^6 target cells were labeled with 100 μCi $Na_2[^{51}Cr]O_4$ for 1 h at 37°C, washed extensively before co-culture with responder cells at the following E:T ratios 10:1, 3:1 and 1:1. Plates were incubated for 4 h at 37°C after which 50 μl supernatant was harvested and radioactivity was measured. The percentage of specific lysis was calculated as follows: Percentage specific lysis = [(experimental release-spontaneous release)/(maximal release-spontaneous release)] × 100.

3. Results

3.1. Isolation and preservation of the HPV16E6 specific T cell receptor

The HPV16E6 specific T cell clone 7E7 used in the current study was generated from a patient suffering from cervical cancer, after short term *in vitro* stimulation of T cells with peptide pulsed irradiated PBMCs followed by limiting dilution cloning. This CTL clone was shown to recognize the HLA-A2 restricted T cell epitope TIHDIILECV corresponding to amino acids 29–38 derived from HPV16E6 as a synthetic 10-mer peptide and as an endogenously processed and presented epitope [13]. Because of its anti-tumor reactivity this T cell clone was considered a promising candidate for the isolation of its TCR for transfer purposes. Tetramer and TCRVβ staining on the original CTL clone 7E7 was positive for tetramers presenting the relevant HPV16E6$_{29–38}$ epitope and TCRVβ1 antibodies (data not shown). Using RT-PCR, we isolated

the TCRα and TCRβ ORFs from this HPV16E6$_{29–38}$ specific T cell clone. Sequence analysis revealed the chains to be TCRVα29J37 and TCRVβ1J2S1 family members. TCRVβ usage as revealed by sequence analysis was in accordance with the results obtained by flow cytometric TCRVβ staining. No antibodies were available to stain the TCRα chain.

3.2. TCR expression and functional activity of HPV16E6 specific TCR transgenic JurMA cells

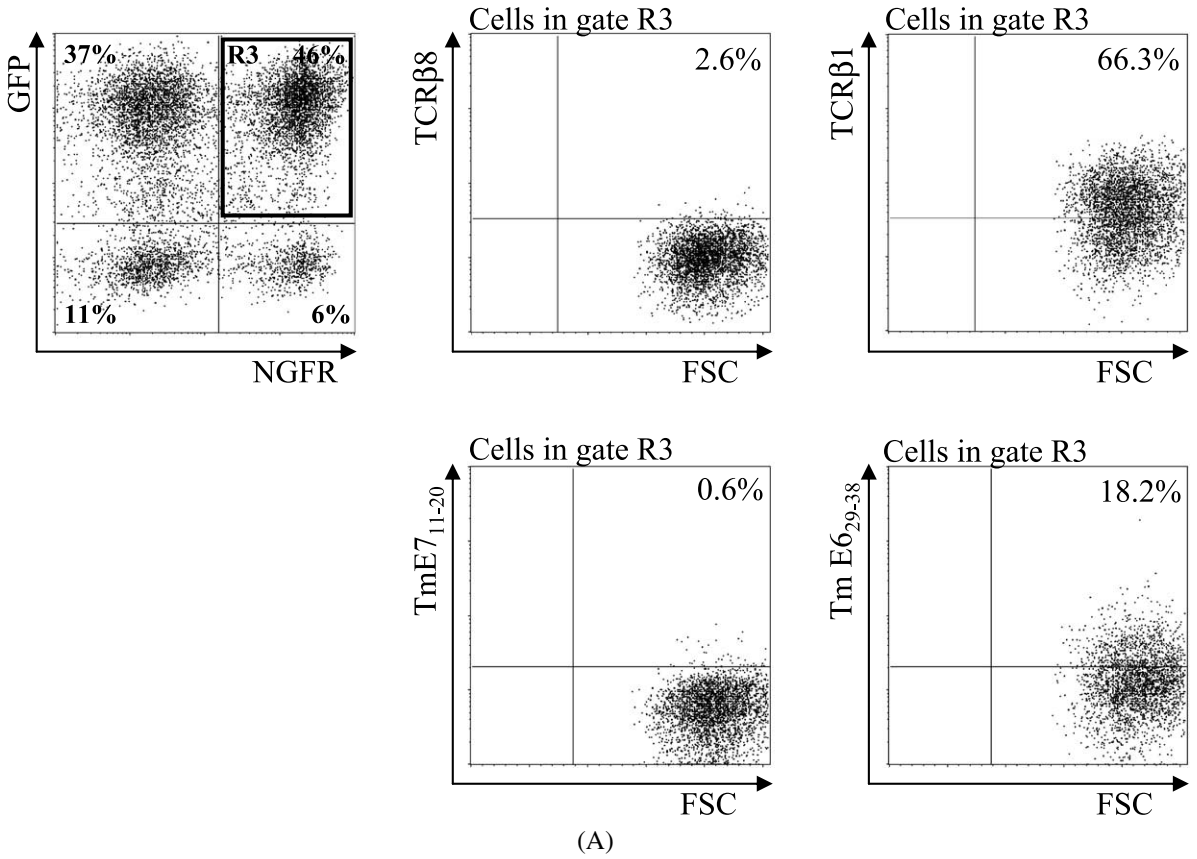
Isolated TCR alpha and beta ORFs from CTL clone 7E7 were cloned into the marker gene containing retroviral vector LZRS as TCRα-IRES-GFP and TCRβ-IRES-NGFR [17]. Next, we tested whether the isolated TCR ORFs were capable of forming a stable $\alpha\beta$TCR complex on the cell surface of JurMA cells. JurMA cells are devoid of endogenous TCRβ chains, but do express a TCRα chain. After retroviral transduction we found co-expression of the markers GFP and NGFR in 46% of the transduced JurMA cells (Fig. 1(A)). TCR cell surface expression was determined using specific TCRVβ1 antibodies and tetramers presenting the relevant HPV16E6$_{29–38}$ epitope. Despite the fact that 66% of the JurMA cells were positive for TCRVβ1 staining in the GFP$^+$/NGFR$^+$ quadrant, only 18% of the JurMA cells in the same quadrant were positive for tetramer staining (Fig. 1(A)). Discrepancies of this kind have been documented by others as well [1,6] and may in part be explained by differences in affinity of tetramers and TCRVβ antibodies. As expected, negligible percentages of TCRVβ1 staining were detected in the GFP$^-$/NGFR$^-$ ($\sim$0.4%) quadrant and GFP$^-$/NGFR$^+$ ($\sim$3%) quadrant (data not shown). This clearly indicates that the introduced TCRVβ1 does not cross-pair to significant levels with the TCRVα8 endogenously present in JurMA cell. As expected, no tetramer binding was observed when using an HLA-A2 tetramer containing a negative control peptide, nor did we see staining with monoclonal antibodies directed against the irrelevant TCRVβ8 family members (Fig. 1(A)).

In order to test functional activity of the transduced TCR alpha and beta chains we made use of the JurMa/NFAT-luciferase system [1,4,41]. TCR transgenic JurMA cells were incubated overnight with the HLA-A2.1 positive B cell line JY loaded with either relevant or negative control peptide. As shown in Fig. 1(B), JY cells loaded with relevant HPV16E6$_{29–38}$ peptide induced luciferase activity in TCR transgenic JurMA cells. As expected, no luciferase activity was induced after incubation of the TCR transgenic cells with JY cells pulsed with negative control peptide. From these experiments we concluded that we had successfully isolated the wild-type TCRα and TCRβ ORFs from CTL clone 7E7 and that this wtTCR is functionally active against peptide loaded target cells as determined in the JurMA/NFAT-luciferase system.

3.3. Comparative analysis of transgenic 7E7-TCR expression in recipient CD8$^+$ T cells

To investigate the application potential of 7E7-TCR transgenic CD8$^+$ T cells in a pre-clinical setting, we introduced the 7E7 derived TCR ORFs in peripheral blood derived CD8$^+$ T cells from unrelated HLA-A2.1 positive donors. In this analysis we compared three different formats (wt, cm and cmCys) to achieve functional expression of the transgenic 7E7-TCR. In order to facilitate analysis of transgene expression we made use of the retroviral vector LZRS, in which the TCRα and TCRβ ORFs were combined with the markers GFP and ΔNGFR, respectively. Co-expression of marker genes in conjunction with TCRα and TCRβ chains allowed for careful analyses of expression patterns in transduced T cells. Figure 2 shows the vector system we developed to accommodate TCR variable regions into vectors already containing the modified constant regions of the TCRα and TCRβ chains.

After transduction of CD8$^+$ T cells with wt, cm or cmCys TCR ORFs, cells were analyzed for the expression of the markers. GFP expression (as a marker for TCRα) indicated in the upper left and upper right quadrants, was found in 47% (24 + 23), 45% (26 + 19) and 51% (28 + 23) of the cells transduced with wt, cm and cmCys 7E7-TCRα-IRES-GFP retroviruses, respectively. NGFR expression (as a marker for TCRβ), indicated in the lower right and upper right quadrants, was found in 45% (22 + 23), 38% (19 + 19) and 42% (19 + 23) of the cells transduced with wt, cm and cmCys TCRβ-IRES-ΔNGFR retroviruses, respectively (Fig. 3). Co-expression of GFP and NGFR was found in 23%, 19% and 23% of the wt, cm and cmCys TCR transduced T cells, respectively (Fig. 3). It should be noted that the transduction efficiency of the wt, cm and cmCysTCR ORFs were comparable, excluding differences in expression level as a result of the number of retroviral insertions [28]. Cells were subsequently analyzed for the capacity to bind tetramers presenting relevant or irrelevant epitopes. Merely 5.4% of the wtTCR transgenic T cells in the GFP$^+$/NGFR$^+$ gate were capa-



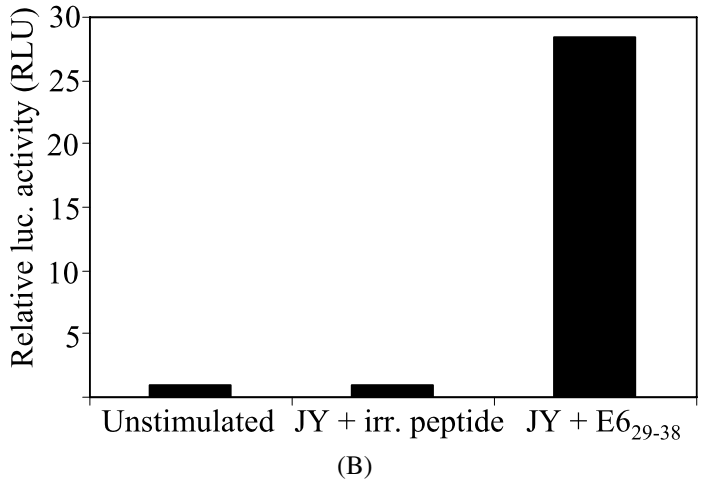


Fig. 1. Phenotypical and functional activity analysis of HPV16E6 specific TCR transgenic JurMA cells. (A) 7E7-TCR transgenic JurMA cells co-expressing GFP and NGFR (gated in R3) are negative for TCRVβ8 but positive for TCRVβ1 staining as indicated by the percentage of TCRVβ positive cells (upper right quadrant). Tetramer analysis of 7E7-TCR transgenic JurMA cells co-expressing GFP and NGFR show binding to relevant but not irrelevant tetramer as indicated by the percentage tetramer positive cells (upper right quadrant). (B) Functional activity of 7E7-TCR transgenic JurMA cells against peptide loaded target cells as determined in a luciferase assay. Luciferase activity in JurMA cells is shown in Relative Luminescence Units (RLU), defined as the ratio of luciferase activity in stimulated versus unstimulated cells. Data presented are representative for 2 different experiments performed.

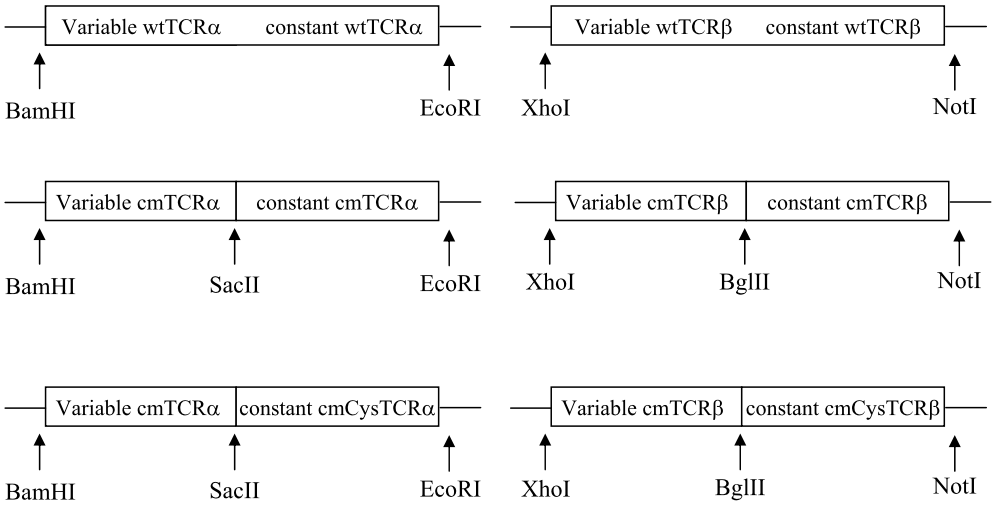


Fig. 2. Schematic representation of retroviral constructs encoding for the wt (A), cm (B) or cmCys (C) TCRα and TCRβ genes. Isolated wt TCRα and wtTCRβ from CTL clone 7E7 were introduced into the multiple cloning sites (mcs) of LZRS carrying the marker genes GFP and NGFR, respectively (B + C), using the indicated restriction enzymes. Variable cmTCRα and cmTCRβ can be easily introduced into the pCRScript vectors containing the cm or cmCys constant domains of the TCR chains. The variable cmTCRα chain can be introduced using BamHI $*$ SacII while the cmTCRβ chain can be introduced using XhoI $*$ BglII. The cm and cmCysTCRα and TCRβ chains can subsequently be introduced into the mcs of LZRS-mcs-I-GFP and LZRS-mcs-I-NGFR, respectively, using BamHI $*$ EcoRI for the TCRα chain and XhoI $*$ NotI for the TCRβ chain.

ble of binding to relevant tetramers (Fig. 3A). To our surprise only 10% of the cmTCR transgenic T cells in the upper right quadrant ($GFP^+/NGFR^+$ cells; gated in R4) were able to bind to relevant tetramers (Fig. 3B). Whereas 22% of the cmCysTCR transgenic T cells in the $GFP^+/NGFR^+$ gate were capable of binding to relevant tetramers (Fig. 3C). Background staining with tetramers containing the relevant peptide was 2–3% in all cases (lower left quadrant; $GFP^-/NGFR^-$ gated in R3). As expected, no tetramer staining was detected in the single positive ($GFP^+/NGFR^-$ or $GFP^-/NGFR^+$) gates or double negative ($GFP^-/NGFR^-$) gate using tetramers presenting the relevant epitope (data not shown). Nor did we see binding in either of the quadrants using tetramers presenting an irrelevant epitope (data not shown).

In conclusion, codon-modification of the TCR ORFs did not lead to highly increased binding of relevant tetramers when compared to wild-type sequences, which is in contrast to previous observations [40]. A further increase in relevant tetramer binding has been observed in cmCysTCR versus cmTCR transgenic cells (Fig. 3). These data suggest that the introduction of extra cysteine residues in the constant regions of the TCR alpha and beta chains indeed allows for improved pairing of the exogenous TCR chains.

3.4. Functional activity of 7E7-TCR transgenic $CD8^+$ T cells

In order to asses functional activity against appropriate target cells, TCR transgenic cells were sorted based on GFP and NGFR expression. The sorted cells were 90–96% positive for both GFP and NGFR (data not shown). Functional activity of 7E7-TCR transgenic $CD8^+$ T cells against peptide loaded target cells was tested in an interferon-γ assay. Cells were incubated with JY cells exogenously loaded with either relevant HPV16E6$_{29–38}$ or irrelevant peptide. Low background levels of interferon-γ production were observed against JY cells loaded with irrelevant peptide. In agreement with tetramer staining profiles (see Fig. 3, right-hand panels), very few wtTCR or cmTCR transgenic T cells in the $GFP^+/NGFR^+$ gate were capable of producing interferon-γ upon stimulation with JY cells loaded with relevant peptide (Fig. 4(A)). In contrast to this, 25% of the cmCysTCR transgenic $CD8^+$ T cells produced interferon-γ. As expected, no interferon-γ production was observed in the double negative and single positive quadrants (data not shown).

Functional TCR avidity of transduced $CD8^+$ T cells was analyzed in response to JY cells exogenously

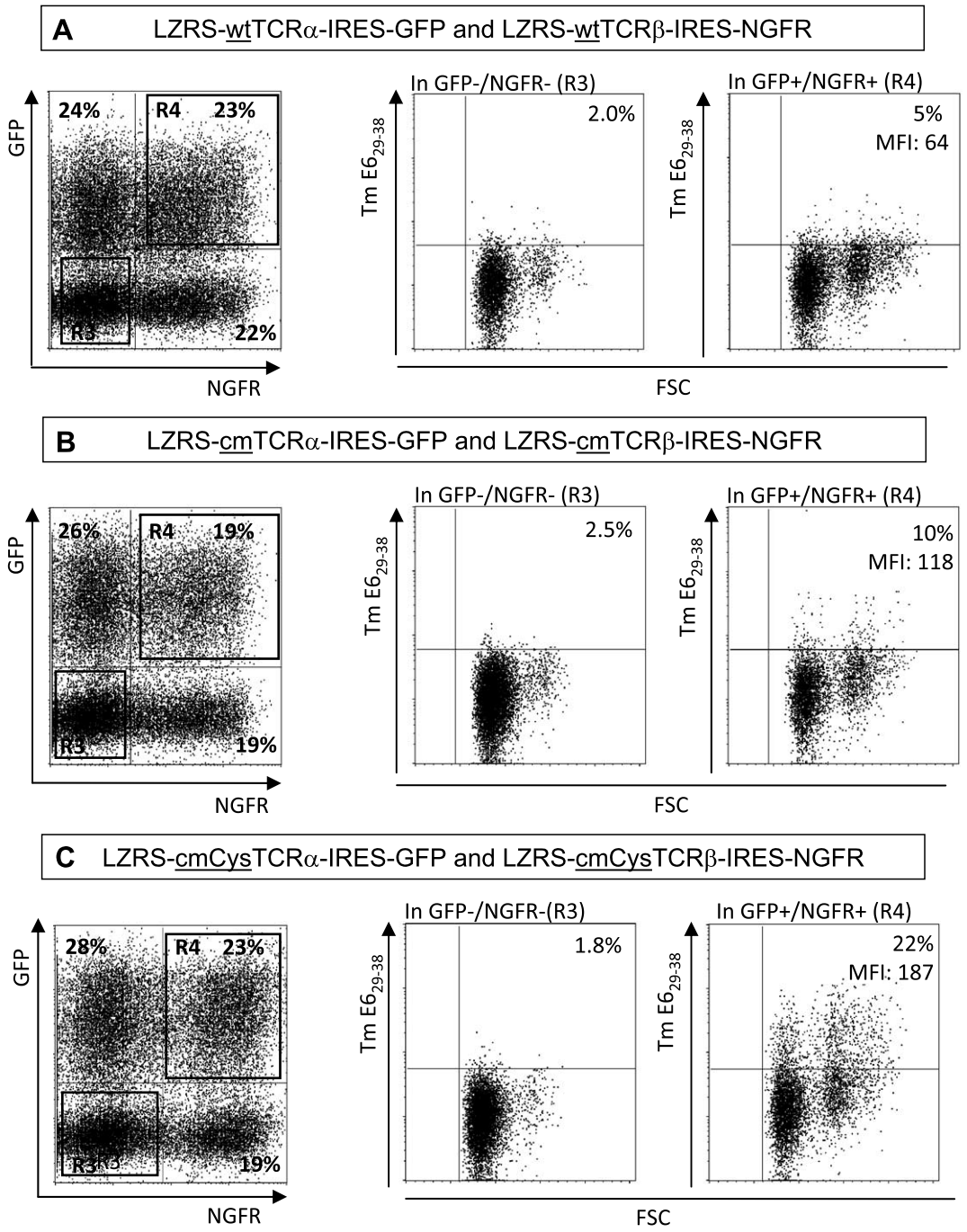


Fig. 3. Tetramer analysis of $CD8^+$ T cells after transduction with wt, cm or cmCyc 7E7-TCR encoding retroviruses. Healthy donor derived $CD8^+$ T cells were transduced with TCRα-I-GFP and TCRβ-I-NGFR encoding retroviruses. Tetramer analysis was performed on T cells with the wt (A), cm (B) and cmCys (C) 7E7-TCR in gate R3 ($GFP^-/NGFR^-$) and gate R4 ($GFP^-/NGFR^+$). Data are representative for 4 different donors tested.

loaded with decreasing amounts of the $E6_{29–38}$ peptide in an intracellular interferon-γ staining. The results show that the cmCysTCR transgenic $CD8^+$ T cells recognize peptide with a half-maximum interferon-γ production between 10–100 nM (Fig. 4(B)) which is indicative of T cells with intermediate avidity [43].

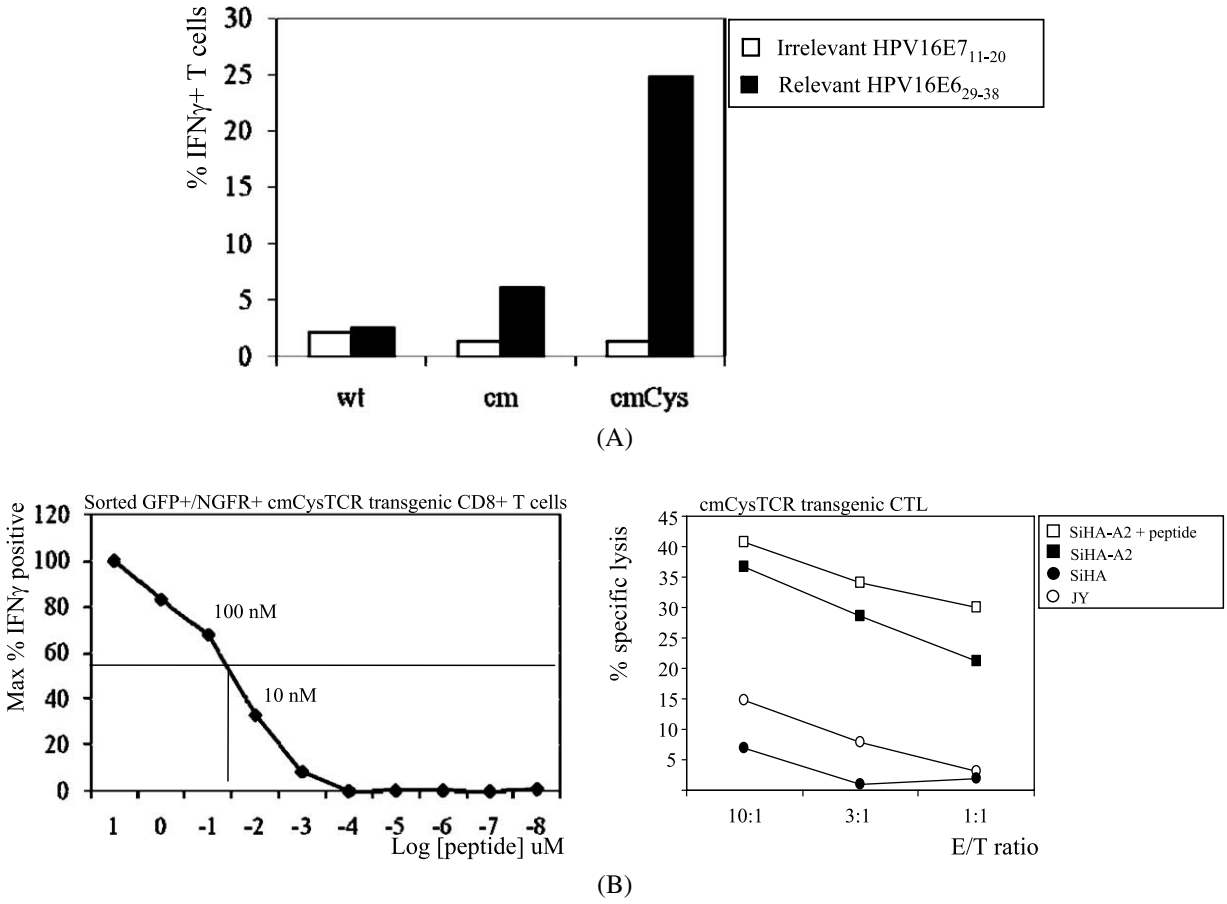


Fig. 4. Analysis of functional activity of $CD8^+$ TCR transgenic T cells against peptide loaded target cells as well as tumor cells. (A) Bulk populations (∼95% $GFP^+/NGFR^+$) wt, cm and cmCys TCR transgenic $CD8^+$ T cells in an intracellular interferon-γ staining against JY cells loaded with irrelevant or relevant peptide. (B) Functional avidity as determined in an intracellular interferon-γ assay. The HLA-A2.1 + cell line JY loaded with decreasing amounts HPV16E6 peptide were used as target cell. The maximal interferon-γ production is set at 100%. The results indicate that 7E7-TCR transgenic $CD8^+$ T cells recognize the $E6_{29–38}$ peptide with a half maximal interferon-γ production of ∼50 nM. Lytic activity of bulk cmCysTCR transgenic $CD8^+$ T cells directed against the HLA-A2.1 negative cervical cancer cell line SiHA (closed circles), SiHA transfected with HLA-A2.1 (SiHA-A2, closed squares) and SiHA-A2 loaded with HPV16E6 peptide (open squares). Percentage specific lysis is shown for the indicated effector to target (E/T) ratio.

To confirm recognition of endogenously processed antigen by TCR transgenic T cells a chromium release assay was performed against cervical cancer cells using cmCysTCR transgenic T cells. As expected TCR transgenic T cells were capable of killing the HLA-$A2.1^+/HPV16^+$ CxCa cell line SiHA-A2, which constitutively expresses the antigen HPV16E6 (Fig. 4(B)). The HLA-A2 negative variant of this cell line (SiHA, Fig. 4(B)) nor the negative control cell lines JY and Caski (data not shown) were lysed by these TCR transgenic T cells. These results are in agreement with the results obtained by Thomas et al. [45] with the original T cell clone.

3.5. *Promiscuous behavior of the HPV16E6 specific TCR beta chain*

Cross-pairing of endogenous TCRα and TCRβ chains with exogenously introduced TCRβ and TCRα chains might lead to unexpected and possibly harmful T cell specificities. Antibodies directed against TCRVβ1 are available and facilitate the analysis of expression of the transgenic TCRβ chain, whereas antibodies against TCRVα29 are unavailable. Co-expression of the marker genes GFP and NGFR with the TCRα and TCRβ chain respectively facilitates detailed analysis of TCR expression patterns. Low per-

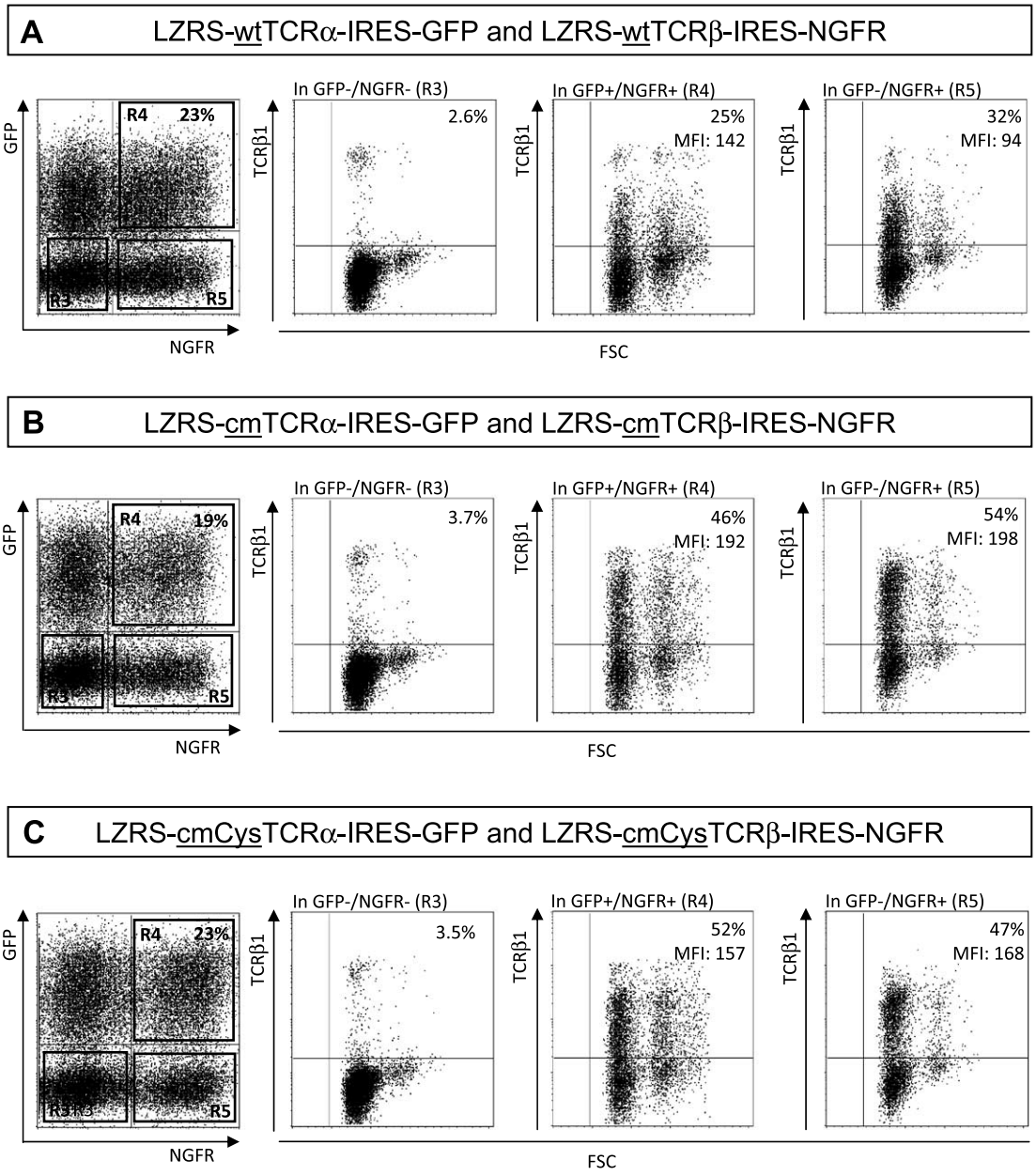


Fig. 5. Phenotypic analysis of CD8$^+$ T cells after transduction with either wt, cm or cmCys 7E7-TCR encoding retroviruses. Healthy donor derived CD8$^+$ T cells were transduced with TCRα-I-GFP and TCRβ-I-NGFR encoding retroviruses. TCRVβ1 analysis was performed on T cells with the wt (A), cm (B) or cmCys (C) 7E7-TCR in gate R3 (GFP$^-$/NGFR$^-$), gate R4 (GFP$^+$/NGFR$^+$) or gate R5 (GFP$^-$/NGFR$^+$). Data are representative for 4 different donors tested.

centages of TCRVβ1 positive cells were detected in the GFP/NGFR double negative quadrant (Fig. 5; gated in R3), representing the percentage of endogenous repertoire of TCRVβ1 positive T cells naturally present in this particular donor. Approximately 25% of the GFP$^+$/NGFR$^+$ double positive T cells (gated in R4) transduced with the wtTCR were positive for TCRVβ1 (Fig. 5A). In the GFP$^+$/NGFR$^+$ quadrant (gated in R4) 46% of the cmTCR transgenic T cells were positive for TCRVβ1 staining (Fig. 5B) while 52% of the cmCysTCR transgenic T cells showed TCRVβ1 staining (Fig. 5C). Additionally, TCRVβ1 expression levels of cmTCR transgenic cells (MFI 192) were higher compared to wtTCR transgenic cells (MFI 142) and

cmCysTCR transgenic cells (MFI 157).

Importantly, approximately 54% of the cells transduced with the cmTCRβ ORF were positive for TCRVβ1 staining in the GFP$^-$/NGFR$^+$ population (Fig. 5B, gated in R5). This percentage is lower in the cmCysTCR transgenic cells in the GFP$^-$/NGFR$^+$ gate where 47% of the T cells show TCRVβ1 staining (Fig. 5C). Biochemical evidence provided by others has shown that TCRβ chains are incapable of cell surface expression without a paired TCRα chain [7]. Our data clearly indicate that the TCRVβ1 isolated from CTL clone 7E7 is promiscuous in binding to different TCRVα family members.

In general the percentage of specific tetramer binding is lower when compared to binding of specific TCRbeta antibodies, as documented previously by others [6,21]. As also seen in JurMA cells, the percentage of TCRVβ1 staining is much higher compared to tetramer staining. In summary, $\sim$20–25% of the wt and cmTCR transgenic T cells positive for TCRVβ1 were also capable of binding relevant tetramers, whereas this is $\sim$40% in the case of cmCysTCR transgenic T cells. This is a clear indication that cross-pairing between endogenous TCR chains and the introduced TCR chains can in part be circumvented by the introduction of an extra di-sulfide bond between the TCRα and TCRβ chains.

4. Discussion

Thus far only few T cell clones directed against HPV16E6 have been described [13,25]. By isolating the TCR from CTL clone 7E7 and its introduction into recipient CD8$^+$ T cells we were able to generate HPV16E6 specific T cells capable of recognizing peptide loaded target cells as well as HPV expressing tumor targets. Next we sought to enhance TCR expression levels by codon-modification [22,40] and to facilitate preferential pairing of the exogenously introduced TCRα and TCRβ chain by cysteinization [8,27, 46] of the codon modified TCRα and TCRβ chains. In the current study we compared TCR expression levels and functional activity of T cells transduced with wild-type, codon-modified or codon-modified/cysteinized TCRs. The combined use of TCRVβ antibodies and tetramers allows for detailed analysis of the introduced TCR chains as reported here. Transduction of wild-type TCRs into CD8$^+$ T cells resulted in low percentages of tetramer positive cells, as has also been documented by us for a TCR with a different specificity [41]. To our surprise and in contrast to our previously published data [40], only low percentages of codon-modified TCR transgenic T cells were capable of binding tetramers presenting the relevant epitope, despite the notion that a high percentage of T cells expressed the introduced TCRβ chain at high levels. Therefore we sought to improve TCR expression by codon-modification in combination with cysteinization. Others have shown previously that cysteinization allows for preferential pairing of introduced TCRs. Indeed the percentage of tetramer positive cells increased from 10% to 22% in the GFP$^+$/NGFR$^+$ quadrant. A substantial increase in mean fluorescence intensity was also observed (Fig. 3). About half (52%) of the cmCysTCR transgenic cells in the GFP$^+$/NGFR$^+$ quadrant were positive for TCRVβ1 staining (Fig. 5), and about a quarter (22%) of the cells in the same quadrant were capable of binding to relevant tetramers (Fig. 3). This probably indicates that tetramer staining is abrogated due to the fact that in a fair portion of these TCR transgenic T cells the exogenously introduced TCRβ much rather pairs with the endogenously present TCRα. From these data it is clear that the formation of mixed TCR dimers is not prevented completely by this combined approach. Besides TCRVβ and tetramer staining we compared the three different platforms for the production of interferon-γ upon stimulation with target cells loaded with synthetic peptide. In agreement with the findings of Cohen et al. [8], we found increased interferon production in transgenic T cells carrying the cysteinized TCRs. Codon-modified/cysteinized TCRs showed improved functional activity when compared to wild-type and codon-modified TCR transgenic T cells. To confirm recognition of endogenously presented antigens we performed a chromium release assay. Whereas the original T cell clone 7E7 was only able to kill CxCa cell lines in 20 hour cytotoxicity assays, 7E7-TCR transgenic CD8$^+$ T cells were capable of killing tumor cells in a standard 4 hour chromium release assay. A possible explanation for these findings may be that the original T cell clone was near exhaustion [10,55], whereas the TCR transgenic T cells were not. Therefore, TCR transgenic CD8$^+$ T cells might still have retained their intrinsic cytolytic capacity.

To determine functional TCR avidity of the 7E7 derived TCR, we stimulated 7E7-TCR transgenic CD8$^+$ T cells with decreasing amounts of relevant peptide. TCR transgenic CD8$^+$ T cells showed a half maximal interferon-γ production at approximately 10–100 nM peptide indicating an intermediate functional avid-

ity [43]. Evans et al. showed that the 7E7 T cell clone had a 50% maximal lysis of approximately 1 nM [13]. Cross-pairing of a substantial number of exogenously introduced TCRbeta chains with endogenous TCRalpha chains, leading to a reduction of the number of HPV16E6 specific TCR on the cell surface may explain these differences in sensitivity.

Alternative approaches have been published recently to prevent the formation of mixed TCR dimers. These approaches include the murinization of TCR constant domains [9], and inter-chain conversion of the constant domains, referred to as the hole-into-knob approach [51]. Here we have engineered a cassette model in which TCR alpha and beta variable regions can easily be incorporated. To date these contain codon modified constant regions as such or combined with the addition of extra cysteine residues. Likewise one could prepare additional cassettes containing murine constant domains, hole-into-knob configurations or the addition of an extracellular tag to the variable region of the TCRalpha chain. Incorporation of marker genes would allow for detailed analysis of expression of individual TCR chains derived from one and the same T cell clone.

As an alternative approach one may choose recipient CTL clones with known specificity [18] or effector cells devoid of endogenous TCRα and TCRβ chains like $\gamma\delta$ T cells [47] or NK cells [34]. Phage display technology could also be applied, not only to improve TCR affinity [59], but also to improve interchain affinity. Careful analysis of different engineering approaches should be performed to determine which is best applicable.

Preclinical studies in mice have shown value with respect to determining the *in vivo* capacity of TCR transgenic T cells to eliminate model tumors and to compare the effectiveness of different TCRs with the same specificity [22]. Along these lines the HPV16E6 specific TCR presented here should be tested along side the previously described HVP16E7 specific TCRs A9 and D4 [29,41].

In conclusion, promiscuous behavior of TCRβ chains can in part be forced back into specific action in TCR transgenic T cells by codon modification in combination with the inclusion of an extra cysteine in the TCR chains.

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