

Characterization and modulation of anti- $\alpha\beta$ TCR antibodies and their respective binding sites at the β TCR chain to enrich engineered T cells

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T cell engineering strategies offer cures to patients and have entered clinical practice with chimeric antibody-based receptors; $\alpha\beta$ T cell receptor ($\alpha\beta$ TCR)-based strategies are, however, lagging behind. To allow a more rapid and successful translation to successful concepts also using $\alpha\beta$ TCRs for engineering, incorporating a method for the purification of genetically modified T cells, as well as engineered T cell deletion after transfer into patients, could be beneficial. This would allow increased efficacy, reduced potential side effects, and improved safety of newly to-be-tested lead structures. By characterizing the antigen-binding interface of a good manufacturing process (GMP)-grade anti- $\alpha\beta$ TCR antibody, usually used for depletion of $\alpha\beta$ T cells from stem cell transplantation products, we developed a strategy that allows for the purification of untouched $\alpha\beta$ TCR-engineered immune cells by changing 2 amino acids only in the TCR β chain constant domain of introduced TCR chains. Alternatively, we engineered an antibody that targets an extended mutated interface of 9 amino acids in the TCR β chain constant domain and provides the opportunity to further develop depletion strategies of engineered immune cells.

INTRODUCTION

The US Food and Drug Administration (FDA) approval of the first engineered T cells expressing chimeric antigen receptors (CARs) has paved the way for new cellular interventions in the clinic.^{1,2} A next wave of cell therapy will come with T cell receptor (TCR)-engineered T cells specific for targets on both solid and hematological malignancies.³ Most clinical trials using $\alpha\beta$ TCR-engineered T cells are directed against cancer/testis antigens, such as NY-ESO-1.⁴ Although the clinical response rates are very encouraging, only a small proportion of the patients benefit from these novel treatments.^{5,6} Disappointing response rates can be partially attributed to the presence of non-engineered and poorly engineered T cells in the administered cell product.⁷ These non-engineered and poorly engineered T cells can hamper the therapeutic efficiency of engineered immune effector cells because of, e.g., insufficient expression of the introduced recep-

tor, mispairing of introduced $\alpha\beta$ TCR with endogenous $\alpha\beta$ TCR,⁸ or by competition for endogenous homeostatic cytokines.^{7,9} Furthermore, in an allogenic setting, the presence of T cells still expressing the endogenous $\alpha\beta$ TCR can lead to severe graft-versus-host disease. Purification of engineered T cells before infusion can overcome these hurdles, ultimately resulting in enhanced *in vivo* activity. Current methods for purification of engineered T cells often depend on the expression of artificial molecules such as truncated CD34¹⁰ or truncated NGFR,¹¹ in addition to the tumor-specific receptor. However, larger transgene cassettes used to introduce multiple proteins are relatively difficult to express, and additional transgenes can add immunogenic properties to the engineered cell product.¹² Besides purification of engineered T cells to increase effectivity, elimination of engineered T cells after adoptive transfer might be needed in case of cytokine release syndrome¹³ or off-target toxicities, e.g., due to peptide mimicry,^{5,14} expression of the antigen at low levels in healthy tissues,¹⁴ or mispairing of introduced with endogenous $\alpha\beta$ TCR chains resulting in unwanted specificities.⁸ A currently explored solution for the elimination of transferred cells is the co-expression of herpes simplex virus thymidine kinase (HSV-TK) along with the transgene of interest,¹⁵ mainly limited by the immunogenicity and relatively large size of the HSV-TK gene.¹⁵ An alternative elegant solution is to introduce a myc-tag into the $\alpha\beta$ TCR sequence itself, followed by *in vivo* depletion through myc-specific antibodies.¹⁶ However, introducing artificial genes into the $\alpha\beta$ TCR might alter downstream signaling by modifying, e.g., its glycosylation.¹⁷ Selection of engineered T cells and subsequent *in vivo* elimination achieved with a single marker, which has previously been described for CD20,¹⁸ would be favorable, due to the relatively small transgene cassette and therefore

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better expression. Even better would be a method where the introduced tumor-specific TCR could also be used for both purification and *in vivo* depletion, and thereby would combine all three properties in one gene: tumor specificity, a selection opportunity of cells expressing the transgene at high levels, as well as an *in vivo* depletion option, which allows for the elimination of the engineered immune cells in case of toxicities caused by the introduced receptor. Within this context we have explored a strategy based on the recent development of purified T cells engineered to express a defined $\gamma\delta$ T cell receptor (TEGs).^{19–29} In this strategy we took advantage of the observation that an anti-human $\alpha\beta$ TCR antibody used for the purification of TEGs does not cross-react with $\gamma\delta$ TCR chains, and it can thereby differentiate between engineered and non-engineered cells. This anti-human $\alpha\beta$ TCR antibody is routinely used to deplete $\alpha\beta$ TCR T cells from apheresis products using CliniMACS depletion before allogeneic stem cell transplantation.^{3,28} In this study, we describe the translation of the TEG purification procedure into a purification procedure for $\alpha\beta$ TCR-engineered T cells. We also provide the rationale for the additional development of elimination strategies of engineered immune cells by further modulating the binding site to be selectively targeted by a second independent antibody.

RESULTS

Anti-human $\alpha\beta$ TCR binds an epitope on the TCR β chain of human $\alpha\beta$ T cells

The good manufacturing practice (GMP)-grade anti-human $\alpha\beta$ TCR monoclonal antibody clone BW242/412 (from now on referred to as anti-human $\alpha\beta$ TCR) recognizes a common determinant of the human TCR α/β -CD3 complex, which has not yet been characterized. In order to allow for further epitope mapping of the interface between the anti-human $\alpha\beta$ TCR clone BW242/412 and a human $\alpha\beta$ TCR, we first tested the antibody's ability to bind to murine $\alpha\beta$ TCRs. Therefore, Jurma T cells, a TCR-deficient T cell line, were transduced with human $\alpha\beta$ TCRs directed against the cancer/testis antigen NY-ESO-1_{157–165}²⁹ or with a murine nonsense $\alpha\beta$ TCR composed of the TCR α chain of an MDM2-specific $\alpha\beta$ TCR,³⁰ and the TCR β chain of a p53-specific $\alpha\beta$ TCR.³¹ Specific binding of the anti-human $\alpha\beta$ TCR was only observed in the human (α HuHu/ β HuHu) but not the murine (α MuMu/ β MuMu) TCR-transduced Jurma cells (Figure 1A). To rule out that parts of the human variable domain of the $\alpha\beta$ TCR bind to the anti-human $\alpha\beta$ TCR antibody, the human NY-ESO-1 $\alpha\beta$ TCR variable domain was grafted on the murine constant domain to create a chimeric $\alpha\beta$ TCR (α HuMu/ β HuMu). Replacing only the human TCR α and TCR β constant domains by murine equivalents completely abrogated binding of anti-human $\alpha\beta$ TCR to levels resembling binding to a fully murine $\alpha\beta$ TCR (α MuMu/ β MuMu). This indicates that the human constant domain contains the binding epitope. Comparable transgenic expression of murine and human TCRs was confirmed by anti-MuTCR β and anti-V β 4, respectively (Figure 1A). Infusion of T cells expressing TCRs with complete murine constant domains into patients can generate immunogenic effects and lead to a decreased persistence of the engineered cells *in vivo*.³² To minimize these undesirable effects, we aimed to map the minimal amount of murine residues needed to disrupt bind-

ing of anti-human $\alpha\beta$ TCR by making use of previously described chimeric TCR α and β chains, with mutational blocks covering all amino acid (aa) differences between the constant regions of human and mouse $\alpha\beta$ TCRs.²⁹ We tested three NY-ESO-1 TCR α chain variants and four NY-ESO-1 TCR β chain variants, with each containing one murine domain, flanked by complete human aa sequences. Every TCR α chain was paired with the fully human TCR β chain (β HuHu) (Figure 1B), and every TCR β chain was paired with the fully human TCR α chain (α HuHu) (Figure 1C) and introduced into Jurma cells, after which binding of anti-human $\alpha\beta$ TCR was determined by flow cytometry. Antibody binding was significantly impaired in T cells expressing the $\alpha\beta$ TCR, which includes murine domain 3 (β HuM3), while none of the other chimeric $\alpha\beta$ TCRs substantially impaired anti-human $\alpha\beta$ TCR binding (Figures 1B and 1C). β HuM3 TCR expression was confirmed by staining for anti-V β 4 and was comparable to α HuHu/ β HuHu (Figure S1). These results indicate that domain 3 of the TCR β chain (β HuM3) dictates the binding of anti-human $\alpha\beta$ TCR.

Anti-human $\alpha\beta$ TCR binding can be abrogated by mutating two residues

Analysis of the sequence of domain 3 of the TCR β chain constant domain revealed 11 residues that are non-homologous between murine and human species (Figure S2). To determine which residues are essential for anti-human $\alpha\beta$ TCR binding, we constructed 11 variants of the TCR β chain, in which each one of the non-homologous aas was replaced by the murine counterpart. These 11 constructs were paired with the completely human α TCR chain (α HuHu), introduced in Jurma cells and tested for binding by the anti-human $\alpha\beta$ TCR antibody. Of the 11 generated mutants, the substitutions of “human” glutamic acid (E108) to the “murine lysine” (K), “human” threonine (T110) to the “murine” proline (P), and “human” aspartic acid (D112) to the “murine” glycine (G) showed a substantial abrogation of anti-human $\alpha\beta$ TCR binding (Figure 2A). However, none of these substitutions was sufficient to induce total abrogation, as shown by the TCR consisting of α HuHu/ β HuM3 (Figure 2A). Therefore, we constructed TCR β chains with a combination of the aforementioned mutations. The TCR β chains with a D112G mutation combined with E108K or T110P were both effective in abrogating binding of the anti-human $\alpha\beta$ TCR antibody (Figure 2B), which can be explained by a substantial decrease in bulkiness, and thus a decrease in size of these residues (Figure 2C; Table S1). For further engineered T cell experiments, the combination of T110P and D112G murinization was selected.

Enrichment of $\alpha\beta$ TCR-engineered T cells utilizing fragments of murine $\alpha\beta$ TCR chains

Murine $\alpha\beta$ TCRs, or residues derived from murine $\alpha\beta$ TCRs introduced into human $\alpha\beta$ TCRs and expressed in human T cells, have been reported to outcompete endogenous human TCR chains.^{29,33,34} These murine and murinized $\alpha\beta$ TCRs preferentially pair with each other, thereby decreasing the occurrence of mispairing with endogenous human $\alpha\beta$ TCRs. Therefore, we utilized single murine aas to enhance the expression of introduced TCRs.²⁹ These “minimally

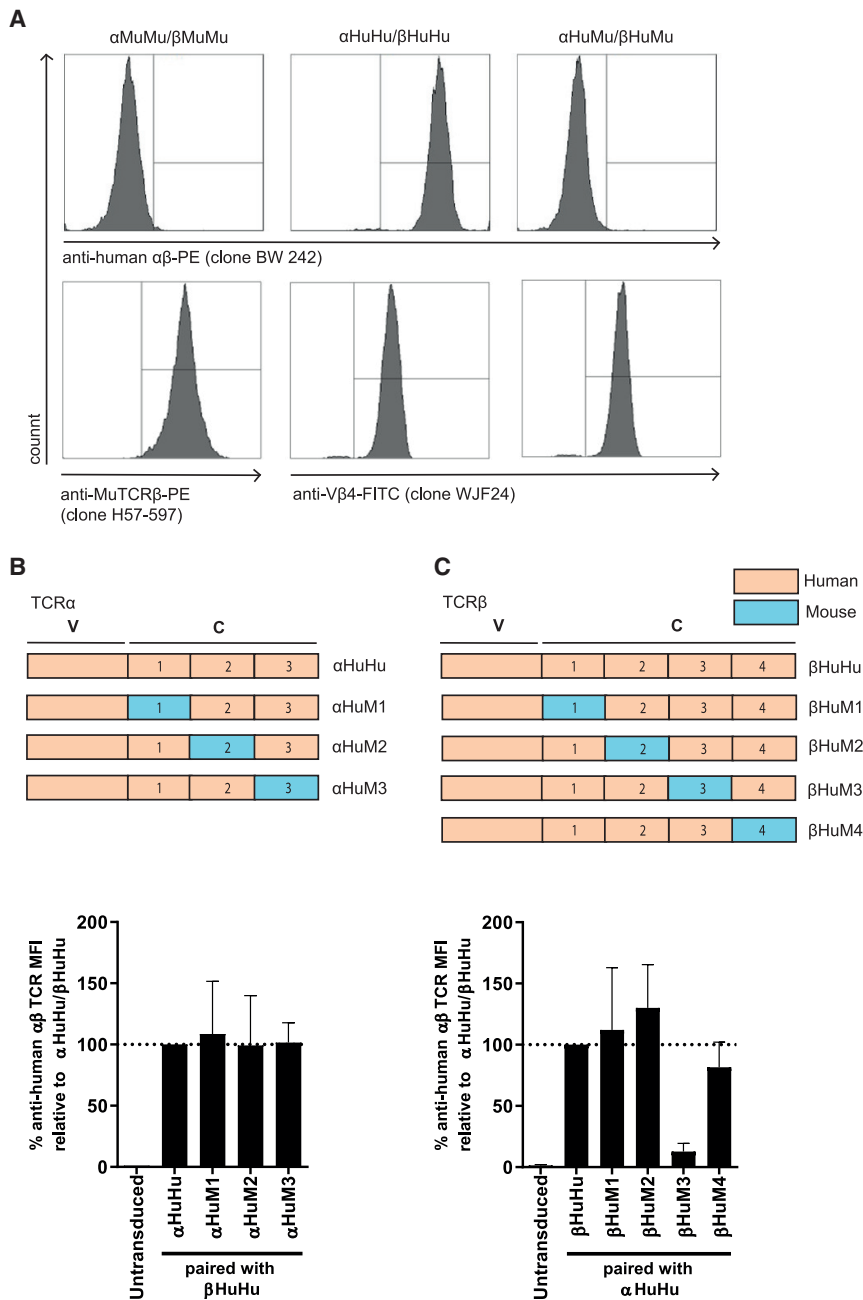


Figure 1. Partial murinization of the TCRβ chain constant domain abrogates binding of the anti-human αβTCR antibody clone BW242/412

(A) Jurma cells were transduced with fully murine (αMuMu/βMuMu), fully human NY-ESO-1-specific (αHuHu/βHuHu) or chimeric αβTCR, in which the α and β constant domains were murine, and the variable domains were human NY-ESO-1 specific. Binding of anti-human αβTCR, anti-MuTCRβ, and Vβ4 was assessed by flow cytometry. (B and C) Schematic representation of the constructed variable (V) and constant (C) domains of αβTCRs that cover all aa differences in the (B) TCRα chain and (C) TCRβ chain (upper panels). The constant domain of the TCRα and β chain have been divided in respectively three or four different regions, based on the comparison of human and murine regions revealing clustered differences flanked by homologous regions as described.²⁹ Jurma cells were transduced with the different murinized αβTCRs, after which anti-human αβTCR antibody binding was assessed by flow cytometry. The bar graphs (B and C, lower panels) show the anti-human αβTCR MFI relative to the fully human TCR. Untransduced Jurma cells served as a negative control. The data correspond to two independent experiments, and a representative figure is shown (A) or as the average with standard deviation (B and C).

depletion using anti-human αβTCR resulted in an increased cell fraction not able to bind anti-human αβTCR after an expansion of 2 weeks, in order to assess stability of the phenotype (Figure 3A). However, we also observed outgrowth of a large fraction of Vβ4- and αβTCR-negative cells, mainly consisting of natural killer (NK) and γδ T cells, as reported previously.²⁰ To further increase purity of engineered immune cells, T cells were selected by CD4/CD8 MACS from peripheral blood mononuclear cells (PBMCs) prior to the transduction. This indeed prevented the outgrowth of NK and γδ T cells after αβTCR depletion and expansion (Figure 3B). Next, we quantified the fraction of NY-ESO-1₁₅₇₋₁₆₅ HLA*02:01 pentamer⁺ cells before and after depletion, showing a significant increase in pentamer⁺ cells after depletion (Figure 3C),

further proving successful enrichment of engineered immune cells when using T110P+D112G-modified αβTCRs.

Enrichment strategy within the context of alternative αβTCR stabilization procedures

Multiple alternative strategies to prevent αβTCR chain mispairing and thereby increase the expression of the introduced tumor specific αβTCR have been reported. For example, adding an additional cysteine residue to introduce a disulfide bridge between the α and β chains has been shown to increase expression and decrease mispairing.⁸ Also,

murinized" constant domain variants (from now on referred to as mm) contain murine aas that are both critical and sufficient to improve pairing between the two chains.²⁹ Next, we introduced the above-identified murine residues (T110P+D112G) in the TCRβ chain constant domain in order to test whether this was sufficient to disrupt the binding of anti-human αβTCR in human primary T cells. To test this concept, healthy donor T cells were transduced with mm NY-ESO-1-specific αβTCRs as a negative control, or mm NY-ESO-1-specific αβTCRs, including the two identified mutations T110P+D112G. Magnetic-activated cell sorting (MACS)

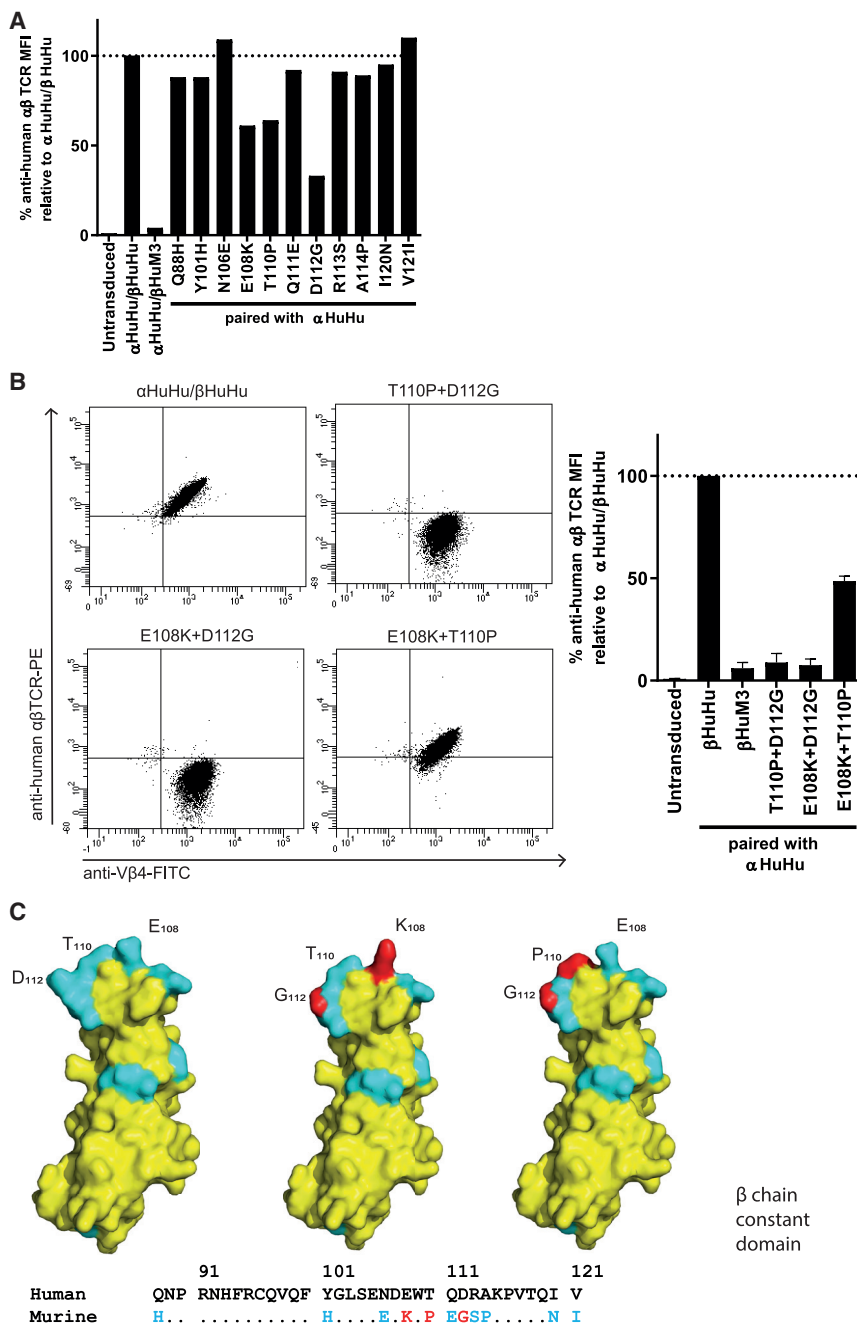


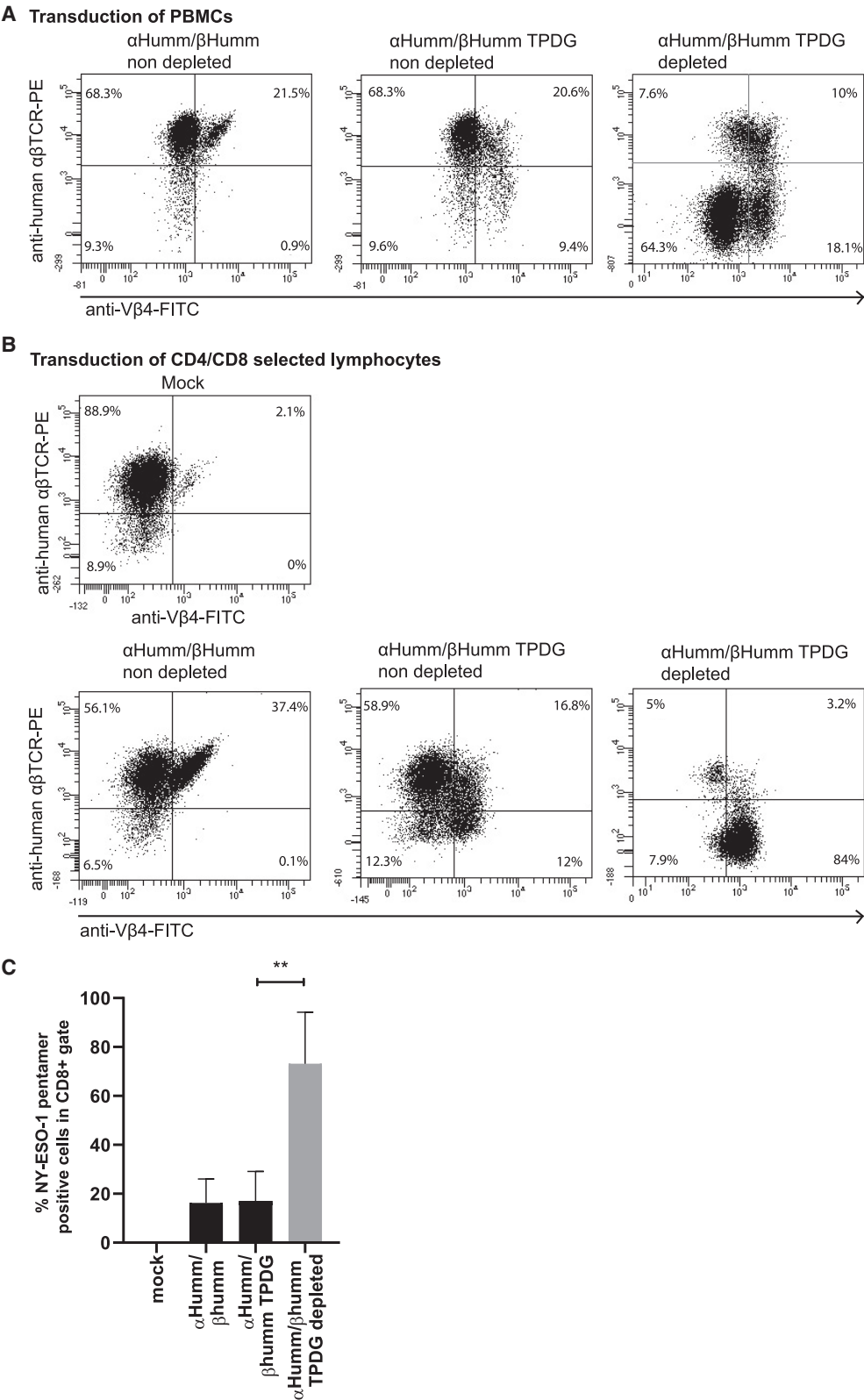
Figure 2. A combination of 2 specific murine aa in the TCRβ chain constant domain is sufficient to abrogate binding of the anti-human αβTCR antibody clone BW242/412

(A) Jurma cells were transduced with αβTCRs containing single murine aa substitutions in the third domain of the β chain, after which binding of the anti-human αβTCR antibody was assessed using flow cytometry. Untransduced Jurma cells served as a negative control, while fully human αβTCR-transduced Jurma cells served as a positive control. (B) Jurma cells were transduced with αβTCRs containing combinations of murine aas in the third domain of the β chain, after which binding of anti-human αβTCR antibody was assessed using flow cytometry. (C) Visualization of the 11 non-homologous aa between human and mouse β chain third domain in cyan using SWISS-MODEL⁵² on the modeled template of the β chain of the human JKF6 T cell receptor (PDB: 4ZDH). Effective single murine aa substitutions are displayed in red. The data correspond to one experiment (A) or two independent experiments shown with a representative image (B) and average with standard deviation (B, bar graph).

domain. These TCRs were compared to the previously used mm TCR strategy (schematic representation, Figure 4A). To later make use of the αβTCR depletion method, we introduced the mutations T110P+D112G in the β chains. We then assessed the expression of the different TCRs in primary T cells by measuring the percentage of Vβ4⁺ and NY-ESO-1₁₅₇₋₁₆₅ HLA*02:01 pentamer⁺ cells within the CD8⁺ population (Figure 4B). All three conditions resulted in a NY-ESO-1₁₅₇₋₁₆₅ HLA*02:01 pentamer⁺ CD8⁺ fraction comparable in size to the Vβ4⁺ CD8⁺ fraction, indicating that in all cases the introduced TCR chains are preferentially paired (Figures 4B and S3). A modest, but significant, increase in expression of the introduced TCR was observed when using a combination of a cysteine bridge and the γδ-transmembrane domain when compared to the mm variant (Figure 4C). The increase in expression of Vβ4 was associated with an increase of the single Vβ4⁺ cells to Vβ4/endogenous αβTCR double-positive cells (Figure 4D),

human γδTCRs introduced in human T cells do not pair with endogenous αβTCRs.³⁵ Therefore, it was attractive to use γδTCR transmembrane domains for engineering αβT cells in a similar way. We tested whether our enrichment strategy could also be combined with these alternative pairing solutions. First, we constructed an NY-ESO-1-specific TCR with an additional disulfide bridge by the mutation of one specific residue in each chain, i.e., T48C in TCRCα and S57C in TCRCβ.⁸ Second, we constructed an NY-ESO-1-specific TCR with the same additional disulfide bridge, and with a human γδTCR transmembrane

indicating that combination of the cysteine bridge and γδ-transmembrane domain was most potent in the downregulation of the endogenous αβTCR. Next, the three different conditions were αβTCR depleted in the same way as before, and the percentage of Vβ4⁺ cells (Figure 5A) and NY-ESO-1₁₅₇₋₁₆₅ HLA*02:01 pentamer⁺ cells within the CD8⁺ population (Figure 5B) was measured by flow cytometry, showing successful enrichment for transduced cells in all conditions. After depletion, however, we did not see significant differences in % Vβ4 or pentamer⁺ cells between the three tested constructs. In



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summary, all three described methods were suitable for creating preferential pairing and subsequent purification by our $\alpha\beta$ TCR depletion method with a slight advantage of combination of the cysteine bridge and $\gamma\delta$ -transmembrane domain when assessed by TCR expression.

Augmented *in vitro* tumor cell recognition by purified engineered T cells

To assess whether purified NY-ESO-1₁₅₇₋₁₆₅ $\alpha\beta$ TCR-engineered T cells were superior in target cell recognition compared to non-purified cells, we pulsed T2 cells with multiple concentrations of NY-ESO-1₁₅₇₋₁₆₅ peptide. Purified engineered T cells showed a stronger response to the peptide-loaded T2 cells than to the non-purified cells. Furthermore, we observed that interferon (IFN) γ release was associated with positivity for the different introduced TCRs (Figure 5C). Purification also resulted in the improved recognition of endogenously processed and presented peptide in the NY-ESO-1-positive tumor cell lines Saos-2 and U226 when assessed by IFN γ release (Figure 5D). As we observed varying and only minor differences between the three strategies (Figures 4 and 5), and wanted to introduce as few changes as possible in engineered TCRs, the mm approach was used in the next set of experiments to prevent mispairing and increase expression of the introduced TCR as reported.²⁹ The placement of these 9 murine aa, not on the surface but rather buried within the TCR, makes it unlikely that they would cause immunogenicity of the mm TCR as suggested by Sommermeyer et al.²⁹

Developing an antibody recognizing the introduced mutated region

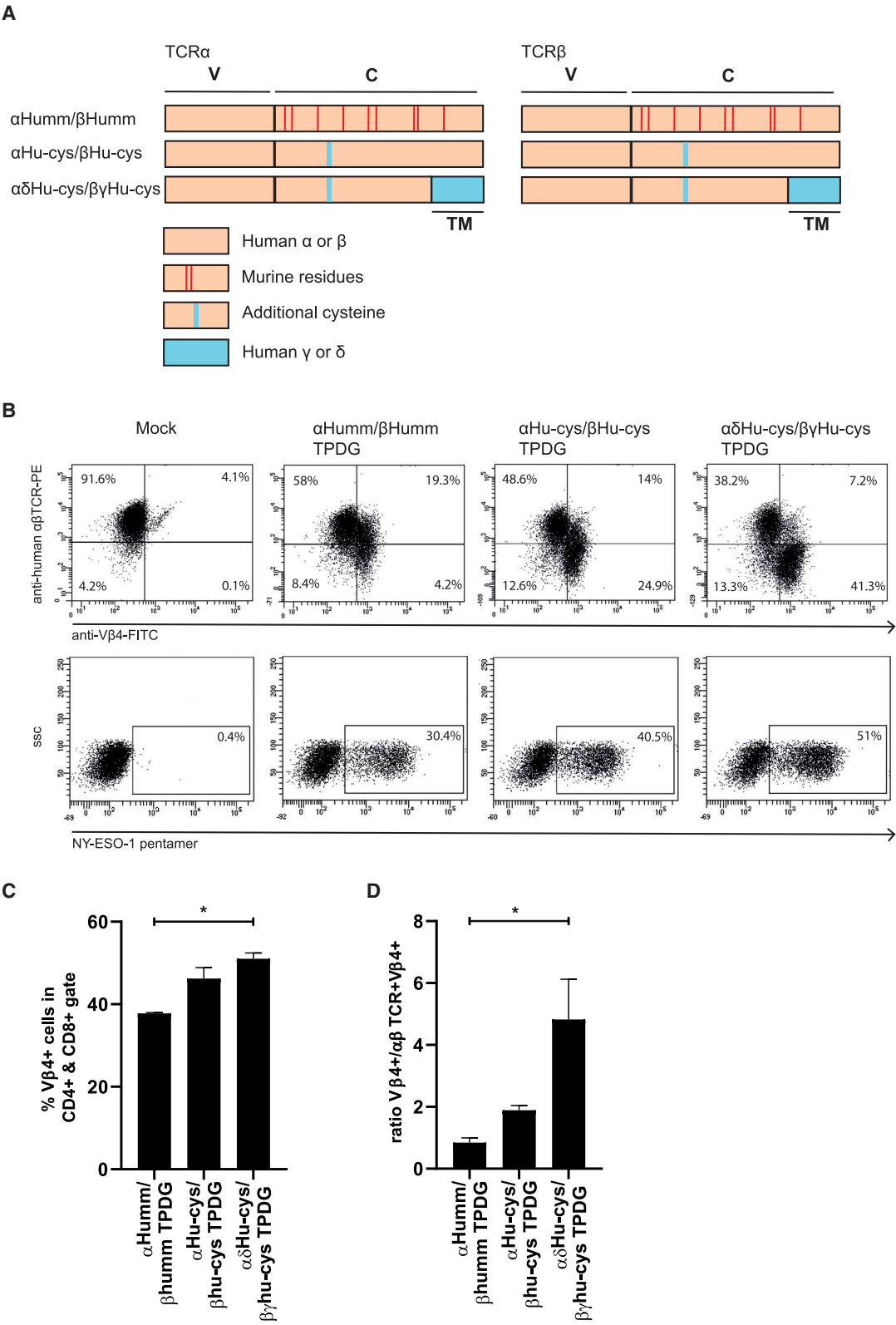
The infusion of engineered T cells can potentially be toxic, due to the occurrence of cytokine release syndrome¹³ or the off-target toxicity of the receptor used.¹⁴ To be able to deplete infused engineered T cells *in vivo* when deemed necessary, we first sought to raise an antibody specific for the T110P+D112G murinized variant of the $\alpha\beta$ TCR by immunizing three Wistar rats with a human-mouse chimeric peptide. Despite that antibodies were formed against the chimeric peptide (Figure S4A), no antibody binding to surface-expressed $\alpha\beta$ TCRs could be detected (Figure S4B). Therefore, we assessed whether the commercially available anti-murine TCR β chain antibody clone H57-597 (from now on referred to as anti-MuTCR β) was able to bind the murinized $\alpha\beta$ TCRs on Jurkat-76 cells generated so far. Jurkat-76 cells expressing the T110P+D112G murinized variant of the $\alpha\beta$ TCR (indicated by β Humm 2/11; i.e., 2 out of the 11 non-homologous aa in the third domain are murinized) were not bound by anti-MuTCR β ; however, Jurkat-76 cells expressing the β HummM3

murinized variant of the $\alpha\beta$ TCR (indicated by β Humm 11/11; i.e., all 11 non-homologous aa in the third domain are murinized) were bound by anti-MuTCR β (Figure 6A). To limit the amount of murine aas introduced, we also constructed a variant in which 9 out of 11 non-homologous aa in the third domain are murinized (Figure S4C). Both 11/11 and 9/11 non-homologous murine aa in the β chain of domain 3 were sufficient to reestablish binding of anti-MuTCR β , but not to the same extent as the HuMu $\alpha\beta$ TCR (Figure 6A). Surprisingly, 9/11 caused a higher mean fluorescence intensity (MFI) than did 11/11. Structural analyses suggested that this differential binding could be a consequence of the fact that 9/11 contains one less negatively charged residue and therefore results in a more focused electrostatic potential to attract the lysine on CDR1 of anti-MuTCR β (Figure 6B). To confirm that the anti-MuTCR β antibody binds to the V β 4⁺ cells, co-staining was performed with both antibodies on transduced primary T cells. The MFI of anti-MuTCR β -PE was plotted for the V β 4⁺ gated cells, which showed that the anti-MuTCR β antibody bound best to the 9/11 or complete murine constant domain (Figure 6C). As expected, there was no binding to the 2/11 variant but surprisingly also not to the 11/11 variant. This might suggest some interference when both antibodies are used in a co-staining, mainly affecting the suboptimal anti-muTCR β binding to the 11/11 variant.

Since the clone of anti-MuTCR β antibody is of Armenian hamster origin and presumably induces severe side effects once administered to humans, comparable to anti-thymocyte globulin,³⁶ we aimed to generate a humanized variant of anti-MuTCR β . We generated chimeric variants of anti-MuTCR β (H57-597, PDB: 1NFD) by exchanging the hamster immunoglobulin (Ig)G2 constant domain for the human IgG1 constant domain (referred to as chimeric anti-MuTCR β). We tested binding of this newly constructed antibody in engineered Jurkat-76 cells, which resulted in specific antibody binding to the 9/11 murinized TCR β chain expressed on Jurkat-76 cells (Figure S5). To determine the capacity of the chimeric anti-MuTCR β antibody to bind to primary T cells expressing the murinized $\alpha\beta$ TCRs, we conjugated this antibody and an isotype control to Alexa Fluor 488 (AF488) and determined binding by flow cytometry. The chimeric anti-MuTCR β antibody was able to bind both 9/11 and 11/11 murinized TCRs and, as shown in Figure 6A, the binding to 9/11 was stronger than to 11/11 (Figure 6D). To assess whether the chimeric variant of anti-MuTCR β was able to selectively deplete engineered T cells *in vitro*, the antibody was coupled to monomethyl auristatin E (MMAE), a cell cycle inhibitor, using the protease cleavable linker VC-PAB³⁷ to create

Figure 3. Primary $\alpha\beta$ T cells engineered with murinized $\alpha\beta$ TCRs can be successfully selected by using anti-human $\alpha\beta$ TCR antibody clone BW242/412 to deplete non-engineered and poorly engineered immune cells

(A) PBMCs were transduced with minimally murinized $\alpha\beta$ TCRs with (middle panel) and without (left panel) the "TPDG" mutations. Primary $\alpha\beta$ T cells with the TPDG mutations were MACS depleted and expanded (right panel). Endogenous $\alpha\beta$ TCR expression and expression of the introduced $\alpha\beta$ TCR without the TPDG mutations were determined by flow cytometry using an anti-human $\alpha\beta$ TCR antibody; expression of the introduced β TCR chain was assessed with an anti-V β 4 antibody. (B) Prior to transduction with minimally murinized $\alpha\beta$ TCRs, T cells were selected from PBMCs using CD4/CD8 MACS selection (CD4/CD8⁺). (C) Expression of correctly paired $\alpha\beta$ TCR chains was assessed before and after depletion and expansion by NY-ESO-1 pentamers (CD8⁺) for both transduction strategies combined. The data correspond to three independent experiments and are shown as a representative figure (A and B) or as average with standard deviation (C). Statistical significance (*p \leq 0.05, **p \leq 0.01) was calculated using a paired t test.



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an antibody-drug conjugate (ADC). Jurkat-76 cells transduced with different murinized TCRs were incubated with multiple concentrations of the ADC. The highest concentration of chimeric H57-MC-VC-PAB-MMAE led to a decrease of V β 4 positivity in the 9/11 condition only (Figure 6E). This specific decrease indicated that the ADC is able to selectively deplete 9/11, and not 11/11, $\alpha\beta$ TCR-engineered Jurkat-76 *in vitro*, most likely due to the weaker binding of the engineered antibody to the 11/11 $\alpha\beta$ TCR (Figure 6D). However, depletion was far from complete, indicating that although this binding site is interesting, it is far from being developed for a kill strategy.

DISCUSSION

The main finding of our study is that replacing only 2 aa within the constant domain of the TCR β chain allows for the purification of $\alpha\beta$ TCR-engineered T cells with GMP-ready tools,³⁸ without the need for additional complex genetic engineering. The very same region on the TCR β chain can also serve as a targeting interface for antibodies, which can be used to develop strategies to eliminate engineered immune cells. These new insights provide the molecular basis for developing select-kill strategies for increasing purity and augmenting the safety of $\alpha\beta$ TCR engineered T cells, with only minor engineering steps.

A sufficient downregulation of the endogenous $\alpha\beta$ TCR chains by the introduced $\alpha\beta$ TCR chains is essential for this method to work. Therefore, strategies interfering with endogenous $\alpha\beta$ TCRs or utilizing knockout of the α or β locus to enhance expression of introduced $\alpha\beta$ TCRs³⁹ will benefit from this strategy. However, engineering T cells via zinc finger nucleases (ZFNs), CRISPR, or transcription activator-like effector nucleases (TALENs)⁴⁰ requires additional engineering steps and therefore is an additional hurdle for GMP-grade production. We accomplished dominance of the introduced receptors by using a previously described method where human residues are replaced by key murine counterparts.²⁹ Furthermore, we successfully assessed whether the introduction of an additional disulfide bridge⁸ or the exchange of the human $\alpha\beta$ TCR transmembrane domain for the human $\gamma\delta$ TCR counterpart¹⁹ could also lead to enhanced expression. Thus, we found, in line with our recently published solution for TEGs,¹⁹ an elegant and minimalistic strategy to purify $\alpha\beta$ TCR-engineered T cells.

We observed, as reported previously for purification of TEGs,^{19,20} that $\alpha\beta$ T cells double positive for endogenous and introduced TCRs are also depleted. This is most likely due to the high affinity of the GMP-grade depletion antibody to the natural β TCR chain. This resulted in a substantial loss of engineered immune cells with residual endogenous $\alpha\beta$ TCR expression. Although the purified popula-

tion represented only a small fraction of the initial population, we have shown when using this process for $\gamma\delta$ TCRs engineered immune cells that the recovery is sufficient to reach therapeutic cell numbers in a full GMP-grade process.²⁰ Furthermore, we observed enrichment of NK and $\gamma\delta$ T cells after depletion, previously reported for $\gamma\delta$ TCR-engineered immune cells²⁰ and transplantation products⁴¹ as well. Therefore, selection of CD4⁺ and CD8⁺ T cells prior to transduction is recommended when applying our strategy. Selection of CD4⁺ and CD8⁺ T cells is used already successfully during the full grade GMP production process of approved CAR T products.⁴² Overall, our strategy can further improve the current practice for infused engineered products that harbor only between 15% and 55% engineered immune cells,^{43,44} since the lack of purity of infusion products can become a major clinical obstacle in terms of efficacy¹⁹ as well as toxicity.^{13,45}

Many tumor-associated antigens targeted by $\alpha\beta$ TCR gene therapy are not exclusively expressed on tumor cells.⁴⁶ Thus, depending on the type of antigen targeted by the introduced $\alpha\beta$ TCR, depletion strategies can be useful. This is illustrated by multiple clinical trials, which have led to devastating results caused by off-target or on-target but off-tumor toxicities.^{5,14} Preclinical strategies to predict off-target toxicities by affinity-enhanced TCRs provide an important tool to minimize these risks.⁴⁷ However, these strategies are not infallible, and therefore an additional safeguard would be extremely valuable when, e.g., targeting novel antigens or antigens that are also partially expressed on healthy tissues. Methods described so far for introducing a safety switch in engineered T cell products rely on the introduction of additional genes for the expression of (truncated) targetable proteins, the introduction of inducible caspase proteins,⁴⁸ or sensitivity to ganciclovir in the case of the widely used HSV-TK suicide gene.¹⁵ The strategy described herein, using minimal murine aa substitutions, is not only suitable for creating an untouched population of purified T cells, but it also has the potential to develop strategies that will allow an *in vivo* depletion when needed. However, to accomplish this goal, the two identified murine aas that enable $\alpha\beta$ TCR depletion needed to be expanded with an additional 7 aa to create a chimeric TCR β chain with a total of 9 murine aa. The major advantage of our strategy, as compared to strategies using, e.g., myc-tags introduced into the TCR α chain,¹⁶ would be its combined property as a selection and a safeguard system, as well as its use of natural $\alpha\beta$ TCR domains, which most likely do not affect signaling or impair pairing. However, a major remaining limitation of our approach at this stage is the reduced binding efficacy of our engineered depletion antibody to the murine mutants when compared to the murine wild-type, implying that further engineering of the TCR domain or affinity maturation of the antibody will be needed to enable translation of this strategy into an efficient killing strategy *in vivo*. As binding of the antibody is

Figure 4. Efficacy of different strategies to induce preferential pairing of introduced α TCR and β TCR chains

(A) Schematic representation of the three different methods for creating preferential pairing between the introduced α TCR and β TCR chains. TM, transmembrane domain; V, variable domain; C, constant domain. (B) Primary $\alpha\beta$ T cells were transduced with the three differentially modified $\alpha\beta$ TCRs as indicated in (A), and expression of the introduced β TCR was determined by an anti-V β 4 antibody. (C and D) Pairing of the introduced α TCR and β TCR chains was assessed by NY-ESO-1 pentamers (C), and the percentage V β 4⁺ cells was quantified for the differently modified $\alpha\beta$ TCR (D) ratio between V β 4 single-positive/V β 4/ $\alpha\beta$ TCR double-positive cells was determined. The data correspond to two independent experiments and are shown as a representative figure (B) or as the average with standard deviation (C and D). Statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$) was calculated using a one-way ANOVA.

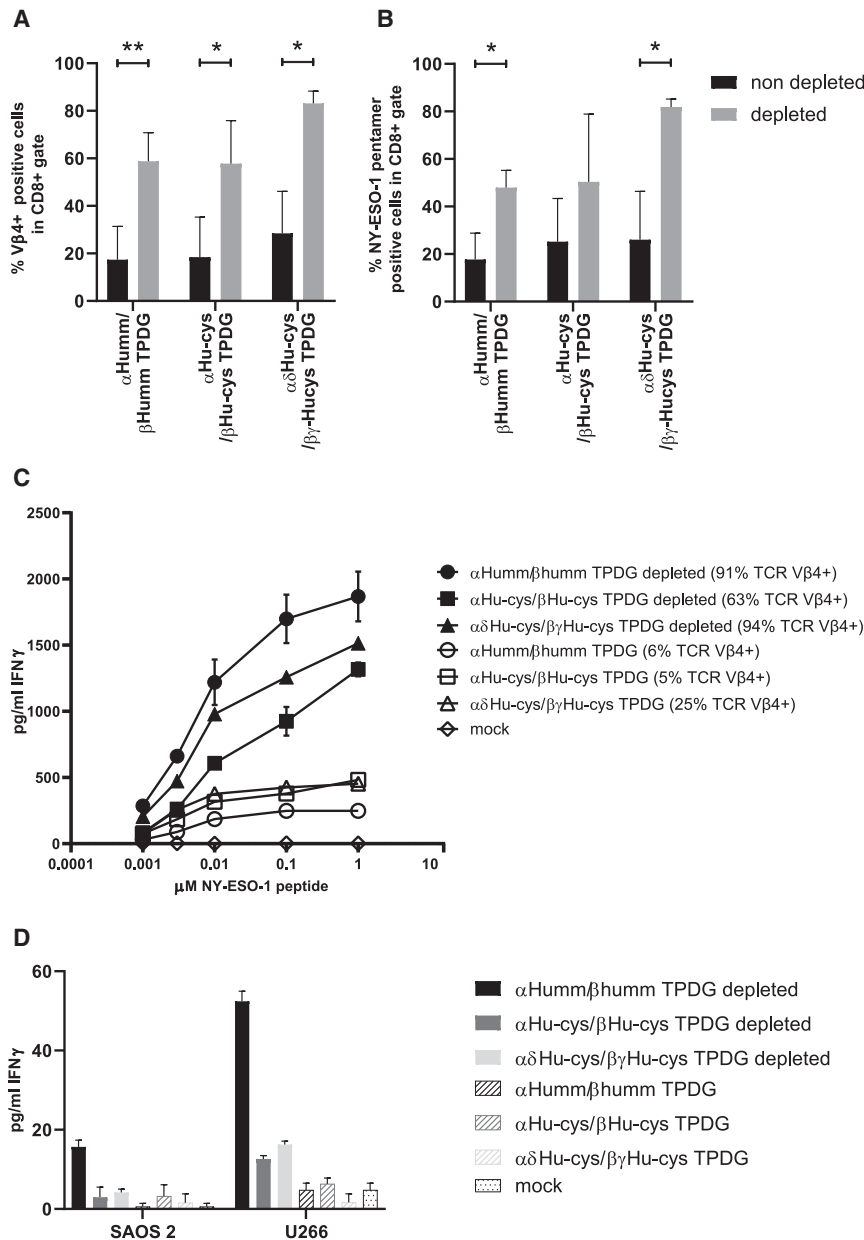


Figure 5. Depletion of non-engineered and poorly engineered T cells within the context of different preferential $\alpha\beta$ TCR pairing strategies

Primary $\alpha\beta$ T cells were transduced with the three differently modified $\alpha\beta$ TCRs as indicated in Figure 4A and depleted with the anti-human $\alpha\beta$ TCR antibody clone BW242/412. (A) Directly before and after depletion, expression of the introduced β TCR was determined by an anti-V β 4 antibody. (B) Expression of appropriately paired introduced α TCR and β TCR chains was determined by NY-ESO-1 pentamers. (C and D) Functionality of purified or non-purified engineered immune cells was assessed in a stimulation assay after co-incubation with NY-ESO-1_{157–165} peptide-pulsed T2 cells (C) or tumor cell lines with endogenous expression of NY-ESO peptide (D). IFN γ production was measured in the supernatant by ELISA. The data correspond to three (A and B) or two (C and D) independent experiments and are shown as average with standard deviation (A and B) or a representative figure (C and D). Statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$) was calculated using a one-tailed paired t test.

engineering of the TCR-antibody interface as well as carefully selecting the appropriate killing mechanism to reach its full potential.

MATERIALS AND METHODS

Cells and cell lines

Phoenix-Ampho cells (CRL-3213) were obtained from ATCC and cultured in DMEM (Thermo Fisher Scientific, Breda, the Netherlands) containing 1% penicillin/streptomycin (Pen/Strep) (Invitrogen by Thermo Scientific, Breda, the Netherlands) and 10% fetal calf serum (FCS) (Bodinco, Alkmaar, the Netherlands). The TCR $\beta^{-/-}$ Jurma cell line (a derivative of Jurkat J.RT3-T3.5 cells⁵⁰), a kind gift from Erik Hooijberg (VU Medical Center, Amsterdam, the Netherlands), TCR $\beta^{-/-}$ Jurkat-76, a kind gift from Miriam Heemskerk (LUMC, Leiden, the Netherlands), and the T2 cell line (ATCC CRL-1992) were cultured in RPMI 1640 + Gluta-

MAX (Thermo Fisher Scientific, Breda, the Netherlands) containing 1% Pen/Strep and 10% FCS. Cell lines were authenticated by short tandem repeat profiling/karyotyping/isoenzyme analysis. All cells were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. In addition, all cell lines were routinely verified by growth rate, morphology, and/or flow cytometry and tested negative for mycoplasma using a MycoAlert mycoplasma kit (Lonza, Breda, the Netherlands). PBMCs were obtained from Sanquin Blood Bank (Amsterdam, the Netherlands) and isolated by Ficoll-Paque (GE Healthcare, Eindhoven, the Netherlands) from buffy coats. PBMCs were cultured using the previously described rapid expansion protocol (REP)³⁵ in RPMI 1640 containing 5% non-typed human serum (Sanquin Blood Bank,

also partially driven by residues in the C β -TCR M1 domain,⁴⁹ additional introduction of several murine aas in this domain could therefore be considered.

In conclusion, the murinization of two specific residues in the TCR β constant domain allows for the untouched isolation of $\alpha\beta$ TCR-engineered T cell products, and it can be easily introduced in existing GMP procedures. When a safeguard of engineered immune cells is required, mutating an additional 7 human aa to murine residues in the TCR β constant domain allows for binding of an antibody, which has the potential to, after further optimization, selectively recognize engineered T cells. However, the second step will require additional

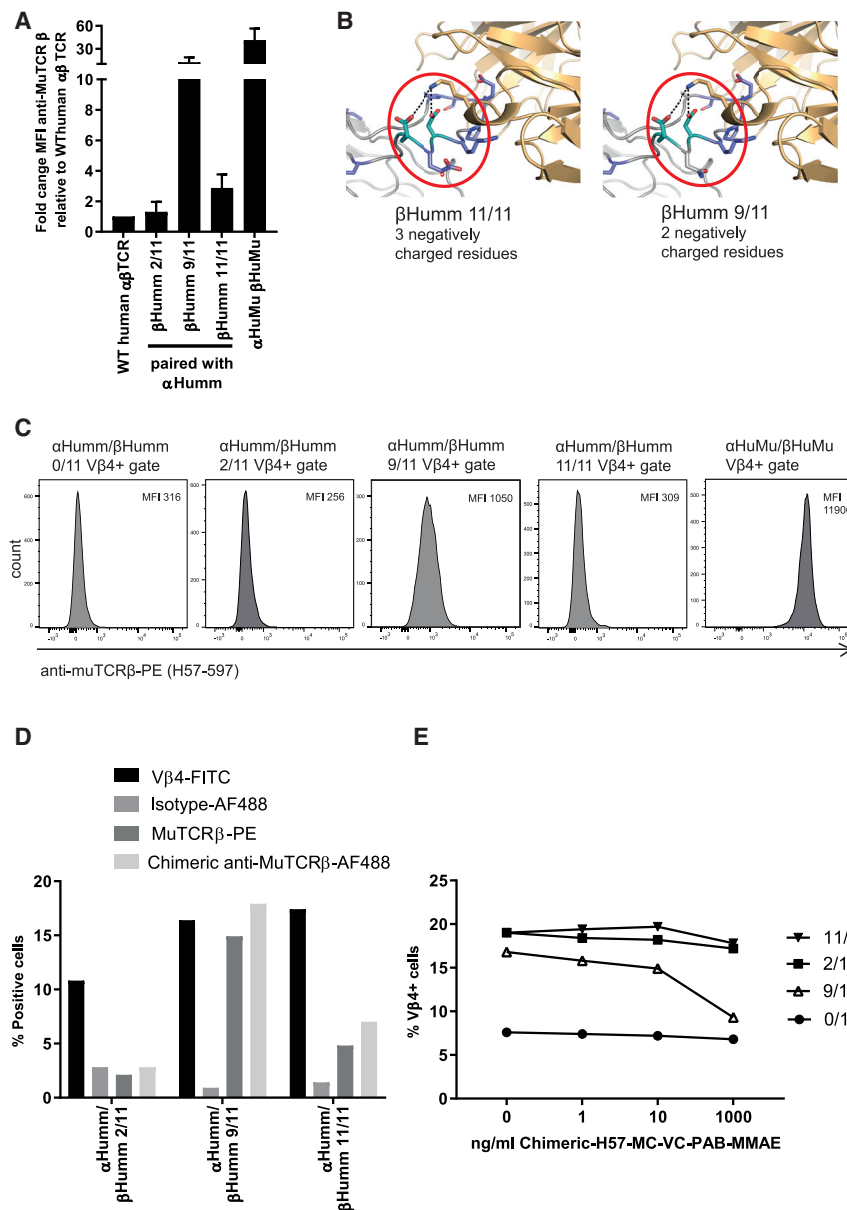


Figure 6. Opportunities for depletion of engineered T cells by using a mutation-specific antibody

(A) Jurkat-76 cells were transduced with five different murinized $\alpha\beta$ TCRs to assess binding of anti-MuTCR β . Wild-type (WT) $\alpha\beta$ TCR-transduced Jurkat-76 cells served as a negative control, while Jurkat-76 cells transduced with a TCR containing a complete murine constant domain served as a positive control. (B) The structure of the murinized constant domains (β Humm 11/11 and β Humm 9/11) when binding of H57-597 was modeled on the template of the β chain of the murine N15 T cell receptor (PDB: 1NFD).⁴⁹ (C) Primary $\alpha\beta$ T cells were transduced with the five different murinized $\alpha\beta$ TCRs, and co-staining was performed with anti-MuTCR and anti V β 4 antibodies. Cells were first gated for V β 4 positivity, and plots of the anti-MuTCR MFI in the V β 4-positive gate are shown. (D) Primary $\alpha\beta$ T cells expressing three different murinized $\alpha\beta$ TCRs were used to assess binding of wild-type and chimeric anti-MuTCR β . Anti-V β 4 and anti-human IgG1-AF488 isotype were included as a positive and a negative control, respectively. (E) Jurkat-76 expressing four different murinized $\alpha\beta$ TCRs were incubated with chimeric H57-MC-VC-PAB-MMAE for 24 h and then stained with an anti-V β 4 antibody. The data correspond to one experiment (C), two independent experiments (D and E) for which a representative figure is shown, or three independent experiments (A) shown in a bar graph representing average and standard deviation.

Amsterdam, the Netherlands), 1% Pen/Strep (Invitrogen by Thermo Scientific, Breda, the Netherlands), and 50 μ M Gibco β -mercaptoethanol (Fisher Scientific, Thermo Fisher Scientific, Breda, the Netherlands) (collectively called HuRPMI).

Cloning of TCR chains into single retroviral vectors

The minimally murinized V α 16.1 and V β 4.1 chains from an NY-ESO1₁₅₇₋₁₆₅/HLA*02-specific TCR, respectively named M2.2.3 and M1.KA.4.1, were generated as previously described.²⁹ Additional partially murinized (regions or single residues) TCR chains were ordered from GeneArt (Life Technologies, Thermo Fisher Scientific, Breda, the Netherlands) or constructed via mutagenesis PCR. Cysteine-modified chains were designed as reported previously.⁸ Var-

iants of chimeric $\alpha\beta/\gamma\delta$ TCRs were composed using the IMGT database (<http://www.imgt.org>). Sequences were codon optimized and ordered in an industrial resistance, gene-harboring vector or as DNA strings (GeneArt Life Technologies, Thermo Fisher Scientific, Breda, the Netherlands). DNA strings were processed using the TA TOPO cloning kit (Thermo Fisher Scientific, Breda, the Netherlands) and cloned into the pCR2.1-TOPO vector, according to the manufacturer's protocol. All TCR chains were cloned separately into the retroviral vector pMP71 between the EcoRI and NotI restriction sites using the indicated restriction enzymes and T4 DNA ligase (all from New England Biolabs, Ipswich, MA, USA). Transformation of ligated constructs was performed in JM109 competent *E. coli* (Promega, Leiden, the Netherlands), and subsequent plasmid DNA isolation was conducted using NucleoBond PC500, according to the manufacturer's protocol (Macherey-Nagel, Düren, Germany).

Retroviral transduction of primary T cells and T cell lines

Phoenix-Ampho packaging cells were transfected using FuGENE-HD (Promega, Leiden, the Netherlands) with env (pCOLT-GALV), gagpol (pHIT60), and separate pMP71 constructs containing α or β chains from a NY-ESO1₁₅₇₋₁₆₅/HLA-A*02-specific TCR (isolated from clone ThP2⁵¹) kindly provided by Wolfgang Uckert,²⁹ or containing TCR γ (G115)-T2A-TCR δ (G115)LM1.¹⁹ PBMCs preactivated

with 50 IU/mL interleukin (IL)-2 (Proleukin, Novartis, Arnhem, the Netherlands) and 30 ng/mL anti-CD3 (clone OKT-3, Miltenyi Biotec, Bergisch Gladbach, Germany), CD4/CD8 T cells selected from PBMCs with a REAlease CD4/CD8 (TIL) MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) preactivated with anti-CD3/CD28 Dynabeads bead to a T cell ratio of 1:5 (Thermo Fisher Scientific, Breda, the Netherlands), and 1.7×10^3 IU/mL MACS GMP recombinant human IL-7 and 1.5×10^2 IU/mL MACS GMP recombinant human IL-15 (Miltenyi Biotec, Bergisch Gladbach, Germany), and Jurma or Jurkat-76 cells were transduced twice within 48 h with viral supernatant in six-well plates (4×10^6 cells/well) in the presence of 50 IU/ml IL-2 (PBMCs only), 1.7×10^3 IU/ml IL-7, 1.5×10^2 IU/ml IL-15, and CD3/CD28 Dynabeads at a 1:5 bead-to-T cell ratio (CD4/CD8-selected T cells only), and 6 µg/mL Polybrene (all) (Sigma-Aldrich, Munich, Germany). After transduction, primary T cells were expanded by the addition of 50 µL/well anti-CD3/CD28 Dynabeads (Thermo Fisher Scientific, Breda, the Netherlands) and 50 IU/ml IL-2 or 1.7×10^3 IU/mL IL-7 or 1.5×10^2 IU/mL IL-15.

Purification of engineered T cells by MACS depletion of poorly engineered and non-engineered immune cells

Transduced primary T cells were incubated with biotin-labeled anti-human $\alpha\beta$ TCR antibody (clone BW242/412; Miltenyi Biotec, Bergisch Gladbach, Germany), followed by incubation with an anti-biotin antibody coupled to magnetic beads (anti-biotin MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany).¹⁹ Next, the cell suspension was applied to an LD column in a QuadroMACS separator. $\alpha\beta$ TCR⁺ T cells were depleted by MACS cell separation according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).

In silico TCR modeling

The structure of different murinized constant domains was predicted using SWISS-MODEL⁵² on the modeled template of the β chain of the human JKF6 T cell receptor (PDB: 4ZDH). The structure of the murinized constant domains when binding H57-597 was modeled on the template of the β chain of the murine N15 T cell receptor (PDB entry code: 1NFD).⁴⁹ Structure visualizations were performed using the PyMOL molecular graphics system (<https://pymol.org/2/>).

Chimeric antibody production and purification

Hamster-human (IgG1) chimeric H57-597 antibody was generated using Lonza expression vectors (pEE14-4-kappaLC, pEE14-4-IgG1).^{53,54} The antibody was produced by transient transfection of HEK293F cells with the heavy chain coding plasmid, the light chain coding plasmid, and pAdVantage (accession no. U47294; Promega, Leiden, the Netherlands) using 293fectin transfection reagent (Invitrogen, Thermo Scientific, Breda, the Netherlands) following the manufacturer's instructions. Antibody-containing supernatant was harvested 4 days after transfection and purified by affinity chromatography using HiTrap protein G HP antibody purification columns (GE Healthcare, Eindhoven, the Netherlands).

Sequencing

DNA sequences of cloning intermediates and final constructs in pMP71 were verified by barcode sequencing (BaseClear, Leiden, the Netherlands). 75 µg of plasmid DNA and 25 pmol of primer specific for the pCR2.1-TOPO vector or pMP71 vector were premixed in a total of 20 µL and sent to BaseClear for Sanger sequencing.

Flow cytometry

Cells were stained with Vβ4-fluorescein isothiocyanate (FITC) (TRBV29-1, clone WJF24; Beckman Coulter, Brea, CA, USA), $\alpha\beta$ TCR-phycoerythrin (PE) (clone BW242/412; Miltenyi Biotec, Bergisch Gladbach, Germany), CD3-Pacific Blue (PB) (clone UCHT1; Becton Dickinson [BD]), CD4-PeCy7 (clone RPA-T4; eBioscience, Thermo Fisher Scientific, Breda, the Netherlands), CD8-allophycocyanin (APC) (clone RPA-T8; BD), CD8-PB (clone SK1; BioLegend, San Diego, CA, USA), or RPE-conjugated NY-ESO-1₁₅₇₋₁₆₅ HLA*02:01 (SLLMWITQV) pentamer (ProImmune, Oxford, UK). Samples were fixed using 1% paraformaldehyde (PFA) in PBS, measured on a FACSCanto II flow cytometer (BD, Eysins, Switzerland), and analyzed using FACSDiva (BD, Eysins, Switzerland) or FlowJo (BD, Eysins, Switzerland) software.

ELISA

Effector and target cells (E:T 50,000:50,000) were incubated for 16 h, after which supernatant was harvested. IFN γ ELISA was performed using an ELISA-ready-go! kit (eBioscience, Thermo Fisher Scientific, Breda, the Netherlands) following the manufacturer's instructions.

MMAE ADC construction

Chimeric H57-MC-VC-PAB-MMAE was constructed using a kit from CellMosaic (Woburn, MA, USA) following the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.3.0 for Windows (GraphPad, La Jolla, CA, USA). Differences between groups was calculated using a one- or two-tailed paired t test (Figures 3 and 5) or a repeated-measures one-way ANOVA (Figure 4). Normal distribution of input data was assumed.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtm.2021.06.011>.

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AUTHOR CONTRIBUTIONS

G.J.J.K., T.S., W.S., Z.S., W.U., E.v.D., P.H.-L., R.O., and J.K. designed or analyzed experiments; G.J.J.K., E.v.D., P.H.-L., A.C.M.d.B., T.A.-R., S.F.J.v.D., D.X.B., E.F., and L.K. performed the experiments; G.J.J.K., E.v.D., P.H.-L., and J.K. wrote the manuscript; all authors agreed on the final manuscript.

DECLARATION OF INTERESTS

G.J.J.K., D.X.B., Z.S., and J.K. are inventors on different patents with $\gamma\delta$ TCR sequences, recognition mechanisms, and isolation strategies. J.K. is the scientific cofounder and a shareholder of Gadeta. The remaining authors declare no competing interests.

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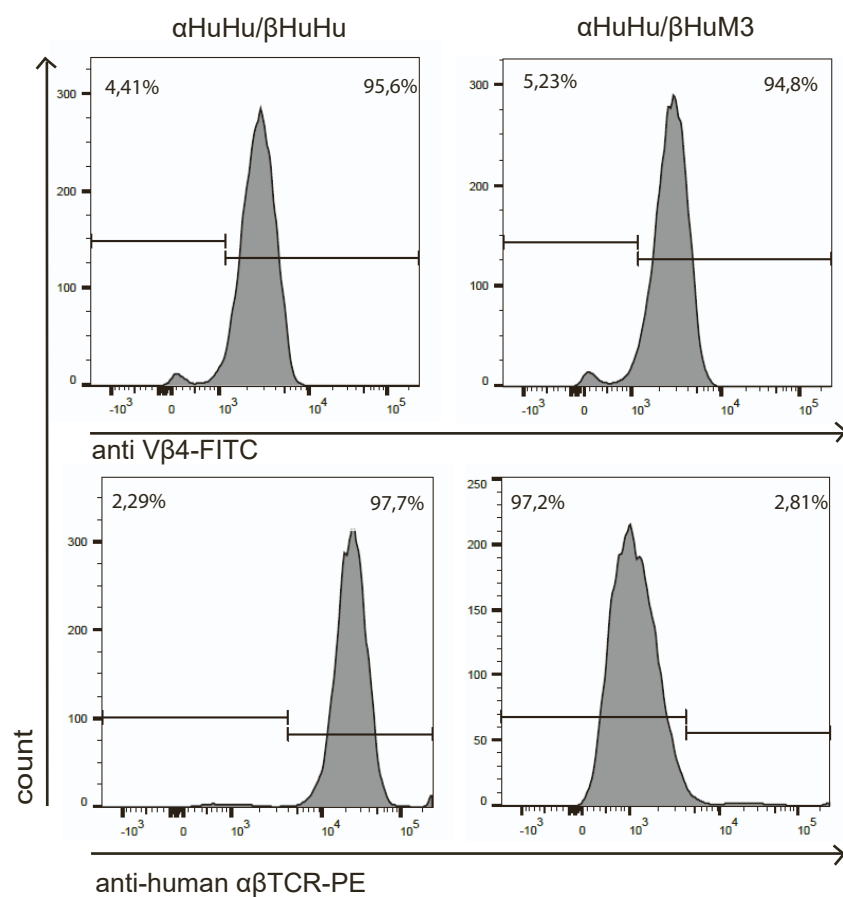
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Supplemental information

Characterization and modulation of anti- $\alpha\beta$ TCR antibodies and their respective binding sites at the β TCR chain to enrich engineered T cells

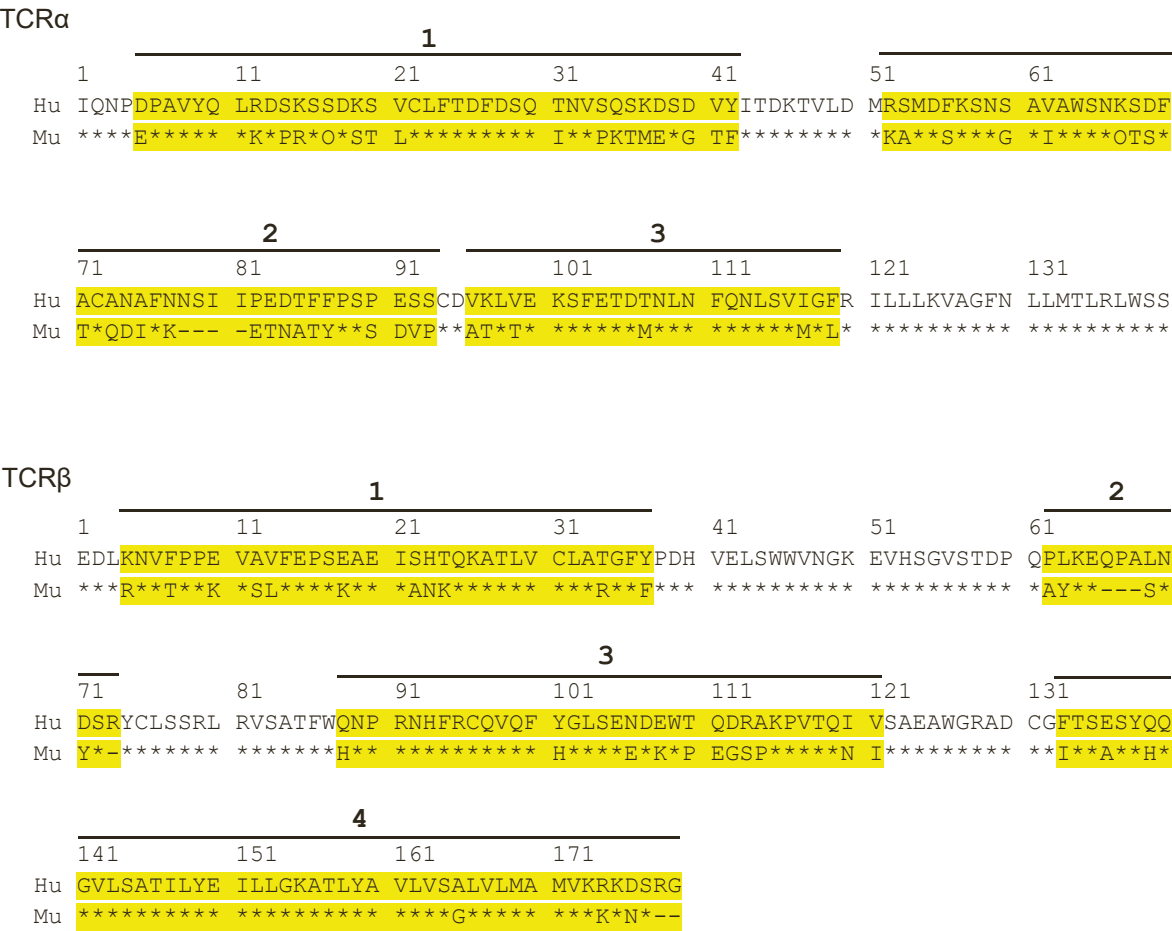
Guido J.J. Kierkels, Eline van Diest, Patricia Hernández-López, Wouter Scheper, Anja C.M. de Bruin, Elselien Frijlink, Tineke Aarts-Riemens, Sanne F.J. van Dooremalen, Dennis X. Beringer, Rimke Oostvogels, Lovro Kramer, Trudy Straetemans, Wolfgang Uckert, Zsolt Sebestyén, and Jürgen Kuball

Supplemental Figure 1



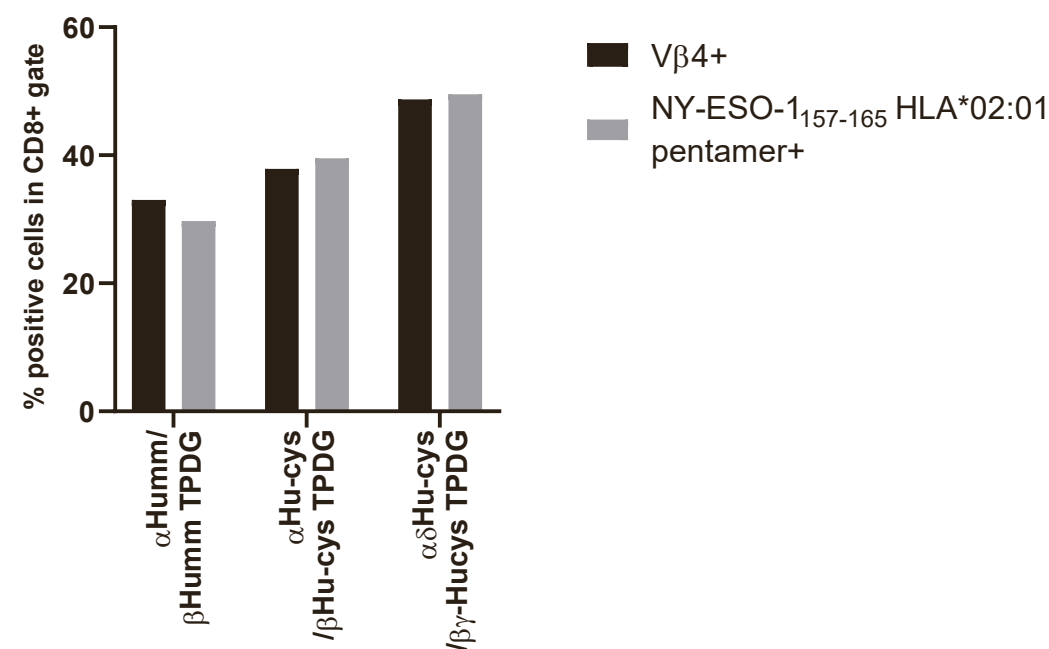
Supplemental Figure 1. α huhu/ β huM3 murinized TCR is expressed at the cell surface. Jurma cells were transduced with the α huhu/ β huhu and α huhu/ β huM3 murinized TCR after which TCR expression was confirmed with an anti-V β 4 antibody (upper panel) and binding of the anti-human $\alpha\beta$ TCR antibody was assessed (lower panel), by flow cytometry. The data correspond to 2 independent experiments and a representative figure is shown.

Supplemental Figure 2



Supplemental Figure 2. Extensive homology between human and murine TCR chains. Sequence alignment of the Human (Hu) and Murine (Mu) TCR α (upper panel) or β (lower panel) constant chains. The three (TCRα) or four (TCRβ) TCR constant regions with clustered Hu-Mu sequence differences are indicated above the alingment.

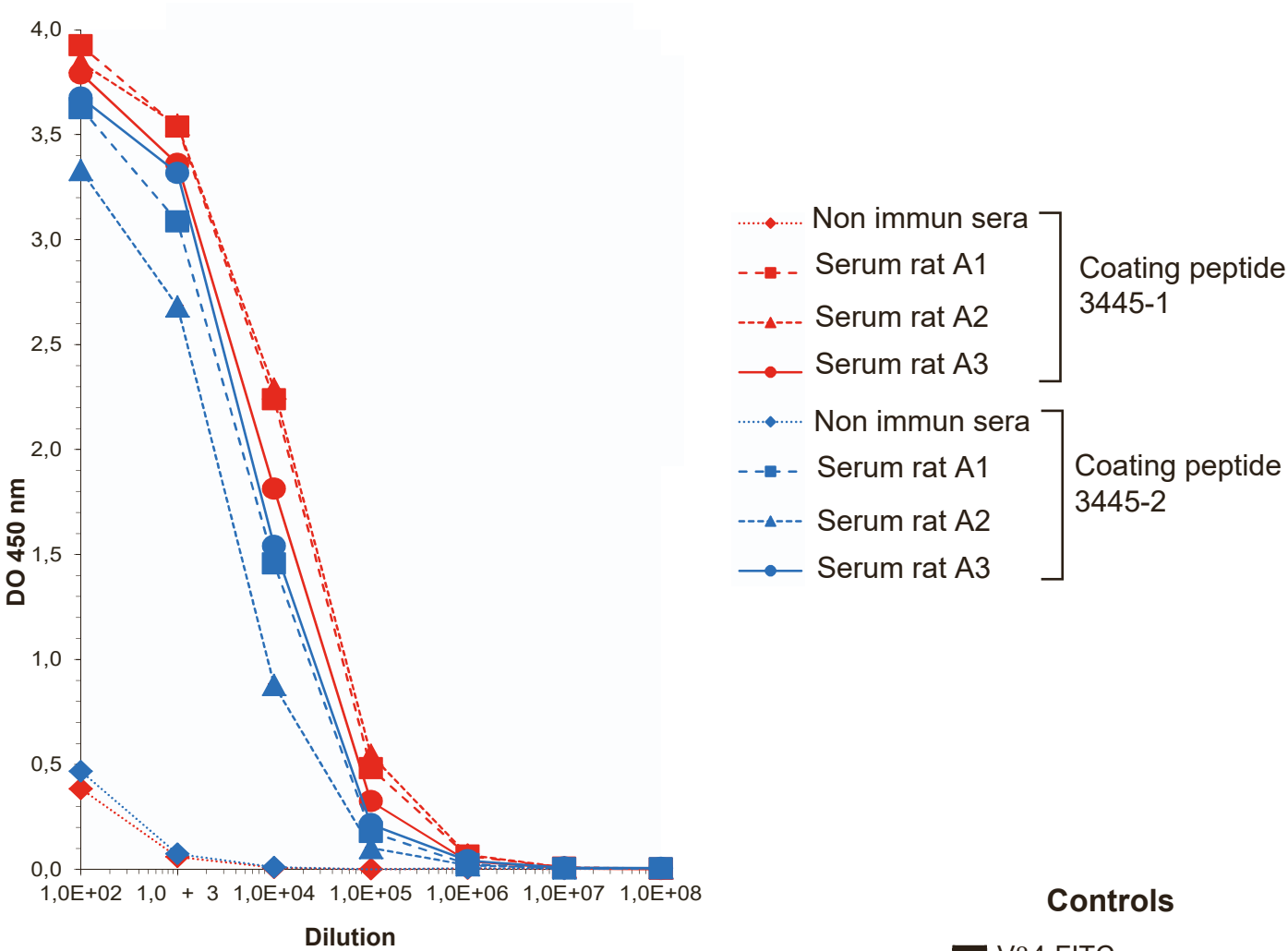
Supplemental Figure 3



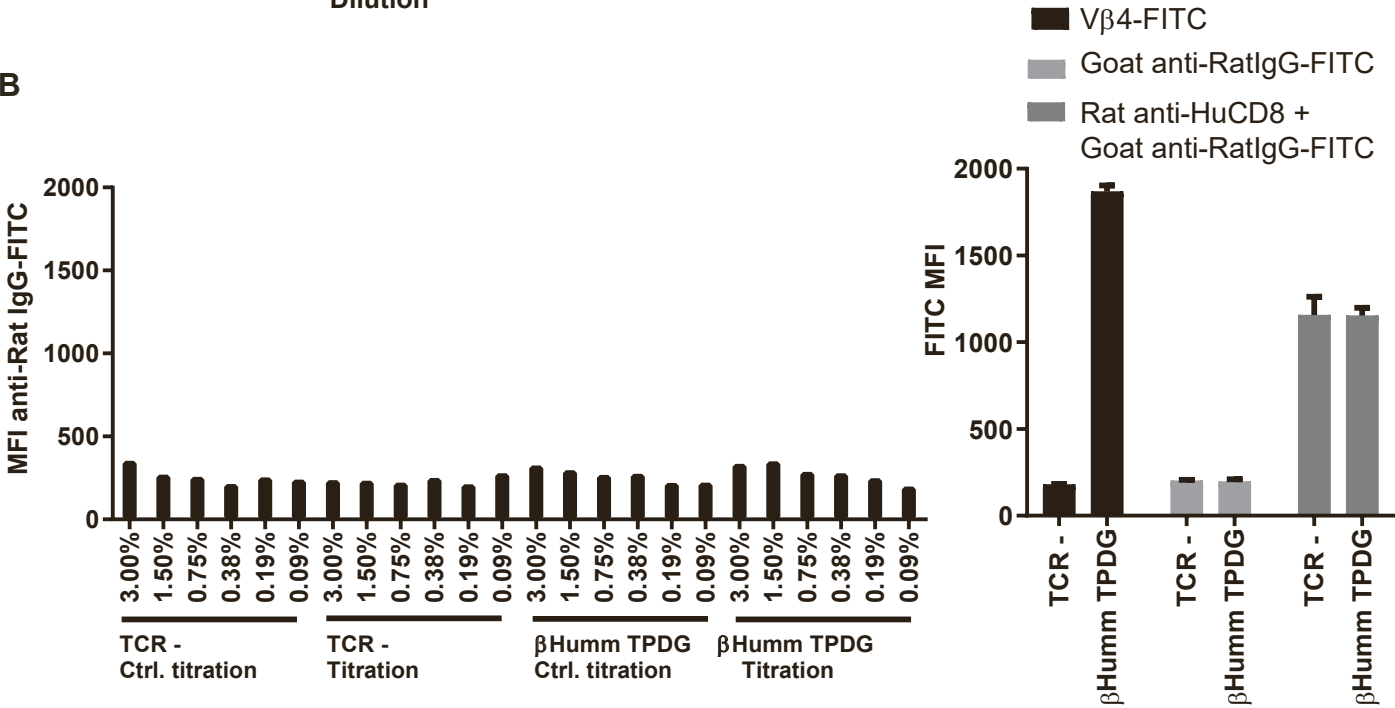
Supplemental Figure 3. Comparable efficacy of different strategies to induce preferential pairing of introduced α and β TCR chains. Primary $\alpha\beta$ T cells were transduced with the 3 differentially modified $\alpha\beta$ TCRs and expression of the introduced β TCR was determined by an anti-V β 4 antibody. Pairing of the introduced α and β TCR chains was assessed by staining with NY-ESO-1 pentamers. The data correspond to 2 independent experiments and a representative figure is shown.

Supplemental Figure 4

A



B

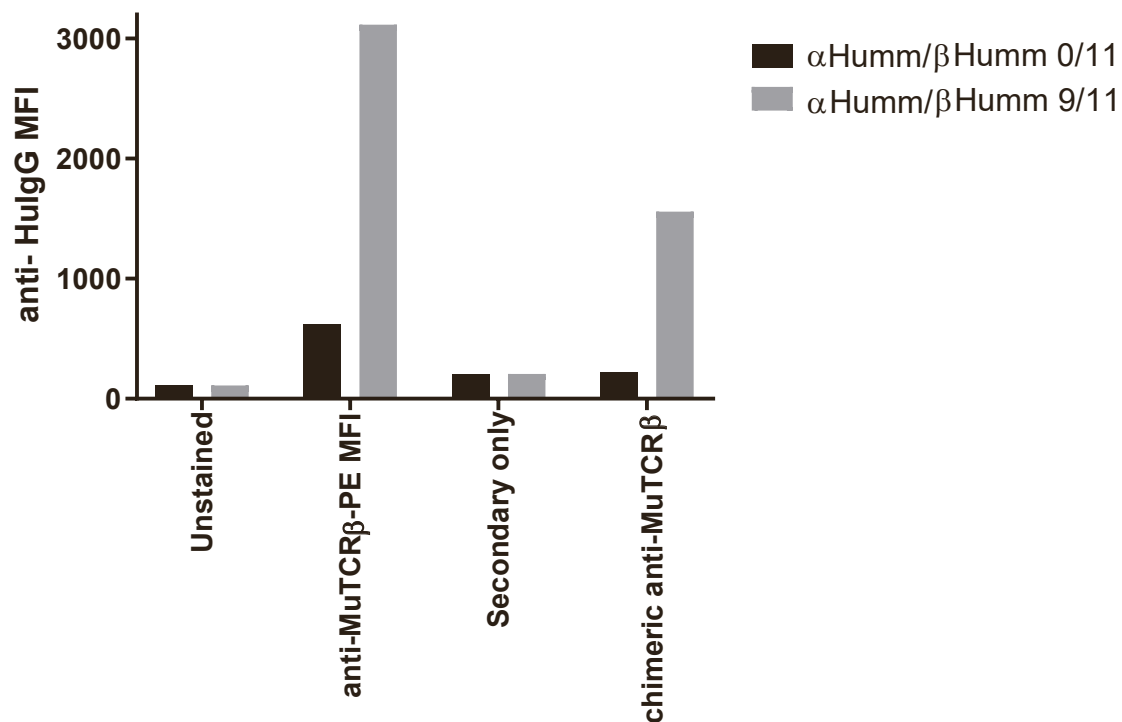


C

		91		101		111		121				
Human		QNP	RNH	FRC	QVQF	YGL	SEN	DEWT	QDRA	KPVT	QI	V
11/11 (Murine)		H..	H....	E.K.P	EGSP	N	I	
2/11		P	.G.			
9/11		H..	H....	K.P	EGS	N	I	

Supplemental Figure 4. Attempting to raise an antibody specific for the T110P+D112G murinized variant of the $\alpha\beta$ TCR by immunizing 3 Wistar rats with a human-mouse chimeric peptide. (A) Determining the presence of peptide-specific antibodies in the serum of the immunized rats. (B) Assessing the ability of the generated antibodies to bind surface-expressed TCRs. aHumm/ β Humm TPDG transduced or non-transduced Jurkat-76 cells were incubated with the indicated percentage of rat serum, after which flow cytometry using anti-RatIgG-FITC was performed. In the controls panel, the functionality of this secondary antibody was confirmed by staining the Jurkat-76 cells with rat anti-HuCD8 followed by anti-RatIgG-FITC. Expression of the TCR was confirmed using anti-V β 4-FITC. (C) Sequence alignment of the human and murine 3rd domain of the TCR β chain and the constructed 2/11 and 9/11 murinized variants.

Supplemental Figure 5



Supplemental Figure 5. Chimeric anti-MuTCR β antibody binds to primary T cells expressing the murinized TCR containing 9 out of 11 murine residues in the 3rd domain of the β chain. Jurkat-76 cells were transduced with 2 different $\alpha\beta$ TCRs, containing 0/11 or 9/11 murine residues in the 3rd domain of the β chain, to assess binding of the newly generated chimeric and CDR grafted anti-MuTCR β antibodies. As negative controls, unstained and secondary antibody only conditions were used. As a positive control, wild-type PE-conjugated anti-MuTCR β was used. The data correspond to 2 independent experiments and a representative figure is shown.

Supplemental Table 1. Differences between the eleven human-mouse non-homologous amino acids in the third domain of the β chain (β M3).

Mutation	Change in		
	Size	Charge	Hydrophobicity
Q ₈₈ H	-	Uncharged to positive charge	Hydrophilic to hydrophobic
Y ₁₀₁ H	-	Uncharged to positive charge	-
N ₁₀₆ E	-	Uncharged to negative charge	-
E₁₀₈K	-	Negative to positive charge	Slightly more hydrophilic
T₁₁₀P	Less bulky	Uncharged	-
Q ₁₁₁ E	-	Uncharged to positive charge	-
D₁₁₂G	Less bulky	Negative charge to uncharged	Less hydrophilic
R ₁₁₃ S	Less bulky	Positive charge to uncharged	-
A ₁₁₄ P	-	Uncharged	Less hydrophobic
I ₁₂₀ N	-	Uncharged	Hydrophobic to hydrophilic
V ₁₂₁ I	-	Uncharged	-