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Minimal Amino Acid Exchange in Human TCR Constant Regions Fosters Improved Function of TCR Gene-Modified T Cells

Daniel Sommermeyer* and Wolfgang Uckert*,†

Adoptive transfer of TCR gene-modified T cells into tumor patients (TCR gene therapy) is a novel approach to specifically eliminate tumor cells (1–9). During the past few years, T cells recognizing various tumor-associated Ags were generated by transfer of TCR genes into T cells. The functionality of TCR gene-modified T cells was confirmed in vitro and in a variety of mouse models. In 2006, the first clinical trial was reported (10), whereby 2 of 17 patients responded to treatment with TCR gene-modified T cells, demonstrating the clinical applicability of TCR gene therapy. However, this study also revealed that gene-modified T cells with the new desired specificity were rarely detected, although reasonable transduction efficiencies were achieved. The most likely explanation for low level surface expression of the transferred TCRs was the occurrence of competition with endogenous TCRs and formation of mixed pairs of endogenous and exogenous TCR chains. Recently, it was shown that different TCRs have different capabilities to be expressed on the cell surface (11, 12), which is likely due to unequal stabilities within the CD3 complex. Only a “strong” TCR—in terms of cell surface expression—is displayed at a level sufficient to endow T cells with the desired Ag specificity. To further improve the expression of strong TCRs and even to enable surface expression of weak TCRs, strategies were developed to optimize TCR expression (13). In a first instance, this was partly achieved by using methods to attain a high-level transgene expression by improving gene transfer systems, most commonly based on retroviral vectors (14–18). Further improvement included optimization of TCR-encoding nucleotide sequences, which resulted in enhanced translation of the transgene (19). However, these approaches only led to higher TCR protein levels but did not impact on preferential pairing of transferred TCR chains. Therefore, strategies were introduced to obtain improved pairing. First, molecular design of the constant (C) region αβ interface was tried by exchanging amino acids between the two chains (20). This resulted in reduced expression of mixed TCRs but did not yield T cells with higher functional avidity as compared with cells transduced with unmodified TCRs. Second, the exchange of the original C domains downstream of the interchain disulfide bridge by the complete human TCRC-chain was used (21). This modification completely eliminated the formation of mixed TCRs, but a functional advantage for the modified cells still needs to be shown. Third, mutations of single amino acids to cysteines in each TCR chain led to formation of an additional disulfide bond connecting the C regions of the TCRα- and TCRβ-chains (22, 23). This reduced mixed TCR pairing and enhanced the functionality of TCR gene-modified T cells. Finally, an increased functional avidity of TCR gene-modified T cells was achieved by removing defined N-glycosylation sites in the TCR C regions (24).

Independent of these modifications to improve the surface expression of transferred TCRs, it was shown that murine TCRs could replace human TCRs on human cells (11, 25). Moreover, it was demonstrated that substitution of human TCR C regions by corresponding murine counterparts increased the cell surface expression of these hybrid TCRs compared with wild-type (wt) human.
TCRs. Furthermore, when murine TCR C regions were replaced by their human counterparts, a decreased expression of the hybrid TCRs was noted in comparison with unmodified mouse TCRs (25, 26). This rather unexpected observation subsequently led to the concept of “murinization,” in which both C regions of human TCRs are exchanged by murine C regions to achieve a higher functional avidity of TCR gene-modified human T cells (26).

The reasons why murinized (mu) human TCRs are expressed more efficiently at the cell surface in comparison with wt human TCRs are not yet clear. One explanation is that mu TCRs compete more effectively for CD3 molecules, which are only available in limited amounts to form the TCR complex. A second explanation is that mu TCR chains preferentially pair with each other and are less prone to form mixed TCRs on human T cells. Thus, murinization of C regions of human TCRs with subsequent transfer of hybrid TCR genes—with or without additional modifications described above—plays an important role in achieving the functional avidity of T cells used in TCR gene therapy. However, an obvious drawback is the potential for increased immunogenicity of hybrid TCRs because of the foreign mouse sequences coupled to the human proteins. Although lymphodepletion of host T cells is applied as a preconditioning of patients prior to the transfer of TCR gene-modified T cells, one cannot exclude that transferred cells will be rejected as has been observed with gene-modified cells given to immunocompromised patients (27). This risk could be diminished if the critical amino acids within the C regions of the murine TCRα- and TCRβ-chain that ensure TCR cell surface expression comparable to full replacement of human C regions could be identified and selectively exchanged.

In this study, we demonstrate that nine amino acids derived from murine TCR C regions are sufficient to significantly improve the cell surface expression of human TCRs and the function of TCR gene-modified T cells. By only using these substitutions, it is possible to achieve levels of TCR expression and functional avidity in recipient T cells that are comparable to fully mu TCRs. This strategy will reduce the risk of TCR transgene immunogenicity in those cases where murinization is required to achieve efficient cell surface expression.

Materials and Methods

Cells
The cell line 293T (ATCC CRL-11268, American Type Culture Collection, Manassas, VA) was cultured in DMEM (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS (Biochrom, Berlin, Germany). The renal cell carcinoma cell line RCC-26 (28) was cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS, 1 mM sodium pyruvate (Life Technologies), and 100 U/ml penicillin/streptomycin.

PBMCs were isolated from blood of healthy donors with donors’ informed consent by Ficoll gradient centrifugation and stimulated in nontissue culture plates at a density of 10^6 per well/ml in the presence of 100 U/ml IL-2 (provided by Chiron, Marburg, Germany). Twelve days after isolation, PBLs were rested for 2 d by reducing the IL-2 concentration to 1 U/ml. Human PBLs and cell lines Jurkat (J76) (29), J76/TCR26 (11), and T2 (ATCC CRL-1992) were cultured in RPMI 1640 medium supplemented with 10% FCS (PAN Biotech, Aidenbach, Germany), 1 mM HEPES, and 100 U/ml penicillin/streptomycin. All cell culture flasks and plates were purchased from BD Falcon (BD, Franklin Lakes, NJ).

Construction of retroviral vectors

wt, mu, and all partially mu, including the final minimally murinized (mm) gene constructs of a NY-ESO-1–specific TCR [NY-TCR, isolated from clone ThP2 (30)], were cloned into the retroviral vector plasmid pMP71 (31) as single TCR chain gene vectors. In addition, wt, mu, and mm TCR chain genes were amplified by a 2A peptide linker (P2A) to generate MP71 vectors expressing both TCR chains simultaneously, as described previously (18). wt, mu, and mm TCR genes of TCR53 (11) were cloned as P2A vectors in different TCR53α combinations (βw/αwt, βm/wmu, and βm/mom). The combinations βw/αwt, βm/wmu, and βm/mom of TCR53 were codon optimized (GENEART, Regensburg, Germany) and then cloned into the vector MP71.

To exchange the first domain of the TCRβ C region, an Eco72I restriction site, located 3’ of this domain, was used. All other changes were cloned using two successive PCR steps. Two fragments of the TCR gene with overlapping regions of 20–30 nt were generated and subsequently connected by annealing of complementary oligonucleotides. The complete TCR gene was amplified via PCR. Finally, the genes were cloned into MP71 using NotI and EcoRI restriction sites (Supplemental Fig. 1 shows the cloning strategy for TCRβ84 as an example). To introduce single amino acid exchanges, mutations were included in the primer sequences. To perform fluorescence resonance energy transfer (FRET) and immunoprecipitation, two myc-tags (amino acid sequence EQKLISEEDL) were added at the N terminus of wt, mu, and mm TCR53α-chains (32). Detailed cloning strategies and primer sequences for the different constructs will be provided on request.

TCR retroviruses and transduction of T cells

For the production of amphotropic murine leukemia virus (MLV)- pseudotyped retroviruses, 293T cells were transfected with the respective TCR-encoding retroviral plasmids and expression plasmids encoding the Moloney MLV gag/pol gene (pcDNA1.3MLVg/p; provided by C. Baum, Medical School Hannover, Hannover, Germany) and the MLV-10A1 env gene [pALF-10A1 (33)]. 293T cells were cultured overnight in 6-well tissue culture plates at a density of 8 × 10^4 per well and transfected with a total amount of 18 μg DNA (TCRα, 3.5 μg; TCRβ, 3.5 μg; gag/pol, 6 μg; env, 5 μg) for single chain vectors; or TCRβ-P2A-TCRα, 6 μg; gag/pol, 6 μg; env, 6 μg for P2A-vectors) by calcium phosphate precipitation. Forty-eight hours after transfection, viral supernatants were filtered (0.45-μm pore size) and used directly for transduction. J76 and J76/TCR26 cells (1.5 × 10^5 per well) were incubated in 24-well nontissue culture plates precoated with RetroNectin (3.5 μg/well) (Takara, Apen, Germany) with 1 ml retrovirus supernatant supplemented with proteamine sulfate (final concentration, 4 μg/ml) (Sigma-Aldrich, Munich, Germany). After addition of supernatant, plates were spinocinated with 800 × g for 1.5 h at 32°C. PBLs were transduced on days 2 and 3 after isolation as described previously (18).

Flow cytometry

Cells were stained using FITC-labeled mAbs directed against human CD8 (BD Pharmingen) and TCRVβ22 (Immunotech, Marseille, France), PE-labeled mAbs directed against human CD3 (BD Pharmingen), TCRVβ4, and TCRVβ20 (Immunotech), and APC-labeled HMC-pentamers (Proimmune, Oxford, U.K.). Fluorescence intensity was measured using a FACScalibur flow cytometer (BD) and CellQuest Pro software (BD). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Flow cytometry-based FRET

PBLs and J76 cells were transduced with wt, mu, and mm TCR53 containing myc-tagged TCRα-chains. One week after transduction, pairing of TCR53α- and TCR53β-chains was analyzed by flow cytometry-based FRET (21). TCR53β-chains were labeled with anti-myc mAbs (hybridoma 9E10, ATCC CRL-1729), washed, and stained with Cy5-conjugated goat-anti-mouse IgG mAbs (Jackson ImmunoResearch Laboratories, Newmarket, U.K.). Afterward, TCR53β-chains were stained with a PE-conjugated anti-TCRVβ20 mAb. Emissions at 570 nm (donor [PE] channel), at 670 nm (acceptor [Cy5] channel), and >760 nm (FRET channel) were measured using a FACScalibur flow cytometer. Data were analyzed with the FLEX software. FRET efficiencies measured with transduced J76 cells were set as 100% because on these cells only correctly paired TCR53αβ combinations could occur. FRET efficiencies analyzed on PBLs were calculated relative to J76 cells.

Immunoprecipitation and Western blot analysis

Transduced J76 cells (1 × 10^6) were lysed as described previously (21). Protein lysates were cleared with protein G-Sepharose (GE Healthcare, Freiburg, Germany) for 2 h at 4°C. Afterward, proteins were immunoprecipitated with protein G-Sepharose coated with anti-myc mAbs or anti-TCRα mAbs (6B10.2; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing, precipitates were separated on a 12% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (GE Healthcare) using a semidy transfer system (1 h, 80 mA). To block unspecific binding, membranes were incubated in 5% nonfat dry milk in PBS supplemented with 0.1% Tween20 (Sigma-Aldrich) for 1 h at room temperature. After washing, membranes were incubated with anti-
Improved expression of mu TCRs is mainly due to a single surface expression of mu TCRs, we first focused on the C region of

Cytokine release assay

Untransduced or TCR-modified PBL (1 × 10^5 per well) were cocultured with 5 × 10^5 target cells in 200 μl medium. As target cells, tumor cell lines or peptide-pulsed T2 cells were used. For peptide loading, T2 cells were incubated with different concentrations of NY-ESO-1 peptide (Biosyntan, Berlin, Germany) in serum-free medium for 2 h at 37°C. Then, cells were washed, resuspended in fresh medium, and distributed into 96-well plates together with PBLs. PBLs cultured without target cells were used as negative controls, and PBLs stimulated with PMA/ionomycin were used as positive controls. Supernatants obtained after 24 h were analyzed for human IFN-γ content by ELISA (BD Biosciences, San Jose, CA). IFN-γ concentrations are given as mean values of duplicates with mean deviation. Additional cytokines were measured using a Cytometric Bead Array (Human Th1/Th2 Cytokine Kit; BD Biosciences), according to the manufacturer’s protocol. Data were acquired using a FACS Calibur flow cytometer and CellQuest Pro software and analyzed using FCAP Array Software (BD Biosciences).

Results

Only mu, but not wt, NY-ESO-1–specific TCR can be expressed on J76/TCR26 cells

Recently, the improved functional expression of human TCRs in which the C regions were replaced by mouse counterparts (mu TCRs) in comparison with wt TCRs was reported (26). For the identification of the amino acids within the C regions of the TCRα- and TCRβ-chains that are responsible for improved expression of mu TCRs, we established a TCR replacement model. It is based on our observations that certain TCRβ combinations, when transferred into T cells expressing endogenous TCRs, are better expressed (strong TCRs) than others (weak TCRs). We created a setting where a NY-ESO-1–specific TCR (NY-TCR) had to compete with the strong TCR26 (11). For this, the TCRβ-deficient T cell line J76 was transduced with TCR26 and enriched for TCR-expressing cells, yielding J76/TCR26 cells (11). These cells were transduced with the NY-TCR, either in a wt or mu form. Staining with mAbs directed against the variable (V) regions of TCR26α (Vβ22) and NY-TCRβ (Vβ4) allowed expression of both TCRs to be determined by flow cytometry. J76/TCR26 cells transduced with the NY-TCRwt (ω^+μ^+β^+wt) showed no or only poor expression of NY-TCR, whereas the expression of TCR26 remained unchanged compared with untransduced cells (Fig. 1A). In contrast, transduction of J76/TCR26 cells with the NY-TCRmut (ω^+μ^+β^+mutu) revealed cells expressing NY-TCR, and in addition, the expression of TCR26 was reduced on this population. These results indicated that the mu variant but not wt NY-TCR could be expressed on J76/TCR26 cells. Gene transfer of combinations of one mu and one wt NY-TCR chain (ω^+μ^+β^+mutu and ω^+μ^+β^+mutu, respectively) did not result in the expression of the NY-TCR, demonstrating that the murinization of both chains was decisive for cell surface expression of NY-TCR on J76/TCR26 cells.

To ensure that the expression level of NY-TCR was not due to differences in transduction, we transduced TCR-deficient J76 cells in parallel. Staining of these cells using anti-CD3 mAbs revealed transduction efficiencies between 78 and 89%, indicating that the different TCR retroviruses possessed equal titers for all combinations (Fig. 1B).

Improved expression of mu TCRs is mainly due to a single exchange from an acidic to a basic amino acid within the TCRβ-chain

For the identification of the amino acids responsible for improved surface expression of mu TCRs, we first focused on the C region of the TCRβ-chain. We defined four domains, which included all differences (38 aa, 21%) between the human and mouse sequences, which were flanked by homologous regions (Fig. 2A). Then, we constructed four NY-TCRβ-chains with different C regions, each containing one human and three murine domains (Fig. 2B). These different NY-TCRβ constructs were transduced together with the completely mu NY-TCRα–chain gene into J76/TCR26 cells. Four days after transduction, cells were stained with Vβ4 (NY-TCR), Vβ22 (TCR26), and CD3, respectively, 4 d after transduction. Untransduced (ut) cells were used as control. Percentages of TCR-positive J76 cells are indicated (B).

FIGURE 1. Murinization of NY-TCR enables expression on J76/TCR26 cells. Different combinations of wt and mu TCRα- and TCRβ-chains of a NY-ESO-1–specific TCR (NY-TCR) were transduced into J76/TCR26 (A) and TCR-deficient J76 cells (B). Cells were stained for the expression of Vβ4 (NY-TCR), Vβ22 (TCR26), and CD3, respectively, 4 d after transduction. Untransduced (ut) cells were used as control. Percentages of TCR-positive J76 cells are indicated (B).

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were combined (m1.2/4.1) (Fig. 3B). In this construct, the number of “murine” amino acids was decreased from 38 to 7 compared with the complete murine Cβ2 sequence, and the remaining mouse amino acids were K-18, A-22, N-23, K-24, I-133, A-136, and H-139.

We continued by mutating each of these seven amino acids of the mouse sequence back to the human sequence. The mutation of the “murine” basic lysine (K-18) to the “human” glutamic acid (E-18) (m1.2KE/4.1) showed the most striking effect, as NY-TCR expression and TCR26 replacement were clearly reduced (Fig. 3B, Table I). The mutation of alanine (A-22) to serine (S-22) (m1.2AS/4.1) caused a smaller effect, whereas the mutations of asparagine (N-23) to histidine (H-23) (m1.2NH/4.1) and lysine (K-24) to threonine (T-24) (m1.2KT/4.1) had only negligible effects (Fig. 3B). For part 4.1 mutations from isoleucine (I-133), alanine (A-136), and histidine (H-139) to the original human residues phenylalanine (F-133), glutamic acid (E-136), and glutamine (Q-139), respectively, yielded a minor decrease of NY-TCR expression for each of the three amino acids (data not shown).

Finally, a NY-TCR β-chain construct was created containing the amino acids K-18, A-22, I-133, A-136, and H-139 of the murine protein. This construct still allowed NY-TCR expression and TCR26 replacement comparable to the completely mu NY-TCR β-chain and was defined as the mm TCR β-chain (Fig. 3C, 3D, Table I). To analyze whether substitution of other basic amino acids at position 18 would lead to enhanced TCR expression, we mutated lysine to arginine (mmKR) and found a similarly enhanced expression of NY-TCR on J76/TCR26 cells (Fig. 3C).

In summary, these studies allowed us to reduce the number of amino acid exchanges from 38 (mu) to 5 (mm) on the TCR β-chain while maintaining nearly the same enhanced level of expression of NY-TCR. The most important difference between the human and murine Cβ2 regions was identified at position 18, where the murine sequence contained the basic amino acid lysine while the human sequence included glutamic acid. The procedures used to identify the relevant positions in the TCR β-chain are illustrated in Figs. 2 and 3.
identify the relevant positions in the TCRβ C region are summarized in Fig. 3E.

Nine “murine” amino acids are sufficient for the improved expression of human TCRs

The final construct of the NY-TCRβ-chain (βmm) was used to determine which amino acids of the mu NY-TCRα-chain contributed to improved TCR expression. We defined three areas for the C region of the TCRα-chain, which covered all differences (50 aa, 36%) between the human and mouse sequences (Fig. 4A). Then, we constructed three NY-TCRα-chains with different C regions, each containing one human and two murine domains (Fig. 4B). The constructs were analyzed as described for the NY-TCRβ-chain, and we identified domain 2 to be indispensable for improved NY-TCR surface expression (Supplemental Fig. 2). Therefore, we generated a construct containing only domain 2 of the murine sequence (αmm2) (Fig. 4D). Analogous to the strategy used for identification of critical amino acids in the C region of the TCRβ-chain, domain 2 was then subdivided into smaller parts, which were subsequently analyzed for NY-TCR expression (Supplemental Fig. 2). Finally, we identified the region containing amino acids serine (S-90), aspartic acid (D-91), valine (V-92), and proline (P-93) as the most important segment. Single mutations of these amino acids did not reveal a preference for any single residue in this region. Thus, we elected to use the construct containing the four amino acids, S-D-V-P, of the murine sequence as the construct with the minimal essential TCRα modifications (αmm). The procedures used to identify the relevant positions in the TCRα C region are summarized in Fig. 4C.

When compared with the combination of the completely mu NY-TCRα-chain (αmu) and the final NY-TCRβ construct (βmm), the mm constructs (αmm+βmm) led to similar NY-TCR expression levels and TCR26 replacement (Fig. 4D, Table I). Moreover, the provision of T cells with the final mm constructs of the TCRα- and TCRβ-chains (αmm+βmm) led to TCR expression that was comparable to that of the completely mu TCR (αmu+βmm) (Fig. 4D, Table I). In summary, we showed that the number of “murine” amino acids included in the sequence of the C regions could be reduced by 90% (from 88 to 9) while retaining the improved surface expression of the modified TCRs and replacement of the endogenous TCRs.

Primary human T cells modified with mm TCRs show increased multimer binding compared with cells transduced with wt TCRs

wt, mu, and mm TCR chains of NY-TCR were linked via the P2A element to ensure simultaneous expression of both TCR chains and transduced into human PBLs. Pentamer staining of transduced PBLs revealed that the usage of mu and mm NY-TCR chains enhanced the expression compared with wt chains (in five of five experiments with different donors; e.g., mean fluorescence intensity of CD8/pentamer-positive cells: wt, 79; mu, 106; and mm, 92) (Fig. 5A). The improvement of TCR expression was relatively modest for both variants but still significant. In average, the enhancement of TCR expression with mm TCR was 50% of completely mu TCR. The same differences for the TCR variants were observed when IFN-γ release was determined after coculture of transduced PBLs and NY-ESO-1 peptide-pulsed T2 cells at different peptide concentrations as exemplarily shown for 1 μM (Fig. 5B).

T cells modified with mm TCRs show improved recognition of tumor cells compared with cells transduced with wt TCRs

To demonstrate that the identified amino acid exchanges, which led to improved expression of NY-TCR, are of general relevance, we mutated a renal cell carcinoma-reactive TCR (TCR53) in the same way. To ensure simultaneous transduction and high expression levels of both TCR chains, TCR53β and TCR53α genes were linked via the P2A element. Retroviral vectors expressing wt, mu, and mm TCR53 chains in different combinations were constructed and transduced into J76 cells and human PBLs. TCR expression, measured by anti-CD3 staining of transduced J76 cells, showed equal TCR cell surface expression levels for all different combinations, except for αwt/βmu, which even on cells lacking endogenous TCR could not be stably expressed (Fig. 6A). Because the antigenic peptide recognized by TCR53 is not yet identified, the transduced PBLs were cocultured with the renal cell carcinoma cell line RCC-26, and IFN-γ release into supernatant medium was measured by ELISA. PBLs transduced with TCR53wt produced 2.1 ng/ml IFN-γ, whereas PBLs transduced with

![Figure 4](http://www.jimmunol.org/Downloadedfrom/fig4.png)

**FIGURE 4.** Enhanced TCR expression after murinization is maintained with only nine “murine” amino acids within the TCR C regions. A, The amino acid sequences of the h and m TCRα C region were compared, and three domains covering all differences were defined (gray boxes). Asterisks represent the same amino acid in the murine sequence. B, Schematic drawing of three different molecularly cloned TCRα C regions consisting of one human and two murine domains. C, Schematic summary of the procedures to identify the relevant amino acid positions in the TCRα C region. D, J76/TCR26 cells were transduced with NY-TCR constructs containing different TCRα and TCRβ C regions and analyzed for surface expression of both TCRs by staining with mAbs directed against Vβ22 (TCR26) and Vβ4 (NY-TCR). *Pictures were taken from Figs. 1A and 3C, respectively, for comparison. h, human; m, murine.
were analyzed.

and concentrations of released cytokines to the J76 cells. Fifteen days after iso-

Percentages of TCR-positive J76 cells are transduction (gray histograms) in com-

Enhanced expression of mm NY-TCR on human PBLs. A, PBMCs were isolated from healthy donors and transduced with vectors containing wt, mu, or mm NY-TCR chains linked by a P2A element. Ten days after second transduction, PBLs were stained with NY-ESO-pentamers and anti-CD8 mAb. Ut PBLs were used as control. Numbers indicate the percentage and mean fluorescence intensity, respectively, of CD8/pentamer-positive cells. B, PBLs were cocultured with T2 cells pulsed with 1 μM NY-ESO-1 peptide, and IFN-γ concentration of supernatants was analyzed by ELISA.

TCR53mu released substantially more IFN-γ (12 ng/ml) (Fig. 6B).

As expected, combinations of one TCR53wt chain and one TCR53mu chain did not yield functional TCR expression and subsequently caused no release of IFN-γ above the background level. PBLs transduced with TCR53mm produced more IFN-γ (9.5 ng/ml) compared with PBLs transduced with TCR53wt and reached ~80% of the amount detected with cells expressing TCR53tmu. Combinations of one TCR53mm and one TCR53wt chain resulted in IFN-γ release comparable to TCR53wt, showing again that amino acid exchanges in both, TCRwt and TCRmu, regions were needed for enhanced functional expression of a TCR. PBLs transduced with TCR53mm also released increased amounts of other cytokines, such as TNF-α and IL-2, as compared with TCR53wt transduced cells (Fig. 6B).

To exclude that the introduced mutations only resulted in an enhanced translation as described for codon-optimized TCRs (19), we completely codon-optimized the three TCR53 variants (wt, mu, and mm). As we found the same functional differences among wt, mu, and mm TCR53-transduced T cells (Supplemental Fig. 3) as before for non–codon-optimized TCRs, we concluded that the increased function of mu TCR is not due to the usage of more optimal codons.

mu and mm TCR chains preferentially pair to each other and result in more stable TCR complexes

To get an insight of which mechanism(s) led to the improved function of T cells transduced with (minimally) murinized TCR, we first analyzed the expression of the TCR53β-chain. Staining with Vβ20-specific mAbs revealed similar levels of TCR53β after transduction with TCRwt, TCRmu, and TCRmm into PBLs (Fig. 7A). Therefore, it seems likely that minimal murinization, like complete murinization, enabled preferential pairing of the transferred TCR53 chains. In contrast, the TCR53wt chains seemed to pair mainly with endogenous TCRα-chains to form mixed TCR heterodimers as nearly no function was seen with TCR53wt, although the TCR53α-chain was expressed. To validate that murinization enabled preferential pairing in comparison with TCRwt, we analyzed pairing of TCR53α- and TCR53β-chains by FRET. Because no specific Ab to label the TCR53α-chain was available, a myc tag was inserted at the N terminus of the TCR53α-chain. TCR53α-chains were labeled with Cy5-conjugated and TCR53β-chains with PE-conjugated mAbs. Measuring the FRET efficiency from PE (donor) to Cy5 (acceptor) by flow cytometry revealed that the efficiency for TCR53mu was highest (69.5%), followed by TCR53mm (46%) and TCR53wt (27%) (Fig. 7B), indicating that TCRmu chains more often paired with each other than TCRwt chains. TCR53mm reached 66% of FRET efficiency of TCR53mu. This result showed that minimal and complete murinization of TCR enhanced preferential pairing of the transferred TCR chains (Fig. 8) and by this most likely contributed to the improved functional avidity of TCR gene-modified T cells.

To investigate whether an improved binding/association of the TCRα- and TCRβ-chains to the invariant chains (CD3γ, CD3β, CD3ε, and ζ) also contributed to higher functional avidities, we performed communoprecipitation experiments. A myc tag-specific mAb was used to precipitate TCR53 complexes from J76 cells transduced with TCR53wt, TCR53mu, and TCR53mm. Western blot analysis using TCR53-specific mAbs showed that the amount of TCR53- (16 kDa) associated with TCR53α increased from wt to mm and mu (Fig. 7C). The same lysates of TCR53wt-, TCR53mu-, and TCR53mm-transduced T cells were used to immunoprecipitate TCR53 chains directly. Staining with TCR53-specific mAb revealed

FIGURE 5. Minimal murinization enhances the tumor recognition of transduced primary T cells. A, J76 cells were transduced with combinations of wt, mu, and mm TCR chains of the renal cell carcinoma-reactive TCR53 and analyzed for TCR expression (CD3) 4 d after transduction (gray histograms) in comparison with ut cells (white histograms). Percentages of TCR-positive J76 cells are indicated. B, PBMCs were isolated from healthy donors and transduced in parallel to the J76 cells. Fifteen days after isolation, PBLs were cocultured with the renal cell carcinoma cell line RCC-26, and concentrations of released cytokines were analyzed.

FIGURE 6. Minimal murinization enhances the tumor recognition of transduced primary T cells. A, J76 cells were transduced with combinations of wt, mu, and mm TCR53-transduced T cells (Supplemental Fig. 3) as before for non–codon-optimized TCRs, we concluded that the increased function of mu TCR is not due to the usage of more optimal codons.

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To get an insight of which mechanism(s) led to the improved function of T cells transduced with (minimally) murinized TCR, we first analyzed the expression of the TCR53β-chain. Staining with Vβ20-specific mAbs revealed similar levels of TCR53β after transduction with TCRwt, TCRmu, and TCRmm into PBLs (Fig. 7A). Therefore, it seems likely that minimal murinization, like complete murinization, enabled preferential pairing of the transferred TCR53 chains. In contrast, the TCR53wt chains seemed to pair mainly with endogenous TCRα-chains to form mixed TCR heterodimers as nearly no function was seen with TCR53wt, although the TCR53α-chain was expressed. To validate that murinization enabled preferential pairing in comparison with TCRwt, we analyzed pairing of TCR53α- and TCR53β-chains by FRET. Because no specific Ab to label the TCR53α-chain was available, a myc tag was inserted at the N terminus of the TCR53α-chain. TCR53α-chains were labeled with Cy5-conjugated and TCR53β-chains with PE-conjugated mAbs. Measuring the FRET efficiency from PE (donor) to Cy5 (acceptor) by flow cytometry revealed that the efficiency for TCR53mu was highest (69.5%), followed by TCR53mm (46%) and TCR53wt (27%) (Fig. 7B), indicating that TCRmu chains more often paired with each other than TCRwt chains. TCR53mm reached 66% of FRET efficiency of TCR53mu. This result showed that minimal and complete murinization of TCR enhanced preferential pairing of the transferred TCR chains (Fig. 8) and by this most likely contributed to the improved functional avidity of TCR gene-modified T cells.

To investigate whether an improved binding/association of the TCRα- and TCRβ-chains to the invariant chains (CD3γ, CD3β, CD3ε, and ζ) also contributed to higher functional avidities, we performed communoprecipitation experiments. A myc tag-specific mAb was used to precipitate TCR53 complexes from J76 cells transduced with TCR53wt, TCR53mu, and TCR53mm. Western blot analysis using TCR53-specific mAbs showed that the amount of TCR53- (16 kDa) associated with TCR53α increased from wt to mm and mu (Fig. 7C). The same lysates of TCR53wt-, TCR53mu-, and TCR53mm-transduced T cells were used to immunoprecipitate TCR53 chains directly. Staining with TCR53-specific mAb revealed
localize the various relevant residues that we identified within the TCR sequence (V-92, D-91, A-22, I-133, A-136, and H-139), which is mainly responsible for the stability of the TCR complex. This stabilizing effect was not restricted to lysine but could also be achieved with arginine as a further basic amino acid. Interestingly, mice seem to be exceptional when compared with other mammals, because they have a further basic amino acid. This exchange from a negatively charged, respectively, this could lead to a more stable interaction of the two TCR chains. However, because the human sequence also has an acidic amino acid at position 91 of the TCR, there is no obvious explanation why it is important that this area of the TCR complex is more stable when mu or mm TCR chains were incorporated.

**Discussion**

One precondition for successful adoptive therapy with TCR gene-modified T cells is a sufficient cell surface expression of the transferred therapeutic TCRs. Many efforts have been made to increase TCR protein levels and to enhance the preferential pairing and stability of transferred TCR combinations (13). One of these strategies evolved from the finding that murine TCRs are more stably expressed on human T cells than human TCRs (11, 25, 26). Therefore, human TCRs were equipped with murine C regions to enhance their expression. This “murinization” resulted in a higher functional avidity of T cells modified with different TCRs (26, 34), but also involves the risk of immunogenicity, because of the mouse gene segments in the TCR gene.

Therefore, we identified the residues within the murine TCRα and TCRβ C regions, which are responsible for the improved functional expression of mu TCRs. In the TCRβ C region, we identified one position (aa 18) to be essential, whereby the human sequence contains an acidic (glutamic acid) and the murine sequence a basic (lysine) amino acid. This exchange from a negatively charged to a positively charged side chain seems to increase the stability of the TCR complex. This stabilizing effect was not restricted to lysine but could also be achieved with arginine as a further basic amino acid. Interestingly, mice seem to be exceptional when compared with other mammals, because they have either lysine (Mus musculus) or arginine (Mus spretus) at this position, whereas other mammals for which TCR sequences are available (chimpanzee, rhesus monkey, rat, rabbit, dog, cattle, sheep, and pig) have glutamic acid at this position. In addition, we found four amino acids (A-22, I-133, A-136, and H-139) in the TCRβ C region that further increased the stability of the TCR. For the TCRα-chain, we identified a segment of four amino acids (S-90, D-91, V-92, and P-93), which is mainly responsible for the enhanced expression of mu TCRs.

We used the structure of the mouse 2C TCR (35) as a model to localize the various relevant residues that we identified within the TCR structure. Interestingly, these amino acids seem to be located in the same area of the TCR. In the structure of the 2C TCR, they are at the base of the crystallized structure at the transition from the Ig domains to the connecting peptides (Fig. 8). For the TCRβ-chain, only the two amino acids K-18 and A-22 are included in the crystallized region of the 2C TCR; the remaining three amino acids of part 4.1 directly follow the crystallized region (orange arrow; Fig. 8) and are, therefore, perhaps also located in the same area as the other important amino acids.

When analyzing the structure of the 2C TCR, it seems plausible that the basic lysine K-18 of the TCRβ-chain and the acidic aspartic acid D-91 of the TCRα-chain interact with each other. As the side chains of these two amino acids can be positively and negatively charged, respectively, this could lead to a more stable interaction of the two TCR chains. However, because the human sequence also has an acidic amino acid at position 91 of the TCRα-chain (E-91), there is no obvious explanation why it is important that this area of the TCRα-chain has to be of murine origin. One could speculate that an altered distribution of in flexible proline residues contributes to the effect, because two prolines at positions 88 and 90 are directly N-terminal to the glutamic acid E-91 in the human sequence. The resultant greater flexibility might facilitate an interaction of a positively charged TCRβ-chain with a negatively charged TCRα-chain. Extracellular binding to CD3 components of the TCR is difficult to predict, because the existing models for CD3 binding are not consistent (36, 37). Moreover, there is no crystal structure of the whole TCR complex, and therefore, a coherent model is still missing. Interactions to CD3 within the membrane are most probably not affected as transmembrane regions are very similar between mice and humans, and decisive residues are conserved across different species (38).

To get an insight into the underlying mechanism(s) of why mu and mm human TCR are superior in comparison with wt TCR, we performed FRET and immunoprecipitation experiments. The FRET data indicate that murinization promotes correct pairing of transferred TCRα- and TCRβ-chains. This holds true—although to a lesser extent—for mm TCR. In addition, we found a stronger association of TCRmu and TCRmm with the invariant TCR chains (as shown for TCRγ binding) in comparison with TCRwt. Whether this is mainly because of the improved pairing of TCRα- and TCRβ-chains to each other (TCR intrinsic effect) or because of a prolonged association of TCRα/β-chains to the invariant TCR chains is not clear. Our data demonstrate that (minimal) murinization did not promote the expression of transferred TCR genes, because 1) similar levels of
TCRβ-chain expression was determined in T cells transduced with TCRwt, TCRmu, and TCRmm, and 2) T cells transduced with codon-optimized TCRwt, TCRmu, and TCRmm revealed the same functional differences in comparison with T cells transduced with non-codon-optimized TCR.

The usage of the mm TCR C regions improved not only the expression of TCRs compared with wt TCRs but also the functionality of transduced PBLs. However, exchange of only nine amino acids did not completely achieve the levels of expression of completely murine C regions. Dependent on the specific TCR, the mm variants yielded between 50 and 85% of TCR expression and function when compared with fully murine TCRs. To achieve effects closer to 100%, it is likely that additional (less prominent) amino acids must also be exchanged. However, when considering clinical application, it is important to balance a somewhat better TCR surface expression against increased risk of immunogenicity because of the insertion of more foreign residues. When considering this point, one could think about further reducing the number of “murine” amino acids within the TCR C region to the single most important lysine at position 18.

The FG loop of the murine TCRβ-chain is a known epitope for Abs (39) and, therefore, most likely also immunogenic in humans. In the mm TCRβ variant, this loop (included in domain β3) was completely humanized, and consequently, the probable immunogenicity of this region could be avoided. In contrast to the FG loop, which is displayed at the surface of the TCR, the identified residues of the murine sequence seem to be buried inside the TCR. Furthermore, this region is covered by the CD3 molecules. Therefore, it is unlikely that the mutated residues in mm TCR serve as an epitope for Abs.

In our experiments, we used the murine TCRβ2 region for substitution because the NY-TCR and TCR53 use a human TCRβ2 region. However, the identified mm TCRβ2 region can also be used to equip human TCRs using a TCRβ1 region. A tyrosinase-specific TCR, which contained a TCRβ1 region, also showed enhanced functionality with the mm C regions (data not shown).

In conclusion, we have identified a set of amino acids in murine TCR C regions which—if used for the replacement of the corresponding counterparts in human TCRs—led to an improved cell surface expression of correctly paired TCR and an increased functional avidity of TCR gene-modified T cells. These results have important implications on the design of TCRs selected for use in TCR gene therapy.

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Disclosures

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References

Cloning strategy for generation of TCRβ variant h4. (A) Two complementary primers binding to the homologue region between domain 3 and 4 (fwd-h4, rev-h4), one primer binding 5’, and one binding 3’ of the TCR gene to pMP71 (fwd, rev) were designed. (B) With these primers and plasmids containing TCRβwt and TCRβmu as templates, two fragments of the TCR with overlapping regions of 29 nucleotides were generated. (C) Subsequently the two PCR products were combined by annealing of the complementary sequences and then the complete TCR gene was amplified via PCR with primers fwd and rev. (D) The gene was finally cloned into the retroviral vector plasmid pMP71 using NotI and EcoRI restriction sites.
Identification of the critical amino acids in the murine TCRα chain. J76/TCR26 cells were transduced with NY-TCRα and TCRβ chains containing different C-regions and analyzed for surface expression of TCR26 (Vβ22) and NY-TCR (Vβ4). Different NY-TCRα constructs contained the following domains of the murine sequence: h1: 52-93 and 96-119; h2: 5-42 and 96-119; h3: 5-42 and 52-93; m2: 52-93; m2.2: 67-93; m2.2.12: 67-87; m2.2.23: 77-93; mm: 90-93.

Due to lower transduction rates compared to the experiment shown in Fig. 1, 3, and 4 also populations of untransduced J76/TCR26 cells are present.
(Minimally) murinized TCR53 chains maintain their improved function compared to human TCR after codon-optimization. Wild type (wt), murinized (mu), and minimally murinized (mm) TCR chains of TCR53 were codon-optimized and transduced into human PBMC. Transduced cells were co-cultivated with RCC-26 cells and supernatants collected after 24 h were analyzed for IFN-γ concentrations.