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

Daniel Sommermeyer and Wolfgang Uckert

*J Immunol* 2010; 184:6223-6231; Prepublished online 5 May 2010;
doi: 10.4049/jimmunol.0902055
http://www.jimmunol.org/content/184/11/6223

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/05/03/jimmunol.0902055.DC1

**References**

This article cites 39 articles, 14 of which you can access for free at:
http://www.jimmunol.org/content/184/11/6223.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Minimal Amino Acid Exchange in Human TCR Constant Regions Fosters Improved Function of TCR Gene-Modified T Cells

Daniel Sommerrmeyer* 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 TCRαβ combination on recipient T cells is 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 therapeutic 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 TCR-C-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

---

*Max Delbrück Center for Molecular Medicine; and †Institute of Biology, Humboldt University Berlin, Berlin, Germany

Received for publication June 29, 2009. Accepted for publication March 16, 2010.

This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich TR36 and Schwerpunktprogramm 1230), the Initiative and Network-Program of the Helmholtz Association within the Helmholtz Alliance on Immunotherapy of Cancer, and the European Union (FP6 Program ATTACK).

Address correspondence and reprint requests to Dr. Wolfgang Uckert, Max Delbrück Center for Molecular Medicine, Robert-Rössle-Straße 10, D-13092 Berlin, Germany. E-mail address: wuckert@mdc-berlin.de

The online version of this article contains supplemental material.

Abbreviations used in this paper: FRET, fluorescence resonance energy transfer; h, human; h1, human domain 1; h2, human domain 2; h3, human domain 3; h4, human domain 4; J76, Jurkat76; mu, murinized; m, murine; MLV, murine leukemia virus; mm, minimally murinized; NY-TCR, NY-ESO-1–specific TCR; ut, untransduced; wt, wild-type.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902055
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—is an important strategy to enhance 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 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 HIV 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 murinized T cells. 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 medium (Life Technologies) supplemented with 10% FCS, 1 mM sodium pyruvate (Life Technologies), and 100 U/ml penicillin/streptomycin. To block unspecific bindings, membranes were incubated in 5% nonfat dry milk in PBS supplemented with 0.1% Tween 20 (Sigma-Aldrich, Munich, Germany). After addition of supernatant, plates were spinoculated with 800 ng/ml of anti-CD3 (1:500 dilution) and 100 μg/ml of anti-CD28 (1:500 dilution) and 100 U/ml of penicillin/streptomycin. After washing, membranes were incubated with anti-mouse IgG (6B10.2; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution. All other constructs were generated by annealing of the complementary sequences. Then, the fragments were cloned using two successive PCR steps. Two fragments of the TCR gene with overlapping regions of 20–30 nt were generated and subsequently combined by annealing of the complementary fragments. The complete TCR gene was amplified via PCR. Finally, the genes were cloned into pMP71 using NotI and EcoRI restriction sites (Supplemental Fig. 1 shows the cloning strategy for TCRβ4 as an example). To introduce single amino acid exchanges, mutations were included in the primer sequences. To perform fluorescence resonance energy transfer (FRET) and flow cytometry-based FRET (21). TCR53 wt, mu, and all partially mu, including the final minimally murinized (mm) TCR genes (wt/mu, mu/mu, wt/mu, and mm/mu) 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 Eco27I restriction site, located 3’ of this domain, was used. All other constructs were cloned using two successive PCR steps. Two fragments of the TCR gene with overlapping regions of 20–30 nt were generated and subsequently combined by annealing of the complementary fragments. The complete TCR gene was amplified via PCR. Finally, the genes were cloned into pMP71 using NotI and EcoRI restriction sites (Supplemental Fig. 1 shows the cloning strategy for TCRβ4 as an example). To introduce single amino acid exchanges, mutations were included in the primer sequences. To perform fluorescence resonance energy transfer (FRET) and flow cytometry-based FRET (21). TCR53 wt, mu, and all partially mu, including the final minimally murinized (mm) TCR genes (wt/mu, mu/mu, wt/mu, and mm/mu) 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 Eco27I restriction site, located 3’ of this domain, was used. All other constructs were cloned using two successive PCR steps. Two fragments of the TCR gene with overlapping regions of 20–30 nt were generated and subsequently combined by annealing of the complementary fragments. The complete TCR gene was amplified via PCR. Finally, the genes were cloned into pMP71 using NotI and EcoRI restriction sites (Supplemental Fig. 1 shows the cloning strategy for TCRβ4 as an example). To introduce single amino acid exchanges, mutations were included in the primer sequences. To perform fluorescence resonance energy transfer (FRET) and flow cytometry-based FRET (21). TCR53 wt, mu, and all partially mu, including the final minimally murinized (mm) TCR genes (wt/mu, mu/mu, wt/mu, and mm/mu) 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 Eco27I restriction site, located 3’ of this domain, was used. All other constructs were cloned using two successive PCR steps. Two fragments of the TCR gene with overlapping regions of 20–30 nt were generated and subsequently combined by annealing of the complementary fragments. The complete TCR gene was amplified via PCR. Finally, the genes were cloned into pMP71 using NotI and EcoRI restriction sites (Supplemental Fig. 1 shows the cloning strategy for TCRβ4 as an example). To introduce single amino acid exchanges, mutations were included in the primer sequences. To perform fluorescence resonance energy transfer (FRET) and flow cytometry-based FRET (21). TCR53 wt, mu, and all partially mu, including the final minimally murinized (mm) TCR genes (wt/mu, mu/mu, wt/mu, and mm/mu) of TCR53 were codon optimized (GENEART, Regensburg, Germany) and then cloned into the vector MP71.

Flow cytometry

Cells were stained using FITC-labeled mAbs directed against human CD8 (BD Pharmingen) and TCRVβ2 (Immunotech, Marseille, France), PE-labeled mAbs directed against human CD3 (BD Pharmingen), TCRVβ4, and TCRVβ20 (Immunotech), and APC-labeled MHC-pentamers (Proimmune, Oxford, U.K.). Fluorescence intensity was measured using a FACS Calibur 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). TCR53α-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-TCRβ20 mAb. Emissions at 770 nm (donor [PE] channel) and 670 nm (acceptor [Cy5] channel) were measured using a FACS Calibur flow cytometer. Data were analyzed with the FLEX software. FRET efficiencies measured with transduced J76 cells were set at 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 × 10⁶) 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-TCR 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 semidry transfer system (1 h, 80 mA). To block unspecific bindings, 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-
TCRα mAbs (dilution 1/1000) and subsequently with goat anti-mouse IgG mAbs coupled to HRP (Santa Cruz Biotechnology) in 5% nonfat dry milk in PBS at room temperature for 1 h for each Ab. Chemiluminescence was measured after adding Western blotting Luminol Reagent (Santa Cruz Biotechnology) with a Lumi-Imager F1 (Roche, Mannheim, Germany).

Cytokine release assay

Untransduced or TCR-modified PBL (1 × 10⁶ per well) were cocultured with 5 × 10⁵ 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 TCRβ26β (Vβ22) and NY-TCRβ (Vβ4) allowed expression of both TCRs to be determined by flow cytometry. J76/TCR26 cells transduced with the NY-TCRwt (αω−βwt) 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-TCRμ (αω+βmu) 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 (αω+βwt and αω+βmu, 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).

Next, we subdivided domains 1 and 4 into smaller parts (part 1.1 included aa 4−13; part 1.2, 18−24; part 1.3, 34−37; part 4.1, 133−139; and part 4.2, 165−179) and replaced each part by the corresponding human sequence. Again, these five constructs were transduced together with the completely mu NY-TCRα−chain gene into J76/TCR26 cells. Flow cytometric analysis revealed that the “murine” amino acids of parts 1.1, 1.3, and 4.2 were not required for an efficient expression of NY-TCR (data not shown). This result was confirmed by the construction of a NY-TCRβ−chain gene in which only parts 1.2 and 4.1 of the murine sequence...
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-TCRβ–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 murine NY-TCRβ–chain and was defined as the mm TCRβ–chain construct (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 TCRβ–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

![](image1)

**FIGURE 2.** Comparison of human and murine TCRCβ2 region reveals clustered differences flanked by homologous regions. **A**, The amino acid sequences of h and m TCRCβ2 region were compared, and four domains covering all differences were defined (gray boxes). Asterisks represent the same amino acid in the murine sequence. **B**, Schematic drawing of four different molecularly cloned TCRβ C regions consisting each of one human and three murine domains. h, human; m, murine.

![](image2)

**FIGURE 3.** Enhanced TCR expression after murinization can be maintained with only five “murine” amino acids of the TCRβ C region. **A–D**, J76/TCR26 cells were transduced with the completely mu NY-TCRα–chain in combination with NY-TCRβ constructs containing different C regions and analyzed for surface expression of TCR26 (Vβ22) and NY-TCR (Vβ4). *D* was taken from Fig. 1A for comparison. **E**, Schematic summary of the procedures to identify the relevant positions in the TCRβ C region.

<p>| Table I. Summary of the fraction of murinization linked to the ability to replace TCR26 |
|------------------|------------------------|------------------------|</p>
<table>
<thead>
<tr>
<th>TCRα + TCRβ Constructs</th>
<th>Murinization (%)</th>
<th>TCR26 Replacement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αwtt+βwtt</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>αmu+βmu</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>αmu+βwt</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>αwtt+βmu</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>αmu+βh1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>αmu+βh2</td>
<td>100</td>
<td>79</td>
</tr>
<tr>
<td>αmu+βh3</td>
<td>100</td>
<td>71</td>
</tr>
<tr>
<td>αmu+βh4</td>
<td>100</td>
<td>79</td>
</tr>
<tr>
<td>αmu+βm1/4</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>αmu+βm1.2/4.1</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>αmu+βm1.2KE/4.1</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td>αmu+βm1.2AS/4.1</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td>αmu+βm1.2NH/4.1</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td>αmu+βm1.2KT/4.1</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td>αmu+βmm</td>
<td>100</td>
<td>13</td>
</tr>
<tr>
<td>αmu+βmmKR</td>
<td>100</td>
<td>13</td>
</tr>
<tr>
<td>αm2+βmm</td>
<td>54</td>
<td>13</td>
</tr>
<tr>
<td>αm2+βmm</td>
<td>8</td>
<td>13</td>
</tr>
</tbody>
</table>

*The TCRβ C region contains an amino acid (arginine at position 18), which is neither of human nor murine origin.*
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 (omm2) (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 TCR modifications (ommm). 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+βmu) (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

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 awtβmu, 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.** 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.
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 TCR53mu. Combinations of one TCR53mm and one TCR53wt chain resulted in IFN-γ release comparable to TCR53wt, showing again that amino acid exchanges in both, TCRα and TCRβ, C 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 TCR53β-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 TCRγ-specific mAbs showed that the amount of TCRγ-chains (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 TCRγ-chains directly. Staining with TCRγ-specific mAb revealed...
cipitates were separated on a SDS-PAGE gel, and the amount of TCR 
munoprecipitated either with TCR 
transduced with TCR53wt, TCR53mu, and TCR53mm were im-
cause of the mouse gene segments in the TCR gene.

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 TCRβ 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 TCR variants and was set as 100%. Shown are relative FRET efficiencies measured on PBLs as percentage of FRET efficiency on J76 cells. C, Protein lysates of J76 cells transduced with TCR53wt, TCR53mu, and TCR53mm were immuno- precipitated either with TCRα (myc)- or TCRζ-specific mAb. Precipitates were separated on a SDS-PAGE gel, and the amount of TCRζ-chains was determined by Western blot analysis.

similar amounts of this protein in all transduced cells. This result indicated that the TCR complex is more stable when mu or mm TCR chains were incorporated.

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 TCRζ 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 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 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 are 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).
TCR53β-chain expression was determined in T cells transduced with TCRwt, TCRmu, and TCRmm, 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 mu C regions. Dependent on the specific TCR, the mm variants yielded between 50 and 85% of TCR expression and function when compared with fully mu 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 immunogenecity 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 immunogenecity 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 functionality with the mm C regions (data not shown).

References


Supplement Fig. 1

A

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>fwd</td>
<td>CCCCTCTTCCAAGCTCAGT</td>
</tr>
<tr>
<td>rev</td>
<td>CAAATTAGGGGATAATGCGCTAAGATGC</td>
</tr>
<tr>
<td>fwd-h4</td>
<td>GCCGAGGCTGCGGCAAGCCAGACCTG7GG</td>
</tr>
<tr>
<td>rev-h4</td>
<td>CCACAGTCTGCTCCGCCAGGCTCGGC</td>
</tr>
</tbody>
</table>

B

PCR 1

PCR 2

C

annealing
+ PCR 3

D

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.