HCV T Cell Receptor Chain Modifications to Enhance Expression, Pairing, and Antigen Recognition in T Cells for Adoptive Transfer

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T cell receptor (TCR)-gene-modified T cells for adoptive cell transfer can mediate objective clinical responses in melanoma and other malignancies. When introducing a second TCR, mispairing between the endogenous and introduced α and β TCR chains limits expression of the introduced TCR, which can result in impaired efficacy or off-target reactivity and autoimmunity. One approach to promote proper TCR chain pairing involves modifications of the introduced TCR genes: introducing a disulfide bridge, substituting murine for human constant regions, codon optimization, TCR chain leucine zipper fusions, and a single-chain TCR. We have introduced these modifications into our hepatitis C virus (HCV) reactive TCR and utilize a marker gene, CD34t, which allows us to directly compare transduction efficiency with TCR expression and T cell function. Our results reveal that of the TCRs tested, T cells expressing the murine Cβ2 TCR or leucine zipper TCR have the highest levels of expression and the highest percentage of lytic and interferon-γ (IFN-γ)-producing T cells. Our studies give us a better understanding of how TCR modifications impact TCR expression and T cell function that may allow for optimization of TCR-modified T cells for adoptive cell transfer to treat patients with malignancies.

INTRODUCTION

The observation of tumor-infiltrating lymphocytes (TILs) present in the tumor lesions has eventually led to the idea of using T cells to target cancer.1,2 Expanding TILs is not feasible for most malignancies, and therefore, an alternative approach in targeting tumor-associated antigens is genetically modifying a patient’s T cells with an antigen-specific T cell receptor (TCR).4–10 Despite the success seen using TCR-gene-modified T cells, there are still hurdles in achieving an effective and safe therapy. When introducing another TCR into a T cell, some of these hurdles include proper TCR expression and function due to mispairing of α and β chains between endogenous and introduced TCRs and proper folding and assembly on the cell surface. Reduced cell-surface expression of the TCR and reduced T cell functionality can also result in impaired therapeutic efficacy against targeted antigens. The chain mispairing can allow for the potential of unanticipated off-target reactivity or autoimmunity, since these TCRs have not been subjected to the process of negative selection.11 Although not seen to date in humans, it has been shown in mouse models that the formation of self-antigen-reactive TCR dimers can result in TCR-gene-transfer-induced lethal graft-versus-host disease.12

One strategy to improve this therapy is to modify the TCR proteins in a way that promotes proper pairing of the introduced TCRs, resulting in a higher level of expression, reduced false pairing, and increased functionality to make a better T cell. Various modifications have been evaluated to directly and indirectly augment proper pairing of introduced TCR chains. These modifications include addition of another disulfide bond in the TCR chains, replacing human constant regions with murine constant regions (murine Cβ1 or murine Cβ2), codon optimization of the transgenic TCR gene, using a leucine zipper fusion protein, and lastly a single-chain TCR that links the variable α domain to the variable β domain followed by the constant β domain.13–18 While all of the TCR modifications explained above have been shown to be successful strategies in promoting proper TCR chain pairing, there has never been a direct comparison of these modifications.

In this study, we can make a direct comparison of these different TCR modifications using our transduction marker, CD34t, as a measure of TCR protein expression. There are no limitations on its cell-surface expression, and consequently, CD34t and the TCR proteins are translated in a 1:1 stoichiometric ratio.20 Using this CD34t transduction marker, we can compare all of the TCR modifications based on this internal reference standard. To determine the optimal TCR modification, we have made modifications to our previously described TCR isolated from an HLA-A2-restricted hepatitis C virus (HCV) NS3:1406-1415-reactive CD8+ T cell clone.21,22 Due to its high

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affinity, in contrast to other TCRs, this TCR can overcome the need for CD8 co-receptor expression.23,24 This is advantageous, because major histocompatibility complex (MHC)-class-I-restricted CD4+ T cells can be generated. Additionally, lower transgene expression is needed to activate the transduced T cells.25

In this study, we made six different modifications to the HCV 1406 wild-type (WT) TCR and determined how each modification impacted the TCR cell surface expression and T cell function compared to the WT TCR. Our results indicate that some modifications do enhance surface expression of the properly paired introduced TCR, and lower transgene expression is needed to achieve proper TCR pairing compared to the WT TCR. It is evident that increased surface expression can result in increased T cell function and this can be attributed to an increase in TCR surface density. Additionally, some modifications lead to an increase in the percentage of transduced T cells exhibiting bi-functionalities, as measured by lytic activity and cytokine release. These results suggest that modified TCRs can be used for gene modifying T cells in adoptive cell transfer to enhance levels of expression of the introduced TCR- and antigen-specific T cell function.

RESULTS
Transduced T Cell Expression of Modified TCRs
One of the challenges in using TCR-modified T cells in adoptive T cell therapy is the potential of TCR chain mispairing between the endogenous and introduced α and β chains. A strategy to promote proper pairing is to make modifications to the TCR genes. Using our HCV 1406 TCR, the following six modifications were made: disulfide bridge (DSB), murine Cb1 and Cb2 (mCb1 and mCb2), codon optimized (CO), leucine zipper (LZ), and single chain (SC) (Figure 1A).13,15,17–19,26 Each modified TCR was inserted into a modified SAMEN retroviral vector (Figures 1B and 1C).10 Peripheral blood mononuclear cells (PBMCs) from three healthy donors were transduced with each of the modified HCV 1406 TCRs. Using CD34 purified transduced T cells, we stained these cells with an anti-CD3 monoclonal antibody (mAb), anti-CD34 mAb, and an HCV 1406 dextramer to measure properly paired TCRs on the cell surface. Flow cytometry plots are shown for one representative experiment (Figure 2); average percentages (three donors and three repeats, nine experiments total) of transduced T cells that express properly paired HCV 1406 TCRs are displayed in Table 1. The double-positive quadrants represent transduced T cells that express properly paired HCV 1406 TCRs. Therefore, by dividing the percentage of double-positive cells by the percentage of CD34+ transduced cells, we calculate the percentage of dextramer-positive cells among the transduced T cells population as a whole. These data reveal that T cells transduced with the DSB TCR and the SC TCR have a lower percentage of properly paired TCRs expressed (averaging 33% and 36%, respectively) than the average WT TCR (42%). T cells transduced with the mCb1 TCR and CO TCR have a percentage of properly paired TCRs expressed (averaging 46% and 42%, respectively) similar to T cells transduced with the WT TCR, while T cells transduced with the mCb2 TCR and LZ TCR have more properly paired HCV 1406 TCRs expressed on the cell surface (65% and 71%, respectively). In addition to comparing the percentages of properly paired HCV 1406 TCRs among transduced T cells, it is important to also determine the relationship between CD34 expression and properly paired TCRs expressed on a per-T cell basis. Therefore, the double-positive quadrant in these flow plots are important, because this population represents transduced T cells that express the properly paired introduced HCV 1406 TCR. To further examine the relationship between transgene expression and proper TCR pairing, we compared all these double-positive quadrants together to compare fitted lines derived
from the compensated log X, Y values collected for each HCV 1406 TCR in all nine experiments (Figure 3). In focusing on the Y-intercepts of the fitted lines for each HCV 1406 TCR, it is evident that mCβ2 HCV 1406 TCRs and LZ HCV 1406 TCRs need much lower transgene (CD34) expression, than the WT HCV 1406 TCRs in order to properly pair on the cell surface (p < 0.0001 for each, compared to WT). For example, at ~5,000 units of CD34, the unit of dextramer (properly paired introduced TCRs) is ~1,000 for the WT HCV 1406 TCR, while at the same 5,000 units of CD34, the mCβ2 HCV 1406 TCRs and LZ HCV 1406 TCRs are ~5,000 units of dextramer. Conversely, the SC HCV 1406 TCR and the DSB HCV 1406 TCR need higher levels of transgene expression to achieve the same levels of properly paired HCV 1406 TCRs on the cell surface. Lastly, mCβ1 HCV 1406 TCRs and CO HCV 1406 TCRs need slightly lower transgene expression than the WT HCV 1406 TCRs in order to properly pair on the cell surface. These data indicate that compared to WT TCRs, mCβ2 and LZ TCR modifications can allow for an increase in properly paired TCRs and lower transgene expression is needed for expression properly paired TCRs on the surface. This is important, because upon introducing a new TCR into a T cell, not all T cells in the population will obtain the same levels of TCR expression. Here, we demonstrate that the mCβ2 and LZ TCR modifications can result in higher levels of surface expression, even at much lower levels of transduction compared to the WT TCR. Therefore, mCβ2 or LZ TCR modifications could compensate for drawbacks such as low transgene expression or transduction efficiency.

Modified TCR Function

Our results indicate that T cells transduced with the mCβ2 TCR or the LZ TCR have an increase in cell surface expression. We predict that this increase in properly paired TCRs would result in an increase in T cell function. Cytokine release assays were done on the same cultures to measure the amount of interferon-γ (IFN-γ) released upon antigen stimulation (Figure 4). Generally, T cells expressing a modified HCV 1406 TCR resulted in T cells that functioned similar or worse than T cells expressing the HCV 1406 WT TCR when cocultured with pulsed T2 cells (Figure 4). One exception is that T cells expressing the mCβ2 TCR displayed an increase in IFN-γ release compared to T cells expressing WT TCR. It appears that increases in properly paired TCRs might not correlate to T cell function; however, these cocultures contain an artificially high level of presented antigen and therefore, tumor cells were used to measure antigen reactivity against physiological levels of antigen (Figure 4). All T cells expressing a modified HCV 1406 TCR functioned better than T cells expressing the WT HCV 1406 TCR. Specifically, T cells transduced the mCβ2 TCR or the LZ TCR secreted the most IFN-γ when stimulated with HepG2:NS3+ tumor cells. It is evident from an artificial peptide presentation system, such as T2, can overcompensate for any reactivity or functional hindrances of the mispaired TCRs. In conclusion, these data indicate that T cells transduced with any of our TCR modifications secrete more IFN-γ and function better against tumor lines presenting naturally processed antigen than T cells transduced with the WT TCR.

Modified TCR Bi-function

It is important to note that preceding data were generated from bulk transduced CD4+ and CD8+ T cell populations and thus do not take into account the functionality of an individual transduced T cell. To further assess the function of these transduced T cells on a per cell basis in regards to CD4+ T cells and CD8+ T cells, we performed a lytic and intracellular cytokine production assay. Each transduced T cell line was cocultured with T2 cells loaded with the HCV 1406 peptide, irrelevant tyrosinase peptide, HepG2:NS3+ tumor cells, or HepG2:NS3+ tumor cells. CD107a expression was measured as a surrogate marker for lytic activity and cells were permeabilized and fixed to measure intracellular IFN-γ production. A representative flow cytometry plot showing CD107a expression and intracellular IFN-γ production (Figure 5A). The values from these four quadrants were used to generate pie charts portraying the percentage of transduced T cells that were non-reactive, lytic only, IFN-γ producing only, and bi-functional (lytic and IFN-γ producing) for CD4+ or CD8+ T cells cocultured with peptide loaded T2 cells and CD8+ T cells cocultured with tumor cells (Figures 5B–5D). Tables S1–S3 summarizes these data among three donors preformed in triplicate, nine experiments total. CD4+ T cells are not depicted because they have low reactivity against the tumor cell line. The data reveals that T cells expressing the mCβ2 TCR or the LZ TCR have a higher percentage of reactive and bi-functional T cells than T cells expressing the WT TCR. This is evident in both CD4+ and CD8+ T cell populations when
stimulated with both peptide loaded targets or tumor targets. T cells transduced with the DSB, mCβ1, CO, or SC TCR generally had an equal or lower percentage of reactive and bi-functional T cells compared to T cells transduced with the WT TCR. These data suggest that on a cell-to-cell basis, T cells transduced with TCRs modified with the mCβ2 or the LZ can result in higher percentages of bi-functional T cells. It is also evident from these data that T cells transduced with the mCβ2 TCR or the LZ TCR demonstrated some cross reactivity against T2s pulsed with tyrosinase or HepG2:NS3 tumor cells. Approximately 3%–9% of transduced T cells demonstrated a basal level of reactivity against media alone depending on the donor (data not shown), which was not reproducible throughout the nine total experiments; however, it is possible that enhanced expression of a high affinity TCR could result in off target reactivities.

Additionally, we examined the impact of TCR expression on T cell function by establishing there CD34 gates that represent high, medium, and low/no levels of transduction (Figure 6). It is evident that the T cell populations in the low levels of transduction gate is still capable of producing IFN-γ, indicating the flow cytometer is not sensitive enough to detect very low levels of transduction, yet these T cells are still functional. CD8+ T cells expressing the mCβ2 TCR or the LZ TCR have the highest percentage of IFN-γ expression in this low/no level of transduction gate, indicating that even T cells with undetectable CD34 expression (due to sensitivity of the flow cytometer) are still capable of being more functional than T cells expressing the WT HCV 1406 TCR. These observations are consistent with the previous studies that have demonstrated that only approximately three to ten peptide MHC (pMHC) interactions are needed for T cell activation. To verify that T cells falling in the CD34− gate could still have undetectable levels of transduction, we examined the median florescence intensity (MFI) of CD34 in the CD34− IFN-γ− and CD34− IFN-γ+ quadrants (Table 2). It is clear that transduced T cells in the CD34− IFN-γ− quadrant do express very low levels of CD34, determined by the enhanced CD34 MFI compared to untransduced T cells. Additionally, transduced T cells in the CD34− IFN-γ− quadrants display an even higher CD34 MFI than T cells in the CD34− IFN-γ+ quadrants, further supporting the hypothesis that even T cells with undetectable levels of transduction can be functional. T cells expressing the mCβ2 TCR or the LZ TCR also had the highest MFI of IFN-γ in the CD34− IFN-γ− quadrant, indicating that on a per-cell basis, these populations were producing the most IFN-γ. In conclusion, the mCβ2 TCR and the LZ TCR modifications result in functional T cells, even with very low undetectable transgene expression.

We next wanted to determine if the increase in bi-functionality in T cells expressing the mCβ2 TCR or the LZ TCR was due to changes in the way the TCR interacted with pMHC or directly due to an increase in TCR surface density. T cells transduced Jurkat E6.1 (JE6.1) cells with the WT TCR, mCβ2 TCR, or the LZ TCR and similarly transduced another JE6.1 population that has been transduced to express the CD8 αβ co-receptor. Each transduced JE6.1 cell line was cocultured with T2s pulsed with HCV 1406–1415 peptide or with alanine substituted peptides. If an alanine was already present at a residue, an isoleucine was substituted. When using a panel of alanine-substituted peptides, changes in T cell reactivity against specific structural changes in the peptide can be observed. We measured IL-2 release via ELISA (Figure 7). It appears that the presence of CD8 increases the magnitude of the IL-2 response but does not alter the ability of TCRs to recognize substituted peptides. It is evident that all recognition is lost upon alanine substitution at position 1, independent of which TCR the T cells are expressing, suggesting that this lysine at position 1 in the peptide is critical for TCR recognition. Additionally, it appears positions 4–7 in the peptide are important for recognition due to the loss of IL-2 release upon substitution. This would be predicted due to the kink in the middle of the HCV 1406–1415 peptide seen in the crystal structure. Overall, there are no changes in the pattern of recognition between T cells expressing with the WT TCR, mCβ2 TCR, or LZ TCR, indicating that the increases seen in T cell functionality in T cells expressing the mCβ2 TCR or the LZ TCR is not due to these modifications possibly altering the TCR/pMHC interaction.

### DISCUSSION

One of the challenges in introducing a new TCR into a T cell is the potential for α and β chain mispairing between endogenous and introduced TCR chains. This study focused on comparing six TCR modifications that have been shown to promote TCR chain pairing. Our data reveal that the mCβ2 TCR and LZ TCR promote proper pairing due to the increased surface expression of HCV 1406 TCRs on transduced cells. Additionally, it appears that the mCβ1 TCR and CO TCR do not have an effect on pairing, since they expressed similarly to the WT TCR. Lastly, it appears that the DSB TCR and the SC TCR do not promote proper TCR chain pairing and could possibly hinder it due to the lower than WT TCR expression levels on transduced T cells. The LZ TCR had the highest level of properly paired TCRs on transduced T cells, and it was evident that low levels of CD34 transgene expression are needed for higher levels of properly paired TCRs on the cell surface. The heterodimerization motifs of the c-Jun and v-Fos proteins present on the C-terminal ends of the α and β chains, respectively, have a high affinity for each other and will result in a favorable heterodimer formation.
In addition to the LZ TCR, the mCβ2 TCR exhibits an increase in TCR cell surface expression in comparison to WT. By substituting human with murine constant regions, a murine TCR chain cannot pair with a human TCR chain, and this essentially can eliminate the chance of potential mispairing. Additionally, it has been shown that murine constant regions have a higher affinity and thus stability with the CD3ζ chain. This can not only stabilize the TCR on the cell surface but also increase the chance this mCβ2 TCR will bind to the CD3 complex in the endoplasmic reticulum. Surprisingly, generally only 45% of the T cells transduced with the mCβ1 TCR expressed the properly paired HCV 1406 TCR. This is significantly less than the mCβ2 and more comparable to T cells expressing the WT TCR. There are five amino acids in the murine β constant region are shown to be important for an increase in TCR surface expression; the Cβ2 construct contains all five amino acids, while the Cβ1 construct contains only three. The decreased levels of TCR cell surface expression seen in the mCβ1 TCR in comparison to the mCβ2 TCR could be a result of lacking the latter three important amino acids. Based on these results, substituting human constants regions with murine constant regions, specifically the Cβ2 region, is an effective way to enhance proper pairing of the introduced TCR chains.

Similar to the mCβ1 TCR, the CO TCR was expressed in levels comparable to the WT TCR. One reason codon optimization could enhance surface pairing is that protein translation will be increased, and therefore more introduced TCR protein will allow for an increase in the likelihood of proper pairing due to more protein being made and thus present in the endoplasmic reticulum. One explanation as to why we did not see increased pairing with this TCR could be that despite the increase in protein being made, there is no driving force to actually sustain proper pairing. Unlike the LZ, murine, and CO TCRs, the DSB and SC TCRs displayed lower levels of TCR expression than the WT TCR. Additionally, it was shown these two modified TCRs needed higher levels of transgene expression to maintain expression levels of properly paired HCV 1406 TCRs that were comparable to the WT HCV 1406 TCR. One explanation for the low DSB HCV 1406 TCR expression could be that the modification is minimal and only changes one amino acid in each α and β chain. Therefore, there is not a large driving force for proper pairing. Also, because there is this additional cysteine in the α and β chains, this cysteine could improperly pair with the cysteine present in the hinge region. This could lead to improper formation of the TCR if the constant region of one chain potentially forms a disulfide bond with the transmembrane domain of another chain via their cysteine residues. The SC TCR links the two variable regions with a 15-residue glycine linker to allow for correct folding. The three-domain TCR (Vα-Vβ-Cβ) is made as a separate protein from the Cζ single domain. The proper folding of this three-domain TCR may not be favorable. Another potential factor that could contribute to expression is TCR protein stability. However, this would still contribute to the net effect of more or less TCR pairing, and while mechanisms of TCR protein stability on the surface might be important, we are focused on examining T cell function for enhancing gene therapy. In conclusion, due to low percentages of T cells expressing properly paired DSB HCV 1406 TCRs and properly paired SC HCV 1406 TCRs, these gene modifications are not an effective way to augment proper TCR chain pairing. We hypothesized that expression levels of the modified TCRs would correlate to T cell function; our results indicate that this is not fully accurate. It is apparent that high levels of antigen presentation could compensate for poor antigen reactivity due to mispaired TCRs (Figure 4). In a lower-antigen-density tumor model, it becomes evident that all the TCR modifications that promote proper pairing can increase T cell function (Figure 4). One surprising result was that T cells expressing the DSB TCR or the SC TCR displayed the lowest levels of properly paired HCV 1406 TCRs on the cell surface; however, these T cells had a slight increase in tumor reactivity above T cells expressing the WT HCV 1406 TCR. The results suggest that large amounts of IFN-γ was being released by a small amount of T cells expressing properly paired DSB or SC HCV 1406 TCRs. For the DSB TCR, the formation of the disulfide bond might not enhance proper chain pairing but properly paired TCRs that do make it to the cell surface have an increase in stability and thus, allows the TCR to consistently function without being degraded. For the SC TCR, the unique TCR structure may not be favorable to form, associate with...
We also determined that this increase in functionality is most functional when expressing either the LZ TCR or the mC TCR. Extremely low and undetectable levels of transgene expression could not pair easily. Consequently, having too many high-affinity TCRs on the cell surface could be detrimental in overall T cell function or could possibly result in cross-reactivity of off targets. Only analysis of other TCRs with different affinities and pairing properties would lead us to know how broadly applicable these conclusions are. Thus, it is critical to examine TCR expression and T cell function independently in the process of comparing these different modifications.

MATERIALS AND METHODS

Cell Lines, Media, and Reagents

T2, HEK293GP, PG13, and HepG2 cell lines were obtained from the American Type Culture Collection. HepG2 tumor cell lines have been engineered to express the full-length HCV NS3 protein. All medium components were obtained from Corning Life Sciences, unless otherwise noted. Jurkat E6.1 cells have been engineered to express the full-length HCV NS3 protein. All medium components were obtained from Corning Life Sciences, unless otherwise noted. Jurkat E6.1 cells have been engineered to express the full-length HCV NS3 protein. All medium components were obtained from Corning Life Sciences, unless otherwise noted. Jurkat E6.1 cells have been engineered to express the full-length HCV NS3 protein. All medium components were obtained from Corning Life Sciences, unless otherwise noted. Jurkat E6.1 cells have been engineered to express the full-length HCV NS3 protein.

TCR Pairing Modifications on Specific Antigen Recognition

Figure 4. Impact of TCR Pairing Modifications on Specific Antigen Recognition

T cells transduced with the WT or modified HCV 1406 TCR were cocultured in a 1:1 ratio with stimulators. IFN-γ release was measured by ELISA. Values represent an average of triplicate wells. One representative experiment and donor is shown (out of nine total experiments). Error bars indicate mean ± SEM. Transduced T cell reactivity against T2 cells pulsed with 10 ng/mL HCV NS3:1406-1415 peptide or control tyrosinase:368–376 peptide (top graph) and transduced T cell reactivity against HepG2 and HepG2:NS3* tumor lines (bottom graph).

The purpose of this study was to compare the six TCR pairing modifications by their surface expression in comparison to the WT TCR, as well as T cell function. By doing this, we could determine if there is an optimal TCR modification that would be advantageous to use in TCR-gene-modified T cells for adoptive T cell therapy. Our study revealed that although some modifications increased properly paired TCRs on the surface and some did not, IFN-γ production upon tumor cell stimulation was increased compared to cells expressing the WT TCR. Additionally, T cells expressing the LZ TCR or the mC2 TCR lead to an increased percentage of antigen-specific bi-functional T cells. We acknowledge that our system uses only one antigen-specific TCR; nonetheless, none of the modifications alter the VJ or VDJ regions of the TCR, and we have demonstrated that the LZ and mC2 TCR modifications do not alter the way in which the TCR recognizes pMHC. By comparing all these TCR modifications, we elucidated modifications that could be beneficial for low-affinity TCRs or TCRs that do not pair easily. Consequently, having too many high-affinity TCRs on the cell surface could be detrimental in overall T cell function or could possibly result in cross-reactivity of off targets. Only analysis of other TCRs with different affinities and pairing properties would lead us to know how broadly applicable these conclusions are. Thus, it is critical to examine TCR expression and T cell function independently in the process of comparing these different modifications.
human IL-15 (rhIL-15; Biologic Resources Branch, NCI, NIH) at 37°C in a humidified 5% CO₂ incubator.

**Peptides**

HCV NS3:1406-1415 (KLVALGINAV) and Tyrosinase: 368-376 (YMDGTMSQV) were obtained from Synthetic Biomolecules and were HPLC purified to 95% purity. Peptides were stored at 5 μg/mL in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) at −80°C.

**TCR Modifications**

Six modified TCRs were made using the WT HCV TCR sequence. A DSB was introduced into the WT TCR in the SAMEN vector by making changes T178C and S721C in the constant region of the α and β chains, respectively by site directed mutagenesis. Primers used for mutagenesis were α forward, 5'-GTATATCAGACAAATGTTGCTAGACATGAGG-3'; α reverse, 5'-CCTCATGTC TAGACACAGTTTGTCTGTGATATAC-3'; β forward, 5'-GTGCA CAGTGGGCTGTGCAGACCCGACGC-3'; and β reverse, 5'-GC TGCGGCTGTGCAGACCCCACTGTCGAC-3'. All other HCV 1406-modified TCRs were synthesized by GenScript and provided in the pUC57 vector (GenScript).

**Retroviral Vector Construction**

MCβ1 and mcβ2, CO, LZ, and SC modified HCV 1406 TCRs were all subcloned from the pUC57 vector to the pCR2.1 vector, containing our CD34t transduction marker, then into the SAMEN vector. Briefly, pUC57 vector DNA was digested with Not I and EcoRI restriction enzymes (Thermo Scientific), and products were separated on a 1% agarose gel. DNA bands corresponding to correct length of the TCR were excised from the gel and purified using a gel purification kit (QIAGEN). The TCR cassettes were ligated into the pCR 2.1 shuttle vector and screened for recombinant clones with the insert in the correct orientation. DNA was digested with Not I and BamHI enzymes (Thermo Scientific), and products were separated on a 1% agarose gel. The TCR/CD34t cassettes were ligated into the SAMEN vector with compatible restriction sites. All HCV 1406 TCR constructs in SAMEN vector were sequenced (Genewiz) to ensure no errors had occurred and insert was in the correct orientation.

**Generating High-Titer Retroviral Supernatant**

Using a HEK293GP packaging cell line, retroviral supernatants for each modified HCV 1406 TCR were prepared by transient transfection, and then these supernatants were used to make stable PG13 retroviral producer cell lines as described previously. Briefly, HEK293GP cells were transiently co-transfected with 20 μg retroviral SAMEN vector DNA and 5 μg of a plasmid containing the vesicular stomatitis virus (VSV) envelope gene using 50 μL Lipofectamine 2000 (Invitrogen). Transfection medium was replaced 6 hr later with 10 mL fresh DMEM medium and incubated for 48 hr at 37°C in 5% CO₂. 48 hr later, HEK293 viral supernatants were harvested, filtered, and used to transduce PG13 cells. Plates were incubated for 72 hr at 37°C in 5% CO₂ and then PG13 cells were stained using anti-CD34-PE mAb (BioLegend) and analyzed for CD34 expression by flow cytometry. CD34-positive PG13 cells were sorted for high and uniform expression using a BD FACS Aria cell sorter (BD Biosciences). To make high-titer retroviral supernatant, HCV 1406 TCR-expressing PG13 cell lines were seeded overnight at 8 × 10⁶ cells/T-175 flask at 37°C in 5% CO₂ on day 1. On day 2, complete Iscove’s DMEM supplemented with 1 mM sodium butyrate (Sigma-Aldrich) and 10 mM HEPES was added to flasks for 8–10 hr to stimulate virus production. T cells transduced for 5 hr in a 1:1 ratio by T2 cells loaded with HCV 1406 peptide. Immunofluorescence analysis analyzed cells for CD34 and intracellular IFN-γ expression. Collected events were gated on CD8+ T cells. High, medium, and low/none subgates indicate levels of transduction by CD34. One representative experiment and donor is shown (out of nine total experiments).
production. Media was then replaced with fresh complete medium and incubated overnight at 37°C in 5% CO₂. Viral supernatants were collected on day 3 and filter sterilized to remove any cellular debris using 0.45-μm filters (Thermo Scientific).

**PBMC and Jurkat E6.1 Transduction**

T cells derived from normal healthy donors were activated prior to transduction using 50 ng/mL anti-CD3 mAb (Miltenyi Biotec), 300 IU/mL rhIL-2 (rhIL-2; Novartis Pharmaceuticals), and 100 ng/mL rhIL-15 (NCI-Frederick) on day 0. T cells, Jurkat E6.1 cells, and CD8⁺ Jurkat E6.1 cells were transduced by spinoculation on day 3 as described elsewhere.10,31 Transduced T cells or Jurkat E6.1 cells were purified by positive selection using anti-CD34 magnetic beads (Miltenyi Biotec) and maintained in complete T cell medium or Jurkat cell medium. The T cells were used in functional assays beginning on day 13.

**Immunofluorescence Staining**

T cell surface markers were stained by immunofluorescence using the following mAbs: anti-CD4-PE/Cy7, anti-CD8-PerCP/Cy5.5, anti-CD3-antigen-presenting cell (APC)/Cy7, and anti-CD34-PE (BioLegend). Surface expression of properly paired HCV 1406 TCRs were stained by using APC-labeled HLA-A*0201 dextramers folded around the HCV NS3:1406–1415 peptide (Immudex). Cells were analyzed by using a BD FACSCanto II instrument (BD Biosciences) by collecting 5 × 10⁴ events and the data was analyzed using FlowJo software (FlowJo Enterprise).

**Cytokine Release Assay**

Antigen reactivity of the HCV 1406 TCR transduced T cells was measured in cytokine release assays as described previously.31 Briefly, all peptide loaded T2 stimulators were pulsed with 10 μg/mL HCV 1406 peptide, alanine-substituted HCV 1406 peptides, or the tyrosinase 368–376 peptide for 2 hr prior to coculture. 1 × 10⁵ washed

![Image](https://example.com/image.png)

**Figure 7. Impact of TCR Pairing Modifications on Structure Changes of the HCV NS3 Peptide**

JE6.1 cells (with or without CD8⁺) transduced with the WT, mCβ2, or LZ TCR were cocultured in a 1:1 ratio with T2s pulsed with HCV NS3 Alanine-substituted peptides or negative control tyrosinase. IL-2 release was measured by ELISA. Values represent an average of triplicate wells. Error bars indicate mean + SEM. One representative experiment is shown (out of three independent repeats).

| Table 2. MFI of CD34 and IFN-γ in CD34⁺ Gates |
|-----------------|-----------------|-----------------|-----------------|
| TCR             | CD34 MFI in CD34⁺ IFN-γ⁺ | CD34 MFI in CD34⁺ IFN-γ⁻ | IFN-γ MFI in CD34⁺ IFN-γ⁺ |
| Untransduced    | 199              | 333              | 774              |
| Wild-type       | 383              | 467              | 2,593            |
| Disulide bridge | 389              | 471              | 2,099            |
| Murinized Cβ1    | 363              | 484              | 2,433            |
| Murinized Cβ2    | 359              | 464              | 3,777            |
| Codon optimized  | 354              | 458              | 2,405            |
| Leucine zipper   | 366              | 445              | 3,234            |
| Single chain     | 385              | 477              | 2,056            |
and re-suspended responder T cells and stimulator cells (peptide-loaded T2 cells or HepG2 tumor cells) were cocultured in a 1:1 ratio in triplicate in 96-well U-bottom tissue culture plates in 200 µL medium. Cocultures were incubated at 37°C in 5% CO2 for 20 hr. PMA (phorbol myristate acetate) was added to cocultures using Jurkat cells at 10 ng/mL. Plates were spun at 1,500 RPM and supernatants were collected. The amount of cytokine release was measured by sandwich ELISA using monoclonal antibodies to IFN-γ or IL-2 (BioLegend).

**CD107a Cytoytic and Intracellular Cytokine Assay**

CD107a expression was used as a surrogate marker to assess the lytic function of HCV 1406 TCR transduced T cells. HCV 1406 TCR transduced T cells were cocultured with stimulators using methods similar to the cytokine release assay described above. Stimulators included T2 cells loaded with HCV:1406–1415 or the tyrosinase: 368–376 peptide and tumor targets (HepG2 or HepG2 expressing the full length HCV NS3 protein). 3 × 10^5 responder and stimulator cells were cocultured in a 1:1 ratio in 96-well U-bottom tissue culture plates in 200 µL complete medium. 5 µL anti-CD107a mAb, 5.0 ng/mL brefeldin-A, and 2.0 nM monensin (BioLegend) were added at the start of coculture. Cocultures were incubated at 37°C in 5% CO2 for 5 hr, and cells were stained for HCV NS3:1406–1415 dextramer for 10 min and cell surface antigens for 20 min at room temperature. Next, cells were fixed in fixation buffer for 20 min, treated in permeabilization and wash buffer (BioLegend) for 20 min, stained for intracellular IFN-γ using anti-INF-γ-BV-421, and analyzed by flow cytometry.

**Statistical Analysis**

Comparisons of modified TCRs versus WT cell percentages were evaluated using random effects linear regression. In Figure 3, for each modified TCR, a linear regression was fit including cell percentages for the WT and the modified TCR of interest as the outcome. Main effects included an indicator of WT versus modified TCR and indicators for replicates. Random effects were included to account for donor. Using the model results, the coefficient for modified TCR (versus WT) represents the difference in cell percentages, adjusted for batch (i.e., replicate) effect and donor. A Wald test was used to generate a p value for the difference between cell percentages. Data shown in (Table 1 and S1–S3) and Figure 4 were analyzed using random effects linear regression to model functional changes in peptide and tumor reactivity (with log fold change as the outcome), with random effects included to adjust for donor.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three tables and can be found with this article online at http://dx.doi.org/10.1016/j.omto.2017.05.004.

**AUTHOR CONTRIBUTIONS**

K.C.F., computation and design, data acquisition, writing and revision of manuscript, development of methodology, analysis, and interpretation of data; T.T.S. and D.C.M., computation and design and data acquisition; K.N., development of methodology; E.G.-M., statistical analysis of data; M.I.N., development of methodology, interpretation of data, writing, review, and/or revision of manuscript, study supervision.

**CONFLICTS OF INTEREST**

The authors have no conflict of interest.

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