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Redirecting Ag specificity by transfer of TCR genes into PBLs is an attractive method to generate large numbers of cytotoxic T cells for immunotherapy of cancer and viral diseases. However, transferred TCR chains can pair with endogenous TCR chains, resulting in the formation of mispaired TCR dimers and decreased or unspecific reactivity. TCR gene transfer into hematopoietic stem cells (HSCs) is an alternative to create T cells with desired Ag specificity, because in this case expression of endogenous TCR chains is less likely owing to allelic exclusion. We generated TCR-transduced T cells from peripheral T cells using the lymphocytic choriomeningitis virus–specific P14 TCR. After transfer of the P14 TCR genes into HSCs and subsequent reconstitution of irradiated mice, TCR-engineered HSC-derived T cells were produced. We then compared the Ag-specific T cell populations with P14 TCR-transgenic T cells for their therapeutic efficiency in three in vivo models. In this study, we demonstrate that TCR-transduced T cells and TCR-engineered HSC-derived T cells are comparable in controlling lymphocytic choriomeningitis virus infection in mice and suppress growth of B16 tumor cells expressing the cognate Ag in a comparable manner. The Journal of Immunology, 2014, 192: 000–000.

Adoptive transfer of Ag-specific CTLs has shown great potential in treatment of cancer and viral diseases (1–4). However, a major obstacle is the generation of sufficient numbers of in vivo functional Ag-specific CTLs because the isolation of tumor-infiltrating lymphocytes is difficult for many tumor entities. Moreover, the isolated lymphocytes are often functionally impaired. Additionally, extensive ex vivo expansion of tumor-infiltrating lymphocytes, which is a prerequisite for a clinical application, promotes T cell exhaustion (5, 6). Therefore, this approach is not suitable for most cancer patients.

The ideal effector T cell for adoptive therapy should be 1) available in sufficient numbers, 2) specific for the targeted Ag, and 3) possess long-lasting in vivo persistence as a prerequisite for successful therapy (7).

The redirection of Ag specificity by transfer of TCR genes into PBLs is an established method to generate sufficient numbers of CTLs with the desired specificity. This approach is currently being applied in several clinical trials (8–11). However, the transfer of TCR genes into terminally differentiated T cells also creates some drawbacks. Ideally, the introduction of a TCR harboring a new Ag specificity into an adult T cell should displace the endogenous TCR from the cell surface (12). When, however, the transferred TCR is not successful in doing so, stimulation of the T cell via its introduced, transgenic TCR can lead to activation of the previously tolerant T cell. This cross-activation may exert unwanted side effects through the endogenous TCR, such as autoimmunity (13). Furthermore, the introduced TCR can dimerize with the endogenous TCR chains to create mispaired TCRs with unknown specificity (12). An in vivo mouse model that closely mimicked the clinical protocol of immunotherapy with TCR-transduced T cells (TCR gene therapy) showed that mispaired TCRs induced lethal autoimmune pathology (7). In an in vitro system, virus-specific human T cells became allo- and self-reactive after transduction with an Ag-specific TCR (14). Recent technical advances aim to reduce these issues to a negligible level. For example, increased surface expression of the transgenic TCR was achieved by codon optimization of the TCR genes (15). Also, improved transgene cassettes in which TCR α- and β-chain genes are linked by 2A peptides instead of an internal ribosomal entry site confer improved TCR function (16, 17). Exchange of amino acids in the C region of human TCR chains by their murine counterpart likewise improved TCR function (18). However, the use of one or a combination of these modifications still cannot completely prevent the formation of mispaired TCRs.

TCR gene transfer into hematopoietic stem cells (HSCs) is another way to create T cells with desired Ag specificity. The feasibility of TCR gene transfer into HSCs was demonstrated by the in vivo generation of TCR-engineered HSC-transplanted mice (21, 22). In such mice, TCR-transduced HSCs mature in the host and give rise to Ag-specific T cells having, theoretically, a lifelong persistence. Expression of endogenous TCR chains is less likely due to allelic exclusion. Even if endogenous TCR chains are coexpressed with transgenic TCR chains (23), the likelihood of uncontrolled autoreactivity is low, as these lymphocytes undergo thymic selection. However, at present, gene transfer into HSCs has one
90 min at 2500 rpm, virus supernatant was aspirated and target cells were disseminated.

Because the generation of T cells derived from adult T cells or HSCs with new Ag specificity has both advantages and disadvantages, in this study we analyzed whether there are decisive differences in the performance of both T cell populations. As a model, we selected the P14 TCR that recognizes the gp33 epitope of the lymphocytic choriomeningitis virus (LCMV) (26). We compared P14 TCR-expressing T cells derived from both sources (adult T cells or HSCs) to T cells derived from P14 TCR-transgenic mice as to their therapeutic efficiency in controlling either an LCMV infection in mice or in suppressing the growth of tumor cells expressing the cognate Ag. We demonstrate that TCR-transduced T cells are comparable in their function to T cells derived from TCR-transduced HSCs.

Material and Methods

Mice

C57BL/6 (B6) and B6.129S7-Rag1<sup>−/−</sup>Mon/J (Rag1<sup>−/−</sup>) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). B6.D2-Tg(Tcr/LCMV)327Dz/JDV3 (P14) mice are transgenic for a TCR, which recognizes the LCMV gp33-41 (KAVYNFATM) in an H<sup>-2</sup><sup>D</sup>-restricted manner (13, 27). P14/Rag1<sup>−/−</sup> mice were obtained by crossing P14 and Rag1<sup>−/−</sup> mice.

Cell lines

Unless otherwise stated, all cell culture reagents were purchased from Charles River Laboratories (Sulzfeld, Germany), 4 g/l dimetridazole (Sigma-Aldrich, Munich, Germany), and 1.5% methoprim, 100 mg/l sulfadoxine (Borgal 24%; Intervet, Unterschleißheim, Germany). Donor and recipient strains were always identical.

Recipient mice received drinking water supplemented with 20 mg/l trimethoprim, 100 µg/ml sulfadoxine (Borgal 24%; Intervet, Unterschleißheim, Germany). Data analysis was performed using FlowJo software (Tree Star, Olten, Switzerland).

Quantitative PCR

To quantify proviral integration events, genomic DNA was isolated using a GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich). Quantitative PCR was based on the woodchuck hepatitis virus post-transcriptional regulatory element (PRE) sequence of the retrovirus vector and was performed on a 7300 real-time PCR system (Applied Biosystems, Darmstadt, Germany) using QuantiTect SYBR Green (Qiagen, Hilden, Germany) and was performed on a 7300 real-time PCR system (Applied Biosystems, Darmstadt, Germany) using QuantiTect SYBR Green (Qiagen, Hilden, Germany), 300 nM primers, and 10 ng DNA. Cycling parameters were: 95°C for 15 min and 50 cycles of 94°C, 60°C, and 72°C (each 30 s in length) in 25 µl reaction mix. The PRE-specific primers (forward, 5′-GAG-GAT-TTG-GCC-GCC-CCTG-3′; reverse, 5′-TGCAGGTTTGGGCAATG-TGCC-3′) amplified a 94-bp fragment. The PRE-specific signal was normalized by the signal of a housekeeping gene (fkl-1 intron enhancer [AP061804], forward primer, 5′-GGT TCT AAC GCT CCT TCA TCC-3′; reverse primer, 5′-CCT TGC CCC AGT CCC AGT TA-3′). Results were quantified using the comparative ΔCT method. Vector copy numbers were calculated for bulk cells, presuming an amount of 6.81 pg genomic DNA per cell. All PCR measures were performed at least in duplicate.

Cytokine release assay

P14 TCR-expressing T cells (1 × 10<sup>5</sup>) were cocultured in 96-well round-bottom plates for 18 h with 1 × 10<sup>4</sup> peptide-loaded splenocytes in 200 µl media. Splenocytes were irradiated with 24 Gy from a 137Cs source beforehand and T cells were equilibrated according to TCR/β<sup>+</sup> or tetramer staining. Supernatants were analyzed for murine IFN-γ by ELISA (BD Biosciences) according to the manufacturer’s protocol.

Virus protection assay

Mice were infected with 200 PFU LCMV strain WE i.e. (30). The following day, mice received P14 TCR-expressing T lymphocytes i.v. Virus titers in the spleen were determined 4 d after infection as previously described (31).
T cells i.v. on the same day. Mice were then monitored for the appearance of palpable tumors.

For suppression of experimental pulmonary metastases, B6 mice received $1 \times 10^6$ B16-gp33 melanoma cells i.v. and $2 \times 10^6$ P14 TCR$^+$ T cells 3 d later. Mice were sacrificed and lungs were excised and analyzed for macroscopically visible foci 14 d (untreated mice) or 21 d after adoptive T cell transfer (treated mice).

Statistical analyses

Statistical significance for comparison of more than two groups was evaluated using the one-way ANOVA F-test, followed by the Tukey-Kramer multiple comparison test. Survival curves were analyzed using the log-rank test. All calculations were performed with Prism4 software (GraphPad Software, La Jolla, CA).

Results

Fixed TCR density and absence of endogenous TCR expression in P14 TCR-engineered HSC-derived T cells

We monitored the TCR expression in the peripheral blood of P14 TCR-engineered HSC-transplanted mice on the Rag1$^{+/+}$ background to determine the time course of repopulation and the stability of TCR expression. Six weeks after HSC transplantation, a proportion of 4–12% of P14 TCR$^+$ T cells could be detected in the blood of individual mice (Fig. 1). The proportion of P14 TCR-expressing T cells then further increased (day 91) and remained stable in comparison with the first analysis at day 42 in most animals for months.

To investigate the robustness of TCR expression in engineered HSC-transplanted mice we analyzed the relationship between retroviral transgene delivery and TCR surface density. We generated three groups of P14 TCR-engineered HSC-transplanted mice on the Rag1$^{+/+}$ background. In one group, undiluted P14 TCR retrovirus supernatant was used for the transduction of HSCs, whereas the second and the third group received 1:10 and 1:20 diluted retrovirus supernatant, respectively. T cells can only develop in Rag1$^{+/+}$ mice when HSCs are transduced with TCR genes. To visualize HSC transduction in this experiment, we used an MP71 vector where GFP was linked by an IRES site to the TCR expression cassette. Flow cytometric analysis at the time of transplantation showed decreasing transduction efficiency with increasing dilution of the retrovirus supernatant (Fig. 2A). This result directly correlated with the copy number per cell of integrated vector genomes (Fig. 2A). The high copy numbers observed at the time of transduction obviously do not reflect stable vector integration, but rather nonintegrated provirus that is still present and trapped together with the genomic DNA (Fig. 2A). The moderate effect of the first dilution step on transgene expression level and retroviral vector copy number indicates a saturation effect when undiluted retrovirus supernatant is used.

Flow cytometric analysis of peripheral blood in engineered HSC-transplanted mice at day 58 after transfer revealed identical TCR density in all groups as quantified by mean fluorescence intensity of anti-TCR V$\alpha$2/V$\beta$8 staining (Fig. 2B). Whereas the proportion of P14 TCR$^+$ cells in blood and the retroviral vector copy numbers differed between groups, the identical TCR density suggests that only T cells with a defined P14 TCR density can pass thymic selection. Fixed TCR density and absence of endogenous TCR expression in engineered HSC-transplanted mice on the Rag1$^{+/+}$ background was collected at the indicated time points after HSC transplantation. PBLs were stained for TCR V$\alpha$2/V$\beta$8 and analyzed by flow cytometry. This plot is representative of at least three independent experiments with slight variations but the same outcome.
likely be explained by a genotoxic impact of multiple vector insertions on cell survival (32). To investigate whether true HSCs with long-term repopulating capacity were transduced, we repeated flow cytometric analysis at day 210 after transfer. Again, we observed identical P14 TCR density in all groups (Supplemental Fig. 1A). Compared with analysis at day 58, the distribution of P14 TCR⁺ cells was similar, albeit the differences between the groups were lower and not statistically significant.

To assess the expression of endogenous TCR chains on the surface of P14 TCR-modified T cells, we maintained P14 TCR⁺/CD8⁺ cells with a panel of commercially available TCR Vα- and VB-chain–specific Abs, except for Vα2 and Vβ8, of which the P14 TCR is composed. T cells taken from peripheral blood of P14 TCR-engineered HSC-transplanted mice both on the Rag1⁻⁻ and the Rag1⁺⁺ background were devoid of endogenous TCR chain expression (Fig. 3). Whereas T cells from P14 TCR-transgenic mice on the Rag1⁺⁺ background expressed a low level of endogenous TCRβ-chain (3%), a remarkable proportion of the P14 TCR-transduced splenocytes expressed endogenous TCRα– (6%) and β-chains (26%) along with the transferred TCR.

**FIGURE 3.** P14 TCR-engineered HSC-derived T cells do not express undesired endogenous TCR chains. TCR-transduced splenocytes and T cells from engineered HSC-transplanted and transgenic mice were stained with gp33 tetramer, anti-CD8, and a mixture of TCR Vα- and VB-chain–specific Abs. Gated on tetramer/CD8⁺ cells, Vα and VB staining indicates coexpression of endogenous TCR chains with the transferred TCR. Cells were sharply gated on small lymphocytes to exclude doublets. Plots are representative of at least three individual mice (engineered HSC-transplanted/transgenic) or two independent experiments (transduced T cells), respectively.

**FIGURE 4.** Comparable cytokine secretion of P14 TCR-transduced, P14 TCR-engineered HSC-derived, and P14 TCR-transgenic T cells. P14 TCR-expressing T cells were cocultured with peptide-loaded splenocytes. IFN-γ secretion was quantified by ELISA. TCR⁺ cells were equilibrated according to TCRα/β or tetramer staining as indicated. T cells were derived from the experiment shown in Fig. 7A. The source of transgenic and engineered HSC-derived T cells was of the Rag1⁻⁻ background. Measurements were performed in duplicate. Mean values are indicated by dots and the error bars indicate the SEM. This plot is representative of two independent experiments.

**Comparable antiviral protection by P14 TCR-transduced T cells and P14 TCR-engineered HSC-derived T cells**

Because the P14 TCR targets the gp33 epitope of LCMV, we first assessed the in vivo recognition of P14 TCR-transduced and P14 TCR-engineered HSC-derived T cells in an LCMV infection model. Splenocytes from B6 mice were retrovirally transduced to express the P14 TCR, whereas splenocytes from P14 TCR-engineered HSC-transplanted mice of the Rag1⁻⁻ as well as of the Rag1⁺⁺ background were isolated and exposed to transduction conditions (mock-transduced). The proportion of P14 TCRα/β⁺ T cells was quantified by FACS analysis (Fig. 5A) and equilibrated accordingly. Day 1 LCMV-infected mice received varying amounts of the different P14 T cell populations. Three days later, spleens were analyzed for P14 TCR-expressing donor cells and viral titers were determined. Adoptively transferred cells expanded well, except for the groups that were treated with 2 × 10⁶ T cells (Fig. 5B). In all treated groups, P14 TCR-expressing T cells diminished the viral load compared with the untreated group (Fig. 5C). The antiviral protection was proportional to the amount of P14 TCR-transduced T cells applied. However, there was no statistical significant difference in viral load (ANOVA, F(2,16) = 2,16) between the groups treated with 1 × 10⁶ P14 TCR⁺ T cells. Mice that received 3 × 10⁶ P14 TCR-transduced T cells or the same number of engineered HSC-derived T cells on the Rag1⁺⁺ background showed a stronger reduction in viral load than did engineered HSC-derived T cells on the Rag1⁻⁻ background. We conclude from these data that TCR-transduced adult T cells and T cells derived from TCR-transduced HSC mediate comparable antiviral protection.

**P14 TCR-transduced T cells and P14 TCR-engineered HSC-derived T cells mediate equal tumor suppression**

To compare the in vivo tumor suppression of P14 TCR-transduced mature T cells, P14 TCR-engineered HSC-derived T cells, and
T cells derived from P14 TCR-transgenic mice in a tumor model, we first tested their ability to suppress the outgrowth of s.c. injected B16-gp33 melanoma cells. Splenocytes from B6 mice were retrovirally transduced to express the P14 TCR, whereas splenocytes from P14 TCR-engineered HSC-transplanted mice and transgenic mice were mock-transduced. TCR+ T cells (5 \times 10^6) were injected i.v. into B6 mice that received 5 \times 10^4 B16-gp33 cells s.c. on the same day. The proportion of TCR+ T cells was quantified by FACS analysis of TCRα/β+ cells and equilibrated accordingly (data not shown). In all groups of mice that received P14 TCR-expressing T cells, a significant delay of tumor outgrowth as compared with the untreated group was observed (Fig. 6, p < 0.0001). Although there was no difference between P14 TCR-engineered HSC-derived and TCR-transduced T cells, the delay in tumor outgrowth was most pronounced in mice that received P14 TCR-transgenic T cells. Nonetheless, the differences between all treatments are not statistically significant (p = 0.0907).

Next, we assessed the therapeutic efficacy of P14 TCR-expressing T cells in a B16-gp33 lung metastasis model. Experimental pulmonary metastases were induced by injecting 1 \times 10^6 B16-gp33 cells into the tail vein of B6 mice. Three days later, mice received 2 \times 10^6 P14 TCR+ T cells. In this experiment, we additionally quantified the proportion of TCRα/β+ T cells by tetramer staining. Although the values of TCRα/β+ T cells between tetramer and Ab staining do not essentially differ between engineered HSC-derived and transgenic T cells (all >90%), there is a discrepancy between both stainings for the transduced T cells (Fig. 7A). In this study, 58% of the transduced splenocytes express TCR Vα2/Vβ8 simultaneously, but only 34% bind the gp33 tetramer. This most probably indicates mispairing of the introduced TCR chains with endogenous TCR chains. To overcome this problem, we equilibrated all T cell numbers according to the tetramer staining. For comparability with previous experiments, we included a group where the transduced T cells were equilibrated according to the Vα2/Vβ8-specific Ab staining. On day 14 after treatment, we analyzed one mouse per group and found only a few lung metastases in the treated animals, whereas the lungs of the untreated animals were covered with >500 metastases. Therefore, untreated mice were sacrificed and the observation of the treated groups was extended for 1 wk. All P14 TCR-expressing T cells caused a drastic and highly significant (p < 0.001 compared with control) reduction of lung surface metastases (Fig. 7B).
Engineered HSC-derived T cells suppress experimental lung metastasis. (A) P14 TCR-transduced, P14 TCR-engineered HSC-derived, and P14 TCR-transgenic T cells were analyzed on the day of adoptive transfer by flow cytometry with TCR Vα2- and Vβ8-specific Abs (upper panel) and gp33 tetramer and CD8-specific Ab (lower panel). These plots are representative of three independent experiments. (B) Pulmonary metastases were induced in mice by injecting 1 × 10⁶ B16-gp33 melanoma cells i.v. Three days later, mice were treated with 2 × 10⁶ P14 TCR-expressing T cells. Fourteen (untreated) or 21 d (treated) after adoptive transfer, lungs were analyzed for macroscopically visible foci on the surface. Dots in the graphs represent values from individual mice. The lines describe the in vitro generation of naive, Ag-specific human T cells have yet to be translated into a clinical application. A few reports describe the in vitro generation of naive, Ag-specific human T cells (16, 36–39). We found that this vector maintained a long-term and stable TCR expression in hematopoietic precursor cells as well as in their differentiated progeny. Using a 2A peptide to combine the TCRα and TCRβ cDNA in the transgene cassette, we achieved an equimolar expression of both chain genes that was high enough to drive T cell development and function. We detected large numbers of P14 TCR-specific T cells even months after the generation of engineered HSC-transplanted mice, which is most probably due to the fact that these cells were derived from long-lived progenitor cells.

In both in vivo models, we found that TCR-engineered HSC-derived and TCR-transduced T cells controlled an LCMV infection and suppressed growth of tumor cells expressing the cognate Ag to a similar extent. Based on the similar therapeutic efficiency of both TCR-modified cell types, it is difficult to categorize which approach is of greater advantage for TCR gene therapy.

Although coexpression of endogenous TCR chains is not a major issue in TCR-engineered HSC-derived T cells and in T cells of transgenic mice, the tendency of lower functional activity of TCR-transduced T cells can most probably be ascribed to the formation of mispaired TCRs, as the quantification of TCR expression by TCRα/β staining resulted in a higher portion of TCRβ cells compared with tetramer staining. Owing to the fact that we used a nonoptimized P14 TCR in these experiments, mispairing can presumably be further reduced by utilizing a codon-optimized and cysteinized TCR (15, 40, 41). The application of more recently described methods such as RNA interference–mediated silencing or zinc finger nucleases–promoted disruption of the endogenous TCRα/β-chain genes could potentially avoid the formation of mispaired TCRs entirely (42, 43) and improve the therapeutic efficiency of TCR-transduced T cells.

Although TCR-engineered HSC-transplanted mice are certainly a valuable tool for basic research in TCR gene therapy (23, 33) and are easier to generate than are TCR-transgenic mice, this approach has yet to be translated into a clinical application. A few reports describe the in vitro generation of naive, Ag-specific human T cells from TCR gene–modified HSCs (44–46). In this case, TCR-transduced HSCs from umbilical cord blood or postnatal thymus
were differentiated to T cells by coculture with OP9 stromal cells expressing the human Notch ligand Delta-like1 (44). HSCs developed into mature, functional T cells, which showed MHC-restricted specific recognition and killing of Ag-expressing cells. Such an in vitro approach would allow the usage of autologous TCR gene-modified HSCs for adoptive T cell therapy in humans. However, there are a few drawbacks associated with the genetic modification of HSCs. First, it would take several weeks until immune reconstitution is mounted, which may be too late for treatment of cancer patients. Second, the induction of neoplasia cannot be excluded when using retrovirus-mediated gene transfer into HSCs. So far, this occurred in several clinical trials using HSCs as target cells and is due to insertional mutagenesis of the retrovirus vector (24, 47, 48). To date, insertional mutagenesis has not been reported for transduction of terminally differentiated T cells (49, 50). Targeted integration by zinc finger nucleases or integrase-defective lentiviral vectors could circumvent this problem in HSCs (51, 52).

In conclusion, we have shown that TCR-transduced adult T cells and T cells derived from TCR-transduced HSCs perform comparably in a virus infection model and in two tumor models. Both and T cells derived from TCR-transduced HSCs perform comparable in HSCs (51, 52).

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Disclosures
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References


**Fixed TCR density on engineered HSC-derived T cells.**

a) Flow cytometric analysis of PBLs from HSC-transplanted mice on day 210 post transfer stained for TCR Vα2/Vβ8. Median fluorescence intensity (MFI) is indicated. There is no statistical significant difference between the three groups (ANOVA: TCR Vβ8: F(2,12) = 0.9512, P = 0.4136; TCR Vα2: F(2,12) = 1.099, P = 0.3644). b) Proportion of TCR⁺ cells in blood (ANOVA: F(2,12) = 0.6088, P = 0.56). a + b) Dots in the graphs represent values from individual mice. The lines indicate the median values. n.s.: not significant.