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Immunotherapy with TCR-Redirected T Cells: Comparison of TCR-Transduced and TCR-Engineered Hematopoietic Stem Cell–Derived T Cells

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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.

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major drawback. The most efficient vector systems, that is, retroviral vectors, used to engineer HSCs bear the risk of vector-induced insertional mutagenesis, which may lead to uncontrolled clonal proliferation and lymphoma development. Indeed, in several clinical trials, where γ-retrovirus vectors were used for the genetic modification of HSCs, severe side effects appeared (24, 25).

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>m01<sup>Mom/1</sup></sup> (Rag1<sup>1−/−</sup>) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). B6.Tg(TcrLCMV)327Dz/JDv3 (P14) mice are transgenic for a TCR, which recognizes the LCMV gp33–41 (KAVYNFATM) in an H2<sup>D<sup>B</sup></sup>–restricted manner (13, 27). P14/Rag1<sup>1−/−</sup> mice were obtained by crossing P14 and Rag1<sup>1−/−</sup> mice.

Cell lines

Unless otherwise stated, all cell culture reagents were purchased from Invitrogen (Darmstadt, Germany). The gp33-transfected B16.F10 melanoma cells (B16-gp33) (28) were cultured in RPMI 1640 supplemented with 10% FCS (Biochrom, Berlin, Germany), MEM nonessential amino acids, and 100 U/ml penicillin/streptomycin and were maintained under G418 selection (1.5 mg/ml).

Retrovirus vector production and transduction

The P14 TCR expression vector MP71-βpro-PRE has been described (16). In experiments presented in Fig. 2 we used a modified vector in which GFP was linked to the TCR expression cassette by an internal ribosome entry site (IRES) site (MP71-βpro-IRES-GFP-PRE). P14 TCR virus supernatant was produced by transient calcium phosphate transfection of the packaging cell line Plat-E (29) with the P14 TCR expression vector. DMEM supplemented with 10% FCS and 100 U/ml penicillin/streptomycin was used to implement media and cultured overnight in RetroNectin/virus-coated 24-well plates. For a second transduction, cells were harvested, resuspended in the same volume of medium supplemented with IL-2, and cultured in a new virus-coated 24-well plate overnight. Cells were then adjusted to 1 × 10<sup>5</sup> ml and cultured for 2 d in media supplemented with 50 ng/ml recombinant human IL-2 and 10 μl/ml mouse T cell activator CD3/CD28 beads (Invitrogen) and cultured overnight in virus-coated 24-well plates. For a second transduction, cells were harvested, resuspended in the same volume of medium supplemented with IL-2, and cultured in a new virus-coated 24-well plate overnight. Cells were then adjusted to 1 × 10<sup>5</sup> ml and cultured for 3 d in media supplemented with 50 ng/ml recombinant human IL-15 (PeproTech). To ensure that spleen cells from HSC-transplanted or transgenic mice are activated in the same way, they were mock-transduced with medium.

Flow cytometry

FITC-labeled anti-CD8, anti-TCR V<sub>b</sub>8.1, and PE-labeled anti-TCR V<sub>c</sub>8.1, V<sub>b</sub>11, V<sub>b</sub>3, V<sub>b</sub>5, V<sub>b</sub>6, V<sub>b</sub>7, V<sub>b</sub>8.1, and V<sub>b</sub>10 Abs were purchased from BioLegend (Fell, Germany). H-2<sup>D<sup>B</sup></sup>-LCMV-gp33 tetramer conjugated to allophycocyanin was purchased from Beckman Coulter (Krefeld, Germany). Mouse Fc Block was used for all stainings to avoid unspecific Ab binding (BD Pharmingen). For staining of blood samples, lysis buffer was used to remove RBCs.

Cells were measured using a FACS-Calibur or a FACS Canto II flow cytometer (BD Biosciences) or a MACSQuant analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Data analysis was performed using FlowJo software (Tree Star, Olenz, 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), 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 for 30 s in length) in 25 μl reaction mix. The PRE-specific primers (forward, 5′-GAG-GAC-GTT-TGG-GCC-GCC-GGT-3′, reverse, 5′-TCG-AAC-GTG-TGG-GCA-TGC-3′) amplified a 94-bp fragment. The PRE-specific signal was normalized by the signal of a housekeeping gene (flk-1 intron enhancer 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), 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 for 30 s in length) in 25 μl reaction mix. The PRE-specific primers (forward, 5′-GAG-GAC-GTT-TGG-GCC-GCC-GGT-3′, reverse, 5′-TCG-AAC-GTG-TGG-GCA-TGC-3′) amplified a 94-bp fragment. The PRE-specific signal was normalized by the signal of a housekeeping gene (flk-1 intron enhancer (Fk-1 intron enhancer [AP061804], forward primer, 5′-GTT-TCT AAC GTC CCG TAG TCT T′-3′, reverse primer, 5′-CCT TGC CCC AGC CCC ATG TA′-3′). Results were quantified using the comparative Ct method. Vector copy numbers were calculated for bulk cells, assuming 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>5</sup> peptide-loaded splenocytes in 20 μl media. Splenocytes were irradiated with 24 Gy from a 140 Cs source beforehand and T cells were equilibrated according to TCR/β 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.v. (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).

Tumor models

For suppression of s.c. growing tumors, B6 mice received 5 × 10<sup>5</sup> B16-gp33 melanoma cells s.c. in the abdominal region and 5 × 10<sup>5</sup> P14 TCR* sugar syrup (Aphonix, Halle, Germany) for 6 wk. All animal work was performed according to local and international guidelines for animal care and protection.
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 transplantation 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 development in engineered HSC-transplanted mice. The lower proportion of P14 TCR$^+$ cells in mice that received undiluted retrovirus supernatant in comparison with mice that received 1:10 and 1:20 diluted supernatant, respectively (Fig. 2C), can most
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 re-
peated flow cytometric analysis at day 210 after transfer. Again,
we observed identical P14 TCR density in all groups (Supple-
mental Fig. 1A). Compared with analysis at day 58, the distri-
bution 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 stained P14 TCR⁺/CD8⁺
cells with a panel of commercially available TCR Vα- and
Vβ-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 en-
dogenous 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.

Identical in vitro peptide sensitivity of transduced, engineered
HSC-derived, and transgenic P14 TCR T cells

To analyze whether the in vitro peptide sensitivity of the P14 TCR
differs in transduced, engineered HSC-derived, or transgenic T cells,
we cocultured equilibrated amounts of P14 TCR-expressing T cells
with gp33 peptide-loaded splenocytes and determined IFN-γ secre-
tion into the supernatant by ELISA. The activation threshold
was between 6.2 and 12.5 pM gp33 peptide, irrespective of the
culture conditions (mock-transduced). The proportion of P14 TCR
transduced T cells that expressed the P14 TCR, whereas splenocytes from P14 TCR-
transduced HSC-derived 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 equili-
brated accordingly. Day 1 LCMV-infected mice received varying
amounts of the different P14 T cell populations. Three days later,
splenes 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
per mouse. In all treated groups, P14 TCR-expressing T cells di-
minished the viral load compared with the untreated group (Fig.
5C). The antiviral protection was proportional to the amount
of P14 TCR-expressing T cells applied. However, there was no sta-
tistical significant difference in viral load (ANOVA,
F(2,16) = 1.190; p = 0.3299) 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⁻/⁻ back-
ground. 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 × 10^6) were injected i.v. into B6 mice that received 5 × 10^4 B16-gp33 cells s.c. on the same day. The proportion of TCR+ T cells was quantified by FACS analysis of TCRαβ 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 × 10^6 B16-gp33 cells into the tail vein of B6 mice. Three days later, mice received 2 × 10^6 P14 TCR+ T cells. In this experiment, we additionally quantified the proportion of TCRαβ T cells by tetramer staining. Although the values of TCRαβ+ 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 or transgenic T cells (both significant (all \( p < 0.05 \)).

The differences between the tetramer-equilibrated groups were not statistically significant (all \( p > 0.05 \)). The lower T cell number given to the transduced TCR\( \alpha/\beta \)-equilibrated group obviously resulted in a higher incidence of lung tumor nodules. Whereas the difference between the two groups that received transduced T cells was not significant (\( p > 0.05 \)), the difference was significant when the TCR\( \alpha/\beta \)-equilibrated group was compared with the groups that received engineered HSC-derived or transgenic T cells (both \( p < 0.05 \)).

**Discussion**

In this study, we generated TCR-engineered HSC-derived and TCR-transduced T cells and compared their therapeutic efficacy in a viral disease and in tumor models using the murine P14 TCR, which recognizes a gp33 Ag of LCMV.

Apart from the body of literature describing TCR gene transfer into T cells, there are only a few reports on the transfer of such genes into mouse HSCs, and a direct comparison of TCR-transduced and TCR-engineered HSC-derived T cells in an immunotherapy setting has not been performed so far (21, 22, 33). Despite the more difficult handling, the genetic engineering of HSCs (compared with T cells) harbors several advantages. Owing to allelic exclusion, it is unlikely that T cells derived from TCR-transduced HSCs express an endogenous TCR with unknown specificity (34). This circumvents the risk of autoimmunity as a result of the activation of the endogenous TCR through the Ag recognition of the transferred TCR. It also circumvents the formation of mispaired TCRs consisting of transgenic and endogenous TCR chains (35). Additionally, this strategy may overcome the problems of a restricted effector function of the TCR-transduced T cell caused by conditions used to activate T cells in vitro for retrovirus-mediated gene transfer as well as prevent unknown in vivo persistence after adoptive transfer.

We used the MP71 retrovirus vector for TCR gene transfer into HSCs, which ensured an efficient TCR gene expression in previous experiments in mouse and 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 TCR\( \alpha \) and TCR\( \beta \) 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 TCR\( \alpha/\beta \) staining resulted in a higher portion of TCR\( \beta \) 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 TCR\( \alpha/\beta \)-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 not been reported for transduction of terminally differentiated T cells (49, 50). Targeted integration by zinc finger nucleases or

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