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Research paper

Enhancing lentiviral transduction to generate melanoma-specific human T cells for cancer immunotherapy

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ABSTRACT

Introduction of a tumor antigen-specific T cell receptor (TCR) into patient-derived lymphocytes has already exhibited promising results for the treatment of melanoma and other malignancies in clinical trials. However, insufficient or unsuccessful ex vivo manufacturing of engineered T cells due to low expansion and/or transduction rate can still be observed in some patients. Thus, we isolated human $CD8^+$ T cells from healthy donors and equipped them with a gp100-specific TCR using a lentiviral construct in combination with a novel chemical lentiviral transduction enhancer (Lentiboost) to increase the rate of transduced cells. Following experiments to determine the ideal multiplicity of infection (MOI) and to analyze the efficacy of the transduction enhancer using a GFP-encoding lentivirus, we analyzed in the next step the transduction rate, cell count, and functionality of gp100 TCR-transduced T cells, i.e. antigen-specific cytokine secretion and lytic capacity. In order to increase the number of transduced cells, antigen-specific stimulation was performed, either once for 1 week (1^{st} activation) or twice for another week (2^{nd} activation). In general, each cycle of antigen-specific stimulation resulted in expansion of TCR-positive cells, while no further significant increase of transduced cells was observed after 2^{nd} activation. Cytokine production pattern of transduced cells after antigen encounter, however, revealed significant antigen-specific secretion of TNF and IFNγ after the 1^{st} as well as the 2^{nd} activation. Furthermore, TCR T cells, either activated once or twice, showed significant cytotoxicity towards antigen-positive tumor cells. Taken together, these results show that it is feasible to transduce human T cells using a lentiviral construct in combination with this novel lentiviral transduction enhancer, which shows potential in the growing field of cancer immunotherapy.

1. Introduction

Tumor antigen-specific cytotoxic T lymphocytes (CTLs) play a key role in tumor immunology (Restifo and Wunderlich, 1996). Thus, the adoptive transfer of ex vivo generated T cells into cancer patients to support the often crippled immune system has been the main aim in several cellular strategies for immunotherapy of melanoma and other malignancies, like lymphomas, leukemias, and sarcomas (Restifo and Wunderlich, 1996). Especially three forms of adoptive T cell transfer (ACT) revealed high potential and durable clinical responses in cancer patients: tumor-infiltrating lymphocytes (TILs), T cells engineered with a tumor antigen-specific T cell receptor (TCR T cells), and T cells engineered with a tumor antigen-specific chimeric antigen receptor (CAR T cells) (Rosenberg and Restifo, 2015). CARs consist of a single chain variable fragment derived from a monoclonal antibody fused to the CD3ζ signaling domain of the TCR complex. In contrast to TCR T cells, the target antigen is recognized independently of major histocompatibility complex (MHC) presentation by CAR T cells (Simon and Uslu, 2018). Recently, two CD19 CAR T cell constructs were approved for patients with therapy-refractory B cell acute lymphoblastic leukemia (B-ALL) and diffuse large B-cell lymphoma (Maude et al., 2018; Schuster et al., 2017), highlighting the strong potential of ACT.

The adoptive transfer of autologous tumor-specific CTLs has been shown to be effective in eradication of tumors in some patients with metastatic melanoma (Parmiani et al., 2003; Rosenberg, 1999; Yee et al., 2002). It was then recognized that transfer of TILs was more

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effective if performed in melanoma patients after a non-myeloablative lymphodepletion (Gattinoni et al., 2006; Dudley et al., 2002; Dudley et al., 2005). Such adoptive T cell trials convincingly demonstrated that T cells are indeed an adequate "tool" to specifically destroy even large tumor masses, partially or completely, in up to 50% of the patients (Gattinoni et al., 2006; Dudley et al., 2002; Dudley et al., 2005). However, the peripheral T cell repertoire is usually devoid of high avidity tumor specific CTLs due to thymic negative selection (Xue et al., 2005). Tumor-specific CTLs are, moreover, often anergic or exhibit a limited life span, making expansion of such T cells to therapeutic doses elaborative and often not feasible (Gattinoni et al., 2006; Xue et al., 2005; Bolhuis et al., 2000). Thus, this treatment strategy, despite its principal efficacy, has only been performed by few centers in a restricted number of patients.

To overcome these hurdles, the ex vivo generation and expansion of tumor-specific T cells by the introduction of an antigen-specificity into bulk T lymphocytes by genetic means was established. Reprogramming of T cells with an anti-viral or an anti-tumor specificity by genetic transduction of full-length TCR α- and β- chains has already been shown in vitro for several antigens (Orentas et al., 2001; Clay et al., 1999; Willemsen et al., 2000; Stanislawski et al., 2001; Morgan et al., 2003; Roszkowski et al., 2005; Cohen et al., 2005; Zhao et al., 2005). TCRs recognize intracellular tumor antigens presented on the cell surface and are therefore dependent on human leukocyte antigen (HLA)-restricted antigen presentation by the cancer cells (Davis and Bjorkman, 1988). A highly investigated melanoma-specific antigen is the glycoprotein 100 (gp100; also known as premelanosome protein, PMEL) (Seiter et al., 2002; Parkhurst et al., 1996; Rosenberg et al., 1998). This transmembrane glycoprotein is enriched in melanosomes, which are involved in melanin synthesis (Watt et al., 2013).

The most commonly used method to reprogram T cells is through stable transduction using retroviral or lentiviral constructs (Morgan et al., 2003; Zhao et al., 2005; Hughes et al., 2005; Bobisse et al., 2007). The introduction of several HLA-restricted viral or tumor antigens into T cells via transduction of the full-length TCR α- and β-chains has already proven to be feasible (Orentas et al., 2001; Clay et al., 1999; Willemsen et al., 2000; Stanislawski et al., 2001; Morgan et al., 2003; Roszkowski et al., 2005; Cohen et al., 2005; Zhao et al., 2005). Regarding melanoma, which still ranks high among tumors with poor prognoses (Bender et al., 2016), there are various tumor antigens that can be used in ACT. The first TCR T cells were clinically used in melanoma patients targeting a HLA-A2-restricted peptide from a melanocytic differentiation antigen (Morgan et al., 2006). Subsequently, highly reactive human TCRs for ACT were identified and used in clinical trials, e.g. TCRs specific for the melanoma/melanocyte differentiation antigen (MART-1) and gp100 epitopes (Johnson et al., 2009). Johnson et al., for instance, engineered genes encoding high-affinity TCRs specific for MART-1 and gp100 into retroviral vectors and transduced autologous peripheral lymphocytes which were then administered to 36 patients with metastatic melanoma (Johnson et al., 2009). 16 of these 36 patients received gp100-specific TCR T cells, while the other 20 patients received MART-1-specific TCR T cells. Objective cancer regressions were seen in 19% and 30% of patients receiving the gp100- and MART-1-specific TCR T cells, respectively (Johnson et al., 2009). Even if some patients receiving gp100-specific TCR T cells exhibited destruction of normal melanocytes in the skin, eye, and ear, these side effects were mainly CTCAE grade 1 and 2 and could be appropriately treated with local steroid administration (Johnson et al., 2009).

Nevertheless, in clinical trials some patients may still not receive ACT due to an insufficient T cell count for the manufacturing of engineered T cells. In a clinical trial performed by Carl June's group at the University of Pennsylvania, three patients out of 28 with therapy-refractory diffuse large B-cell lymphoma could not be treated with CD19-redirected CAR T cells, as ex vivo T cell generation was unsuccessful due to insufficient expansion rate of tumor-reactive cells (Schuster et al., 2017). Thus, in such cases, improvement of transduction and/or expansion rate for generation of a sufficient amount of engineered T cells is needed. One option could be the additional use of transduction enhancers. For instance, LentiBOOST™ (established by Sirion Biotech GmbH, Planegg-Martinsried, Germany) represents a non-cytotoxic, chemical transduction enhancer, which can be used in combination with a lentivirus in order to obtain a higher number of transduced cells (Hauber et al., 2018; Delville et al., 2018; Anastasov et al., 2016). It contains the transduction-promoting agents poloxamer P338 and the polycation polybrene (Hauber et al., 2018; Delville et al., 2018; Anastasov et al., 2016). In previous studies, it was already shown that the additional use of LentiBOOST™ could significantly improve lentiviral $CD34^+$ hematopoietic stem and progenitor cell transduction protocols, as well as the transduction rate of murine T cells and murine hematopoietic stem cells (Hauber et al., 2018; Delville et al., 2018).

The aim of this study was the equipment of human $CD8^+$ T cells with a gp100-specific TCR via lentiviral transduction. In order to enhance the transduction efficacy, LentiBOOST™ was used in combination with the gp100-encoding lentivirus. First, we performed a series of pilot experiments to determine the ideal multiplicity of infection (MOI) and to analyze the efficacy and the potential effect on T cell viability of the transduction enhancer LentiBOOST™ using a GFP-encoding lentivirus. In the next step, experiments were performed with gp100 TCR-transduced T cells to examine the transduction rate, cell count, and functionality of engineered cells, notably their antigen-specific cytokine secretion and cytolytic capacity. To increase the number of transduced cells, antigen-specific stimulation was performed using A375M melanoma cells pulsed with the HLA-A2-restricted $gp100_{280–288}$ peptide, either once for 1 week or twice for another week.

2. Materials and methods

2.1. Cells and reagents

Healthy donor blood was obtained after informed consent and approval by the institutional review board of the Friedrich-Alexander-University (FAU) of Erlangen-Nürnberg (Reference Number: 65_16 B). In the first step, Lymphoprep reagent (Axis-Shield, Oslo, Norway) was used to isolate peripheral blood mononuclear cells (PBMCs) and subsequently $CD8^+$ T cells were extracted via magnetic-activated cell sorting (MACS) according to manufacturer's instructions (Miltenyi, Bergisch-Gladbach, Germany). Purified T cells were then cultured in X-Vivo 15 medium that already contained L-glutamine, gentamycin, and phenol red (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). As target cell lines the TxB cell hybridoma T2.A1 (HLA-$A2^+$, $gp100^-$ (Uslu et al., 2016); kind gift from Prof. Dr. Schulz, Nuremberg, Germany) and the melanoma cell line A375M (HLA-$A2^+$, $gp100^-$ (Uslu et al., 2016); kind gift from Dr. Aarnoudse, Leiden, Netherlands; ATCC CRL-3223) were used. Before target cells were co-incubated with T cells, they were cultured in R10 medium, which consisted of RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine (Lonza), 100 IU/ml penicillin (Lonza), 100 mg/ml streptomycin (Lonza), 10% (*v*/v) heat-inactivated fetal calf serum (PAA, GE healthcare, Piscataway, NY, USA), 2 mM HEPES (PAA, GE healthcare, Little Chalfont, UK), and 2 mM β-mercaptoethanol (Gibco, Life Technologies, Carlsbad, CA, USA). Where indicated, target cells were loaded with the HLA-A2-restricted peptide $gp100_{280–288}$ (YLEPGPVTA) for 1 h as previously described (Harrer et al., 2017). Cells were peptide-loaded in DC-medium, consisting of RPMI 1640 (Lonza), 1% heat-inactivated human serum (Sigma-Aldrich, Taufkirchen, Germany), 2 mM L-glutamine (Lonza), and 0.04% of 20 mg/l gentamycin (Lonza).

2.2. Lentiviral transduction of T cells

Both the GFP and the gp100 TCR-expressing lentiviruses (backbone vector pcLV-EF1a-MCS-WPRE) were designed by and purchased from Sirion Biotech, Planegg-Martinsried, Germany. The expression of GFP

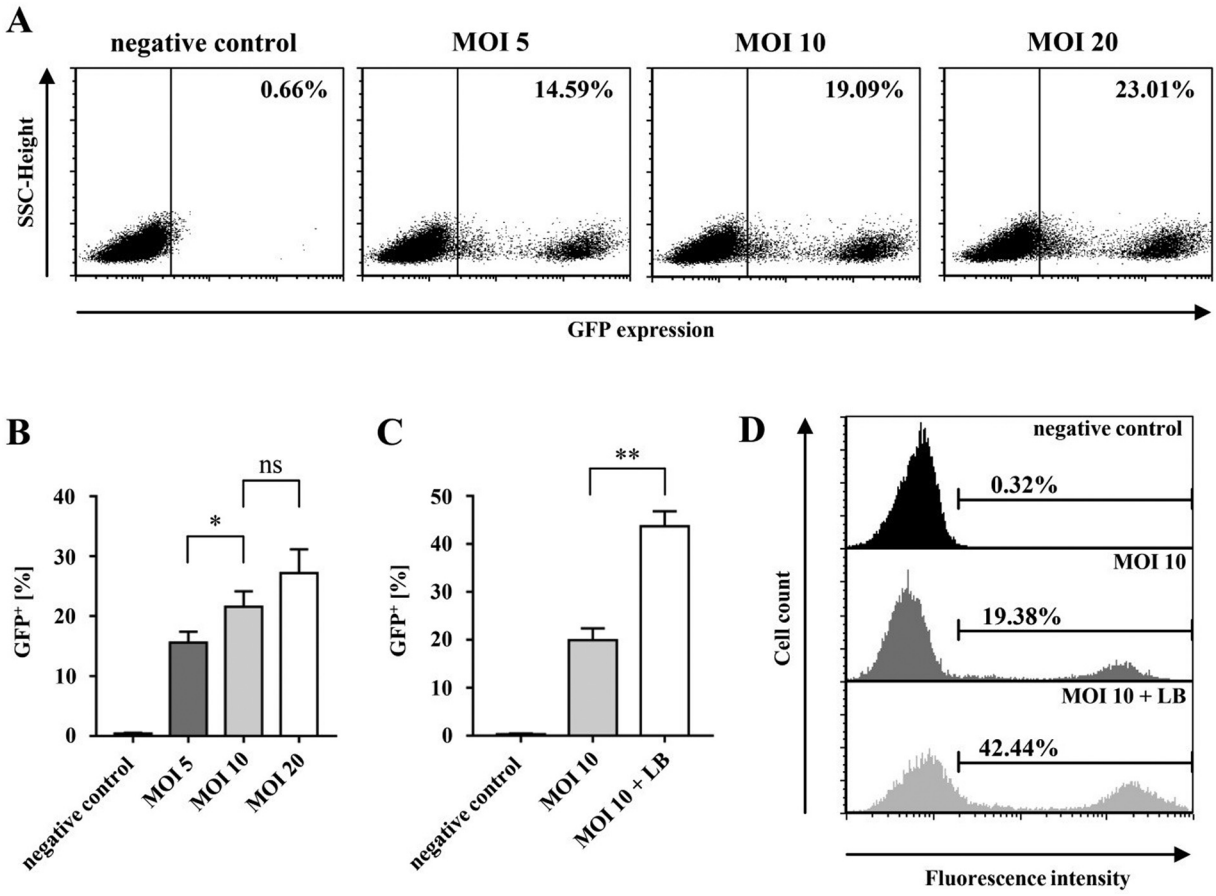

Fig. 1. MOI titration with GFP virus. (A + B) T cells were lentivirally transduced with a GFP virus using different multiplicity of infection (MOI), as indicated. Non-transduced cells were used as negative control. Transduction efficacy of T cells was measured via flow cytometry 3 days after lentiviral transduction. (A) Dot plots of one representative out of 3 independent experiments and (B) average values of GFP-positive cells of 3 independent experiments with SEM are shown. (C + D) T cells were lentivirally transduced with a GFP virus using a MOI of 10 together with the lentiviral transduction enhancer LentiBOOST™. Non-transduced cells served as negative control. Transduction efficacy was analyzed via flow cytometric approach at 3 days after lentiviral transduction. (C) Mean percentages of GFP-positive cells with SEM and (D) representative histograms out of 4 independent experiments are shown. The *p*-values were calculated by paired Student's *t*-test and are listed in Table S1.

or the gp100 TCR α- and β-chains was controlled by an EF1a promotor. For the preparation of lentiviral transduction, MACS-isolated $CD8^+$ T cells were first stimulated with 0.1 µg/ml anti-CD3 antibody OKT-3 (Orthoclone OKT-3; Janssen-Cilag, Neuss, Germany), 0.25 µg/ml anti-CD28 antibody (BD Biosciences, Franklin Lakes, NJ, USA), and 1000 IU/ml interleukin-2 (IL-2) (Proleukin; Novartis, Nuremberg, Germany). After 2 days, T cells were lentivirally transduced with either the GFP- or the gp100 TCR-expressing lentiviral vector together with 1000 IU/ml IL-2 (Novartis) using spinoculation (800 g for 90 min). For GFP transduction a range of different MOI was used (MOI 5, MOI 10 or MOI 20), whereas transduction with the gp100-specific TCR was performed with MOI 10. At the same time, the transduction enhancer LentiBOOST™ (containing 1000 µg/ml P338 and 10 µg/ml polybrene; Sirion Biotech) was added, where indicated. As negative control, only IL-2 was added to the cells before spinoculation. On the next day, culture medium of T cells was replaced by fresh X-Vivo 15 medium and 5 ng/ml IL-7 (PeproTech, Rocky Hill, NJ, USA), 5 ng/ml IL-15 (Miltenyi), and 1000 IU/ml IL-2 (Novartis) were added. After 3 days, lentiviral transduction efficacy of transduced cells was measured. Afterwards, the gp100 TCR-transduced T cells were antigen-specifically stimulated with irradiated (140 Gy for 6 min) and gp100 peptide-pulsed A375M target cells for 1 week and subsequently for another week. Negative control cells were re-stimulated with 0.1 µg/ml OKT-3 (Janssen-Cilag), 0.25 µg/ml anti-CD28 antibody (BD Biosciences) and 1000 IU/ml IL-2 (Novartis). On day four after antigen-specific stimulation, fresh culture medium and IL-2 was added to the cells. Lentiviral transduction efficacy of gp100 TCR-transduced cells was analyzed 3 days after transduction, 1 week after the first antigen-specific stimulation, and again 1 week after the second antigen-specific stimulation, as described below.

2.3. Analysis of receptor expression

A MHC Dextramer (HLA-A*0201/YLEPGPVTV; Immudex, Copenhagen, Denmark) directed against the gp100-specific TCR was used to examine the TCR expression on the cell surface of transduced T cells. Cells were additionally stained with an anti-7-AAD antibody (BD Biosciences) to exclude nonviable cells. For the analysis of T cell subsets, cells were double stained for CD62L (BD Biosciences) and CD45RA (BD Biosciences). In addition, expression of the exhaustion marker PD-1 (BD Biosciences) was examined via flow cytometric approach. The detailed procedure of cell surface staining was previously described (Schaft et al., 2005). Immunofluorescence of Dextramer staining was detected via FACS Calibur (BD Biosciences, Heidelberg, Germany) equipped with CellQuest Pro software (BD Biosciences) and staining of CD62L, CD45RA, and PD-1 was measured via FACS Scan (BD Biosciences, Heidelberg, Germany) equipped with CellQuest software (BD Biosciences). Data were analyzed using FCS Express software, version 5 (DeNovo Software, Glendale, CA, USA).

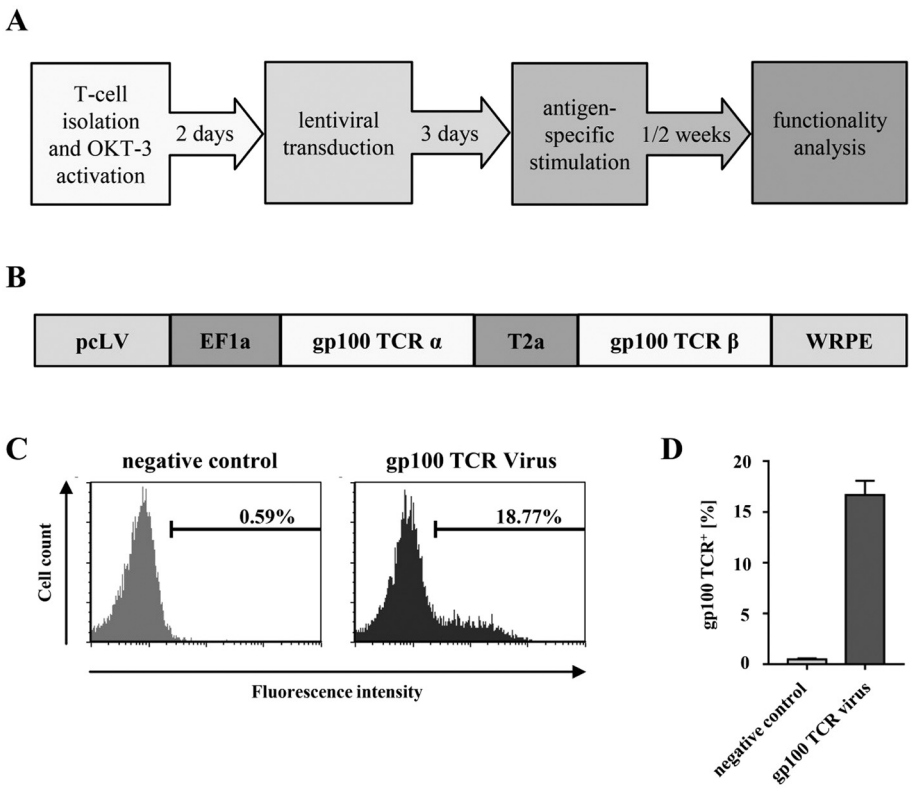

Fig. 2. T cells can be efficiently transduced with a gp100-specific TCR. (A) Schematic of the experimental procedure. First, human T cells were isolated from PBMCs and activated with an anti-CD3 antibody, IL-2, and an anti-CD28 antibody. On day two, T cells were transduced with the TCR viral construct together with the lentiviral transduction enhancer LentiBOOST™. Following 3 days, transduced cells were antigen-specifically stimulated with gp100-peptide loaded A375M cells. After 1 or 2 weeks of antigen-specific activation, the gp100 TCR-transduced cells were used for functionality assays. (B) Schematic of the gp100-specific TCR viral construct. The vector plasmid pcLV included the EF1a promoter, the α- and β-chains of the gp100 TCR fused by T2a, as well as the woodchuck hepatitis virus posttranscriptional regulatory element (WRPE). (C + D) $CD8^+$ T cells were lentivirally transduced with the gp100-specific TCR using a MOI of 10 together with the lentiviral transduction enhancer LentiBOOST™. Non-transduced cells were used as negative control. Percentages of transduction efficacy of T cells were obtained via flow cytometry 3 days after lentiviral transduction. (C) Histograms of one representative and (D) average percentage of gp100 TCR-positive cells with SEM of 5 independent experiments are shown.

2.4. Cytokine production

Analysis of cytokine secretion of T cells was performed as previously described (Simon et al., 2018a). In brief, transduced T cells were stimulated overnight with the target cell lines T2.A1 and A375M (either unpulsed or gp100-peptide loaded) at a 1:1 ratio. Cytokine concentrations in the supernatants were detected using the Th1/Th2 Cytometric Bead Array Kit II (BD Biosciences) according to manufacturer's instructions. Immunofluorescence was measured using the FACSCanto II (BD Biosciences) equipped with FACSDiva software (BD Biosciences). Data were analyzed via FCS Express software, version 5 (DeNovo Software).

2.5. Cytotoxicity

The cytolytic actitivty of transduced T cells was analyzed with a standard 4–6 h ^{51}chromium-release assay, as previously described (Simon et al., 2018b). First, target cell lines T2.A1 and A375M were labelled with 20 µCi of $Na_2{}^{51}CrO_4/10^6$ cells (Perkin Elmer, Waltham, MA, USA) for 1 h. After that, an aliquot of the target cells was loaded with the gp100 peptide for another hour. Transduced T cells were added to labelled target cells to obtain following “effector to target cell ratios” (E:T): 60:1, 20:1, 6:1, and 2:1. Released chromium concentrations in the supernatants were detected with the Wallac 1450 MicroBeta plus Scintillation Counter (Wallac, Turku, Finland). The percentage of lysis was calculated using following formula: [(measured release – background release)] / [(maximum release – background release)] × 100%.

2.6. Figure preparation and statistical analysis

Graphs were created and statistical analysis was performed using GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA, USA). The *p*-values were analyzed using the paired Students *t*-test, assuming a Gaussian distribution. * indicates $p \leq .05$ and ** indicates $p \leq .01$.

3. Results

3.1. Analysis of appropriate MOI and LentiBOOST™-mediated transduction efficacy using a GFP virus construct

In order to determine the ideal MOI, human T cells were short-time activated with an anti-CD3 antibody, IL-2, and an anti-CD28 antibody and subsequently transduced with a range of different MOI of a lentiviral construct expressing GFP (MOI 5, MOI 10, and MOI 20). GFP expression was assessed on day three after lentiviral transduction via flow cytometry.

Non-transduced cells, which served as negative control, showed no GFP expression, as expected (Fig. 1A and B). The use of MOI 5 to transduce T cells revealed a clear GFP-positive cell population. The transduction of T cells with MOI 10 could significantly increase the rate of GFP-expressing cells (Fig. 1A and B and Table S1). While a further increase in GFP-positive cells was observed when a MOI of 20 was used, the difference in transduction rate was not significant when compared to T cells transduced with a MOI of 10 (Fig. 1A and B and Table S1). Therefore, MOI 10 was used for the transduction of T cells in the following experiments. In order to further improve the transduction efficacy of T cells, LentiBOOST™ was used in combination with the lentiviral construct. The transduction rate could be more than doubled through the application of the LentiBOOST™. (Fig. 1C and D and Table S1). Moreover, the additional use of the transduction enhancer did not result in an increase of non-viable cells, indicating that there is no toxic effect when used in human T cells (Table S2 and S3).

Altogether, human $CD8^+$ T cells could be successfully transduced using the GFP-encoding lentivirus and the rate of transduced cells could be further increased significantly with the application of LentiBOOST™, while the viability of cells was not affected.

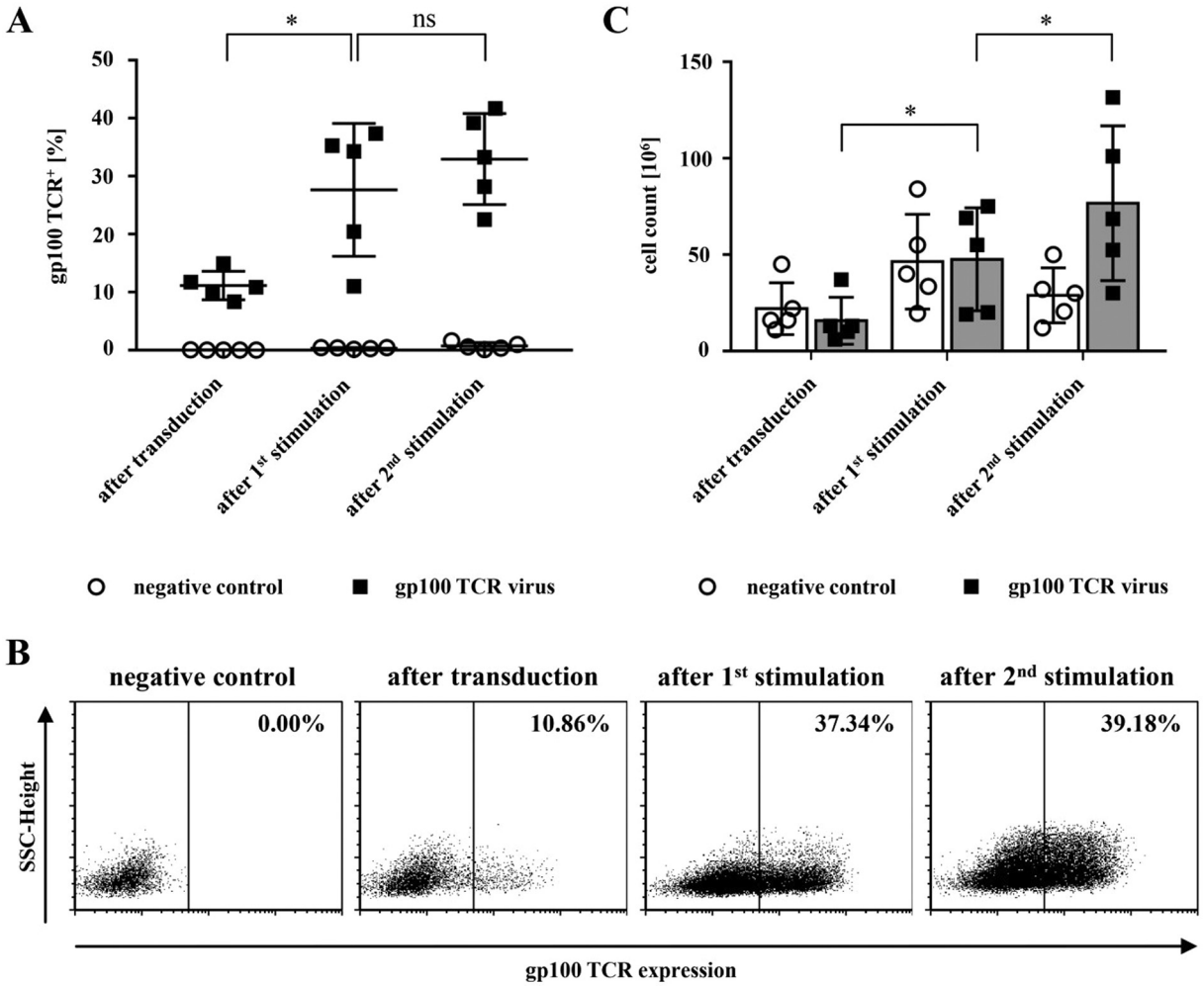

Fig. 3. Antigen-specific stimulation of gp100 TCR-transduced cells. $CD8^+$ T cells were lentivirally transduced with the gp100-specific TCR using a MOI of 10 in combination with the lentiviral transduction enhancer LentiBOOST™. Transduced T cells were either antigen-specifically stimulated once with gp100 peptide-loaded A375M melanoma cells for 7 days or twice for another week. Non-transduced cells served as negative control. (A + B) Transduction rate was assessed via flow cytometric approach at following time-points: 3 days after transduction, 7 days after first stimulation, and 7 days after second stimulation. (A) Mean percentages of gp100 TCR-positive cells ± SEM of 5 independent experiments, each depicted as individual symbols, are shown. (B) Dot plots of one representative out of 5 independent experiments are depicted. (C) Total amount of T cells (transduced and non-transduced cells) were counted at abovementioned time-points. Average values ± SEM of 5 independent experiments, each depicted as individual symbols, are shown. The *p*-values were calculated by paired Student's *t*-test and are listed in Table S4.

3.2. T cells can be efficiently transduced with a lentivirus expressing a gp100-specific TCR in combination with the lentiviral transduction enhancer LentiBOOST™

After evaluation of the ideal MOI, T cells were transduced with a lentiviral construct expressing a gp100-specific TCR. Therefore, T cells were isolated from PBMCs and subsequently activated with an anti-CD3 antibody, IL-2, and an anti-CD28 antibody (Fig. 2A). On day two, cells were transduced with the lentiviral construct containing the gp100-specific TCR in combination with LentiBOOST™. Following another 3 days, transduced cells were antigen-specifically stimulated for one or 2 weeks (Fig. 2A). The lentiviral vector plasmid pcLV contained the α- and β-chains of the gp100-specific TCR fused by a T2a link (Fig. 2B). The expression of the gp100-specific TCR was controlled by the EF1a promotor and was further enhanced by the woodchuck hepatitis virus posttranscriptional regulatory element (WRPE) included in the vector plasmid (Fig. 2B).

Three days after transduction of T cells, transduction efficacy was determined using flow cytometry. The expression of the gp100-specific TCR was confirmed using an MHC-Dextramer (HLA-A*0201/YLEPGP-VTV). Non-transduced cells displayed no expression of the gp100-specific TCR (Fig. 2C and D). Lentivirally transduced cells clearly exhibited a gp100 TCR-positive population (Fig. 2C and D), revealing that T cells can be efficiently transduced using the gp100-encoding lentivirus together with LentiBOOST™.

3.3. Antigen-specific stimulation of gp100 TCR T cells

To increase the number of gp100 TCR-positive cells after lentiviral transduction, T cells were antigen-specifically activated with human melanoma A375M cells pulsed with the HLA-A2-restricted peptide $gp100_{280–288}$. This antigen-specific stimulation was performed either once for 7 days or twice for another week and transduction rate as well as cell count were determined after each activation round.

Following 1 week of stimulation, the percentage of gp100 TCR-positive cells expanded significantly (Fig. 3A and B and Table S4). After the second stimulation, an additional increase of transduced cells was observed (Fig. 3A and B and Table S4 and S5). However, no further significant increase of gp100-specific T cells were seen between first and second stimulation (Fig. 3A and Table S4). Concerning total cell count (including non-transduced cells), each stimulation round displayed a significant increase (Fig. 3C and Table S4). Of note, a higher

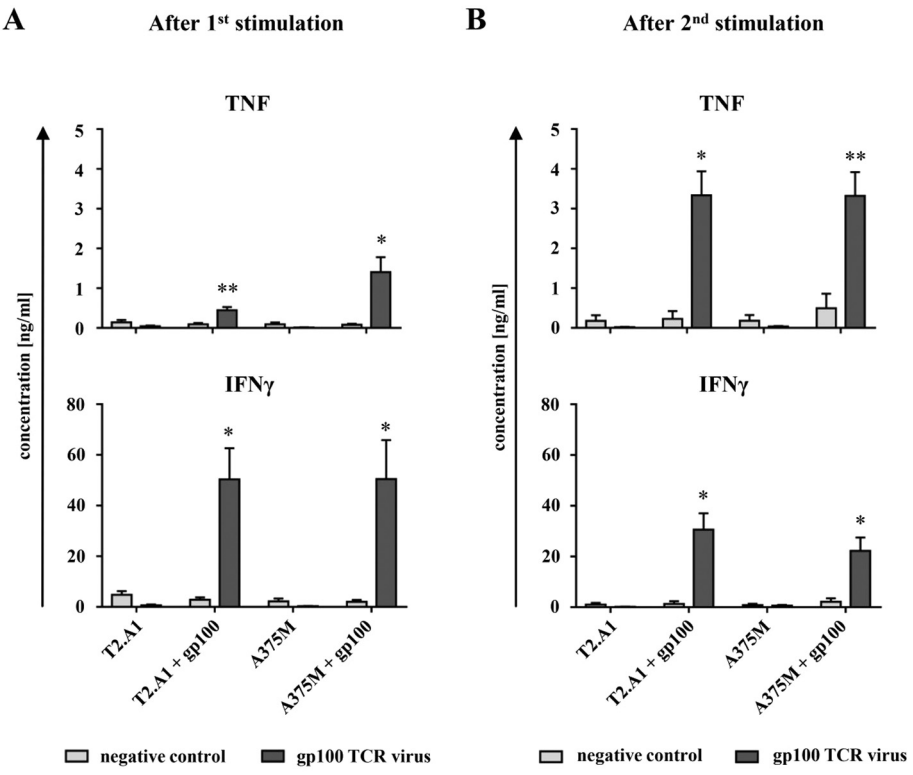

Fig. 4. Gp100 TCR T cells specifically produce cytokines after stimulation with antigen-positive tumor cells. (A + B) CD8$^+$ T cells were lentivirally transduced with the gp100-specific TCR using a MOI of 10 together with the lentiviral transduction enhancer LentiBOOST™. Then, transduced T cells were stimulated with gp100 peptide-loaded A375M cells for 1 week (A) or for another 7 days (B). Non-transduced cells were used as negative control. Following overnight stimulation of T cells with either gp100 peptide-loaded or unpulsed T2.A1 (HLA-A2$^+$, gp100$^-$) and A375M (HLA-A2$^+$, gp100$^-$) tumor cells, the production of TNF and IFNγ was measured in a cytometric bead array (CBA). Mean values of 4 (A) and 5 (B) independent experiments with SEM are shown. The *p*-values were calculated by paired Student's *t*-test and are listed in Table S6.

donor variability was seen following the second stimulation of transduced cells (Fig. 3C and Table S5).

Analysis of proliferation capacity of gp100 TCR-transduced cells revealed a slightly higher effector T (T_{EM}) cell phenotype and a lower percentage of naïve T cells after the second stimulation compared to one stimulation round (Fig. S1A). In addition, staining of the molecule programmed cell death protein 1 (PD-1) on the surface of cells revealed low levels of exhaustion (Fig. S1B and C).

The comparison of 1 week versus 2 weeks of antigen-specific stimulation of gp100 TCR-transduced T cells suggests that (depending on the starting cell count and tranduced cells needed) 1 week of activation already results in an appropriate rate of transduced cells, while the cell number further increased significantly after the second round of activation.

3.4. Gp100 TCR-transduced T cells produce cytokines after antigen encounter

In the next step, functionality analysis was performed to examine the antigen-specific cytokine secretion of transduced cells. Therefore, T cells were co-incubated overnight with tumor cells, either unloaded or pulsed with the HLA-A2-restricted peptide $gp100_{280–288}$. Target cell lines included the human TxB cell hybridoma (HLA-A2$^+$, gp100$^-$) and the human melanoma cells A375M (HLA-A2$^+$, gp100$^-$). In the next step, the production of cytokines by T cells was assessed in a cytometric bead array. Non-transduced T cells served as negative control.

Incubation with unloaded tumor cells showed no or only little background secretion of TNF and IFNγ after each stimulation round in all T cell conditions (Fig. 4A and B). Following 1 week of antigen-specific activation, TCR-transduced cells revealed a significantly higher antigen-specific TNF and IFNγ production after stimulation with gp100-peptide pulsed tumor cell lines compared to non-transduced cells (Fig. 4A and Table S6). Similar results were obtained after 2 weeks of antigen-specific stimulation (Fig. 4B and Table S6). Comparison of one and two stimulation rounds displayed a significantly higher production of TNF by twice stimulated gp100 TCR-transduced cells following co-incubation with gp100-peptide loaded T2.A1 target cells (Table S7).

In addition, the production of IL-2 and IL-4 were analyzed (Fig. S2 and Table S8). Transduced T cells revealed high secretion of IL-2 after antigen encounter independent of the number of antigen-specific stimulations performed (Fig. S2). Analysis of the anti-inflammatory cytokine IL-4 showed very low production levels with values below 100 pg/ml in all T cell conditions (Fig. S2).

Altogether, TCR T cells showed a high secretion of pro-inflammatory cytokines. Comparable results were observed after 1 week and 2 weeks of antigen-specific stimulation of transduced cells.

3.5. Gp100 TCR T cells specifically lyse antigen-positive tumor cells

To investigate whether the transduced cells are also able to specifically lyse antigen-positive tumor cells, a cytotoxcitiy assay was performed comparing gp100 TCR-transduced T cells after the first and the second antigen-specific stimulation cycle to non-tranduced cells. T cells were co-incubated with cell lines at the following effector to target cell ratios (E:T) 60:1, 20:1, 6:1, and 2:1. As target cells, the abovementioned human TxB cell hybridoma (HLA-A2$^+$, gp100$^-$) and the human melanoma cells A375M (HLA-A2$^+$, gp100$^-$), either unloaded or pulsed with the HLA-A2-restricted peptide $gp100_{280–288}$, were used.

Non-transduced cells, which were used as negative control, showed no or only little background lysis of target cells, as expected (Fig. 5A and B). Gp100 TCR-transduced cells displayed a significantly higher antigen-specific cytotoxicity towards gp100-peptide loaded T2.A1 and A375M cells after first antigen-specific stimulation (Fig. 5A and Table S8). Lentivirally transduced cells that were antigen-specifically activated twice exhibited similar results (Fig. 5B and Table S9). When comparing one and two stimulation rounds, gp100 TCR-transduced cells revealed a significantly higher lysis of gp100-peptide pulsed target cells following second antigen-specific stimulation (Table S10).

Thus, gp100 TCR-transduced cells revealed a strong lytic capacity after antigen encounter following one cycle as well as two cyles of antigen-specific stimulation.

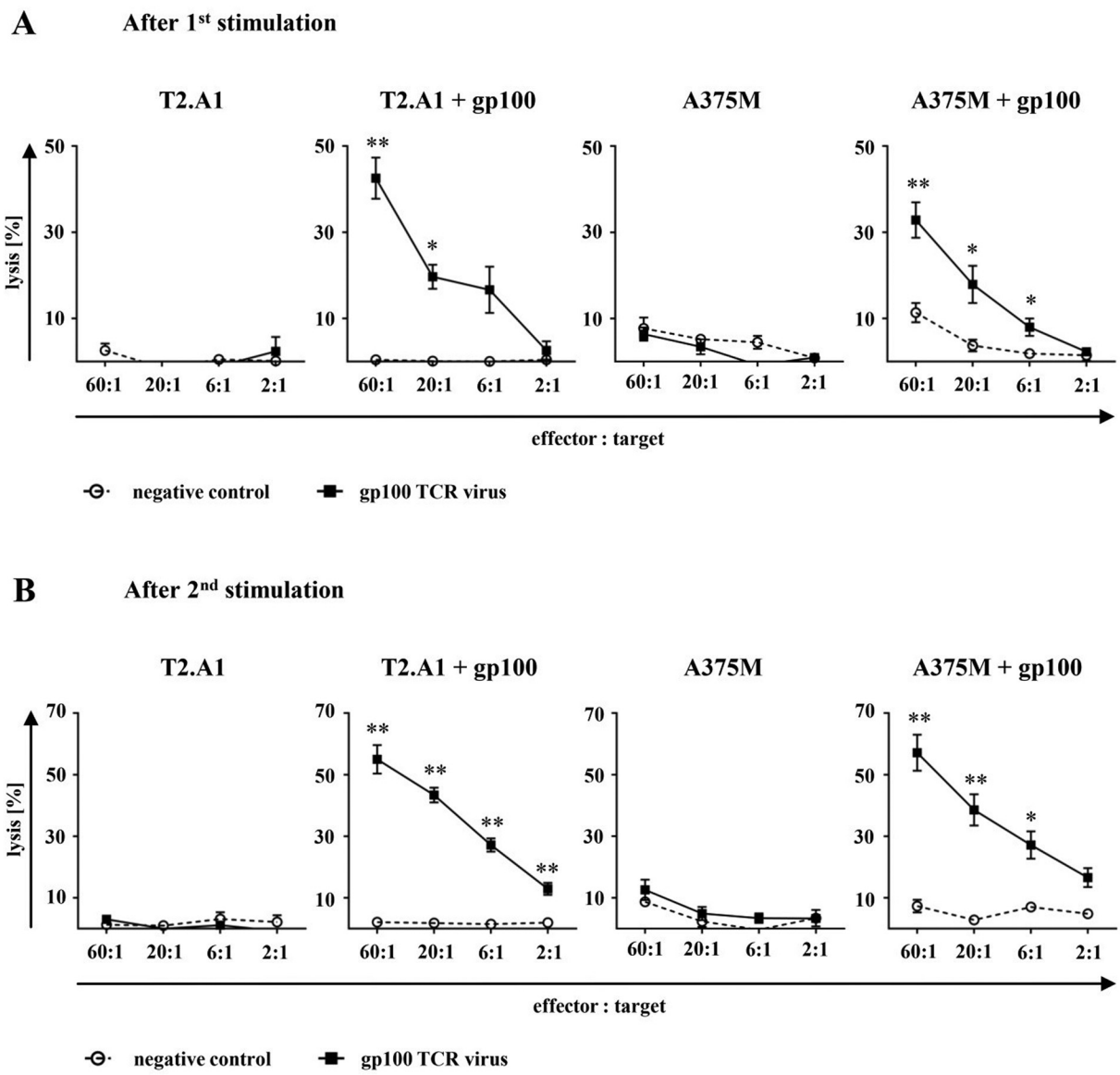

Fig. 5. Gp100 TCR T cells lyse tumor cells after antigen encounter. $CD8^+$ T cells were lentivirally transduced with the gp100-specific TCR using a MOI of 10 in combination with the lentiviral transduction enhancer LentiBOOST™. Transduced T cells were antigen-specifically stimulated with gp100 peptide-loaded A375M cells either for 7 days (A) or for another week (B). Non-transduced cells served as negative control. T cells were co-incubated for 4–6 h with the target cell lines T2.A1 ($HLA\text{-}A2^+$, $gp100^-$) and A375M ($HLA\text{-}A2^+$, $gp100^-$), which were either used unpulsed or loaded with gp100 peptide beforehand. Cytotoxicity of T cells was assessed in a ^{51}chromium-release assay and the percentages of lysed cells were determined at following effector cell to target cell ratios (E:T): 60:1, 20:1, 6:1, 2:1. Average percentages of 4 (A) and 5 (B) independent experiments ± SEM are shown. The *p*-values were calculated by paired Student's *t*-test and are listed in Table S9.

4. Discussion

The adoptive transfer of tumor antigen-specific T cells represents a valuable and promising strategy for the treatment of different malignancies. However, the application of engineered cells can be hampered due to low transduction and/or expansion rate of engineered cells (Schuster et al., 2017). In our study, we could efficiently transduce $CD8^+$ T cells with a lentiviral construct together with the transduction enhancer LentiBOOST™. No effects on cell viability were observed. Thus, the use of LentiBOOST™ may represent an effective tool to improve lentiviral transduction of human T cells in order to generate tumor antigen-specific TCR T cells.

The comparison of the 1st and the 2nd antigen-specific stimulation of gp100 TCR-transduced cells revealed superior effector functions regarding cytokine secretion and cytolytic capacity after two cycles of stimulation. While the total cell count showed a significant increase after each stimulation round, the rate of transduced cells remained at a comparable level, indicating that (depending on the starting cell count of cells to be transduced), 1 week of activation might be sufficient to obtain an appropriate transduction rate, cell number, and effector functions. Regarding a potential clinical application of antigen-specific stimulation, the generation of autologous antigen-presenting cells (APCs) might be an appropriate alternative to the A375M cells we used for this study. APCs, e.g. dendritic cells or monocytes, can easily be isolated from the patient and loaded with GMP-grade gp100 peptide.

Lentiviral vectors are suitable and safe tools to transduce a broad range of cells (Kafri, 2004; Wong et al., 2006; Dull et al., 1998). Moreover, one of the recently approved CD19-directed CAR T cells are generated using lentiviral receptor transfer (Maude et al., 2018; Schuster et al., 2017). An effective strategy to enhance the gene transfer represents, for instance, transduction-promoting polyglycans (Cornetta and Anderson, 1989; Wurm et al., 2010). However, these enhancers could also have toxic effects to some cells, which might limit their use (Lin et al., 2011). The addition of LentiBOOST™ to improve

transduction efficacy of engineered T cells relies on the effects of the included poloxamer P338 and the polycation polybrene (Anastasov et al., 2016). Both agents lead to a reduction of repulsion forces between the cell and the virus and support the binding of viral particles to the cell surface (Anastasov et al., 2016; Swaney et al., 1997; Denning et al., 2013). P338 has a low toxicity and its clinical use is already approved by the U.S. Food and Drug Administration (FDA) (Anastasov et al., 2016). For the use in clinical trials, the application of polybrene may be a problem due to its toxic effects on different cell types (Cornetta and Anderson, 1989). As an alternative, the polycation protamine sulfate represents a suitable alternative to polybrene, as it is classified as an approved drug by the FDA and as it shows similar improvement of viral transduction efficacy (Cornetta and Anderson, 1989; Seitz et al., 1998). In our study, no cytotoxic effect was observed, as no difference in cell viability was seen when LentiBOOST™ was used in addition to the lentivirus.

When using lentiviral transduction, which represents a stable DNA-based receptor transfer into T cells, potential on-target/off-tissue and off-target toxicities induced by engineered T cells attacking non-malignant host cells should not be underestimated and thus, this remains a feared side effect in ACT (Curran et al., 2012). This is caused by various mechanisms including the (unexpected) expression of tumor antigens on healthy tissue (Lamers et al., 2006; Lamers and Sleijfer, 2013; Morgan et al., 2010; Parkhurst et al., 2011) or the cross-reaction of tumor-specific receptors with host molecules (Cameron et al., 2013; Linette et al., 2013). Another challenge is posed by possible random formation of new specificities originating from an α/β-chain-mispairing of the introduced α/β TCR with the endogenous α/β TCR, which could create a reactivity for host molecules (Debets et al., 2002; Zhang et al., 2004). Furthermore, T cell on-target over-activation can result in a cytokine release syndrome (van den Berg et al., 2015). In addition, the overall anti-tumor effect of ACT may not only depend on the functionality of the adoptively transferred T cells, but also on maintaining a sufficient concentration of engineered cells in the patients' blood (Maus et al., 2014; Robbins et al., 2004). Nevertheless, viral DNA-based transduction of T cells is the method of choice used in most clinical studies showing promising results. To counteract potential α/β chain mispairing with the endogenous α/β TCR, or a potential reactivation of dormant self-reactive T cells, the use of engineered alternative cell populations, like human γ/δ T cells, may be another option, as the endogenous TCR of this cell population does not pair with α- or β-chains (Harrer et al., 2017; Koning et al., 1987; Saito et al., 1988). However, the constant improvement in, e.g. engineering of T cells, genetic editing, and the selection of effective cell populations, will certainly further increase the power and safety of ACT (June and Sadelain, 2018; June et al., 2018) in the near future.

Taken together, the results of our study show that it is feasible to generate gp100-specific TCR T cells using a lentiviral construct in combination with the transduction enhancer LentiBOOST™. The transduction rate was significantly improved while no cytotoxic effects were observed on human T cells. In addition, gp100 TCR-transduced cells displayed robust effector functions after one or two rounds of stimulation. Thus, the additional use of LentiBOOST™ for enhanced lentiviral transduction of human T cells may represent a promising tool for the potential use in cancer immunotherapy.

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Conflicts of interest

Christian Thirion is a founder and shareholder of Sirion Biotech GmbH. The other authors have no conflicts of interest to declare.

Author's contribution statement

BS performed the experiments, analyzed and illustrated the data and wrote the manuscript. DCH and CT provided valuable technical advice for conducting the experiments and critically revised the manuscript. DCH participated in data analysis and interpretation. BST and GS influenced the outline of the experimental study design and critically revised the manuscript. UU masterminded the study, planned the experimental setting, participated in the data analysis and interpretation, and critically revised the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.06.015.

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