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Witte, M.A. de

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Chapter 6

Requirements for effective anti-tumor responses of TCR transduced T cells

Moniek A. de Witte*, Annelies Jorritsma*, Marly D. van den Boom, Maarten Dokter,
John B.A.G. Haanen and Ton N.M. Schumacher

(* these authors contributed equally to this work)

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Chapter 6

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John B.A.G. Haanen and Ton N.M. Schumacher

Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands. *These authors contributed equally to this work

Adoptive transfer of T cell receptor gene-modified T cells has been proposed as an attractive approach to target tumors for which it is difficult or impossible to induce strong tumor-specific T cell responses by vaccination. Whereas the feasibility of generating tumor antigen-specific T cells by gene transfer has been demonstrated, the factors that determine the *in vivo* effectiveness of TCR modified T cells are largely unknown. We have analyzed the value of a number of clinically feasible strategies to enhance the anti-tumor potential of TCR modified T cells. These experiments reveal three factors that contribute greatly to the *in vivo* potency of TCR modified T cells. First, irradiation-induced host conditioning is superior to vaccine-induced activation of genetically modified T cells. Second, increasing TCR expression through genetic optimization of TCR sequences has a profound effect on *in vivo* anti-tumor activity. Third, a high precursor frequency of TCR modified T cells within the graft is essential. Tumors that ultimately progress in animals treated with this optimized regimen for TCR-based adoptive cell transfer invariably display a reduced expression of the target antigen. This suggests TCR gene therapy can achieve a sufficiently strong selective pressure to warrant the simultaneous targeting of multiple antigens. The strategies outlined here should be of value to enhance the anti-tumor activity of TCR-modified T cells in clinical trials.

Introduction

Adoptive cell therapy (ACT) with TCR modified T cells is no longer a mere preclinical strategy but is now analyzed in phase I clinical trials. The rationale behind the development of TCR modified T cell therapy is persuasive. For tumor-associated antigens for which the endogenous T cell repertoire is limited in size or activity due to self-tolerance, it seems reasonable to supply this repertoire by infusion of genetically engineered tumor specific T cells¹. The status of the field can be summarized as follows. First, TCR modified T cells can reliably be generated against a large number of tumor-associated antigens²⁻⁷. Second, engineering approaches such as optimization of TCR gene sequences^{8,9}, inclusion of murine constant domains¹⁰, or inclusion of an engineered disulfide bond^{11,12} can be utilized to enhance the expression of the introduced T cell receptor. These latter two approaches can also suppress the formation of mixed TCR dimers that are composed of endogenous and exogenous TCR chains, likely contributing to the safety of the therapy¹³. Third, TCR modified T

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cells are functional *in vivo*. A first set of studies that focused on the feasibility of TCR gene transfer in murine models demonstrated that TCR modified CD8⁺ and CD4⁺ T cells react to antigen encounter *in vivo*¹⁴⁻¹⁶, even when the endogenous T cell repertoire is non-responsive (chapters 4¹⁷ and 5). More recent work has provided first evidence for the clinical potential of TCR gene therapy¹⁸. In this phase I clinical trial, patients with metastatic melanoma were treated with autologous T lymphocytes engineered to express a TCR specific for the melanocyte differentiation antigen MART-I. Notably, following T cell infusion, tumor regression was observed in 2 patients and these clinical responses appeared to correlate with the magnitude of the TCR modified T cell response upon infusion.

While these preclinical and clinical data suggest that the underlying rationale behind this therapy is valid, it is important to emphasize that substantial improvements are required to transform TCR gene transfer into a clinically meaningful strategy. Specifically, the clinical data obtained to date have shown that persistence of TCR gene modified T cells in individual patients is variable, and that the expression of the introduced MART-I-specific TCR was markedly lower than TCR expression from the endogenous loci¹⁸. Perhaps because of this, with a response rate of 2/17, clinical effectiveness of TCR gene transfer was clearly less than that of prior trials by the Rosenberg group that involved infusion of *ex vivo* expanded tumor-infiltrating lymphocytes^{19,20}. The results from murine studies support the notion that the current protocols for adoptive therapy with TCR modified T cells are still suboptimal. Specifically, while infusion of TCR modified T cells can be used to halt the outgrowth of transplantable¹⁷ and spontaneously developing tumors (chapter 5) in otherwise self-tolerant situations, complete remissions are achieved only rarely¹⁷.

Based on these preclinical and clinical data we concluded that, while the genetic engineering of T cell specificities can now be achieved, the functional activity of the resultant cells requires a substantial improvement. Within this study, we set out to examine a set of parameters that could influence the anti-tumor activity of TCR modified T cells *in vivo*. We reasoned that improvements in TCR gene therapy could involve one of either three factors: First, alterations within the format of the introduced TCR genes; Second, modification of the cell graft; Third, adjustment of the host environment that the gene modified T cells encounter upon infusion. Within this study we chose to analyze one parameter representing each of these three different aspects.

Materials & Methods

Mice. RIP-OVA^{hi} mice²¹ were obtained from the Experimental Animal Department of The Netherlands Cancer Institute. All animal experiments were performed in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of The Netherlands Cancer Institute.

Retroviral constructs, T cell transduction and tumor experiments. The pMX-OT-I α -IRES-OT-I β retrovirus encoding the non-modified OT-I TCR genes (pMX-OT-I_{wt}) has been described¹⁷. Optimized OT-I TCR genes were produced by GeneArt (GeneArt GmbH, Regensburg, Germany) and cloned into the retroviral vector pMX to create pMX-OT-I α _{opt}-IRES-OT-I β _{opt} (pMX-OT-I_{opt}). Mouse splenocytes were modified by retroviral transduction as described previously¹⁴. The B16-OVA cell line expressing the C-terminal part of ovalbumin (OVA) (aa 161-385) and the murine CD4 molecule as a marker gene product¹⁷ was cultured in RPMI supplemented with 10% FCS and 100 U/ml penicillin and 100 μ g/ml streptomycin. Prior to inoculation, cells were washed three times with HBSS (Gibco, Auckland, New Zealand) to remove serum components and 1×10^5 cells were injected subcutaneously in the right flank. Tumors were measured with calipers and mice were killed once tumors reached an average diameter of 10mm. For *ex vivo* analysis of antigen expression, sliced tumors were incubated in medium supplemented with collagenase IV (0.2 mg/ml; Worthington, Lakewood, NJ) and DNaseI (25 μ g/ml; Roche, Mannheim, Germany) for 20-30 minutes at 37°C. Single cell suspensions were generated with the aid of a cell strainer (BD Biosciences, Erembodegem, Belgium). Erythrocytes were removed by NH₄Cl treatment, and cells were subsequently cultured in RPMI supplemented with 10% FCS and antibiotics. After 1-3 days of culture, expression levels of the CD4 marker gene product on cells recovered from tumor material were measured as a surrogate marker of OVA expression, and were compared to CD4 expression levels on cultured B16-OVA and B16 cell lines.

Flow cytometry. Surface TCR expression was measured 24 hours after retroviral transduction by flow cytometry. Cells were stained with FITC- or PE-conjugated anti-TCR V α 2 and anti-TCR V β 5 mAbs (the V α and V β segments used by the OT-I TCR), and APC-conjugated anti-CD8 α mAb (Pharmingen). Propidium iodide (Sigma) was used to select for live cells. For the measurement of T cell responses, 25 μ l of peripheral blood was collected in heparin-coated vials (Microvette CD 300 Li-Heparine, Omnilabo, Breda, The Netherlands) at the indicated days post-transfer. Following removal of erythrocytes by NH₄Cl treatment, the cells were stained with the indicated antibodies and analyzed by flow cytometry. Data acquisition and analysis was done on a FACS Calibur (Becton Dickinson, MountainView, CA) with CellQuest and FCS express (De Novo Software, Thornhill, Ontario, Canada) software.

Irradiation-induced host conditioning and viral vaccination. Irradiation-induced host conditioning was achieved by 5 Gy total body irradiation (TBI) with a radiobiology constant potential X-ray unit (Pantak HF-320; Pantak Limited, Reading, United Kingdom), one day before adoptive cell transfer. For viral vaccination, mice were infected intraperitoneally at the indicated timepoints with 1×10^6 PFU of a recombinant vaccinia strain that expresses ovalbumin (rVV-OVA)²².

Measurement of blood glucose levels and treatment of diabetes. To monitor the onset and severity of diabetes, mice were weighed regularly throughout experiments and in case of weight loss, blood

glucose levels were monitored by Accu-Check Compact (Roche Diagnostics, Germany) measurement. Mice were considered diabetic when blood glucose levels reached > 20 mmol/L. To allow long-term follow-up, diabetic mice were treated by subcutaneous introduction of insulin implants according to the manufacturer's protocol (©LinShin Canada, Inc.).

Statistics. Survival curves were compared using a Log-rank (Mantel-Cox) test. Immune responses were compared using a Student T-test. P values less than 0.05 were considered significant.

Results

ACT with TCR transduced T cells upon irradiation-induced host conditioning. Two fundamentally distinct strategies can be used to drive the expansion of adoptively transferred T cells *in vivo*. When the cognate antigen of the introduced T cells is provided by vaccination, TCR triggering is induced and the resulting T cell proliferation and T cell differentiation parallels that seen during physiological T cell responses. As an alternative to antigen-specific vaccination, host conditioning regimens such as non-myeloablative chemotherapy or irradiation can be utilized to promote the outgrowth of infused T cell populations. The mechanisms that drive T cell proliferation and differentiation in the latter case are thought to be substantially more diverse. First, the reduction in T cell and NK cell numbers that is achieved by host conditioning leads to an enhanced availability of IL-7 and IL-15, cytokines that can induce T cell proliferation independent of the presence of cognate antigen. In addition, depletion of regulatory T cells and release of adjuvants from intestinal bacteria may further drive T cell activation. Finally, in case tumor-specific T cells are infused into tumor-bearing hosts, release of cognate antigen as a consequence of tumor cell death may be an added contributing factor. Importantly, due to the fact that T cell expansion upon vaccination and host conditioning is driven by distinct mechanisms, both the persistence and functional properties of the induced T cell population can differ^{23,24}. Specifically, while vaccination results in the rapid emergence of a highly differentiated pool of effector T cells²⁵, T cell populations induced by host conditioning display properties of memory T cells²³, possibly translating in an enhanced capacity for long-term persistence.

Irradiation- and chemotherapy-induced host conditioning prior to adoptive T cell transfer has been used to enhance the *in vivo* expansion and anti-tumor effect of TCR-transgenic T cells in mouse models²⁶ and of tumor-infiltrating lymphocytes (TIL) in melanoma patients¹⁹. Likewise, in the phase I TCR gene therapy trial by Morgan and colleagues, chemotherapy-induced host conditioning was used with the aim to facilitate engraftment of the infused TCR modified T cells. However, in preceding preclinical studies of TCR gene transfer in mouse models^{14,17}, vaccination rather than host conditioning has been used to drive activation and expansion of the transferred TCR modified T cells, and a comparison of the two strategies has not been made. It is noted that the identification of optimal engraftment regimens for TCR modified T cells is of particular relevance as prior experiments have

revealed that when TCR-modified T cells are infused into unconditioned hosts in the absence of vaccination, only negligible numbers of TCR modified T cells can be recovered (M. Coccoris, J. Immunology, *in press*).

To first develop a mouse model that allows a comparison of the relative value of host conditioning regimens, vaccination regimens and other variables in TCR gene transfer, RIP-OVA^{hi} were injected subcutaneously with B16 tumors expressing OVA. As documented previously, RIP-OVA^{hi} mice are tolerant towards the self antigen ovalbumin. As a consequence, the endogenous T cell repertoire is unable to influence the outgrowth of B16-OVA tumors, even upon vaccination¹⁷ and this model thereby forms a stringent test of the value of different approaches for ACT. In a first set of experiments, RIP-OVA^{hi} mice were challenged with B16-OVA tumor cells. Subsequently, mice were either left untreated, or were treated by 5 Gy total body irradiation (TBI, leading to sublethal lymphodepletion) on day 6, plus transfer of 1×10^6 of either OT-I TCR transduced or mock transduced CD8⁺ T cells the following day. Infusion of mock-transduced cells in mice conditioned by TBI at day 6 post tumor inoculation had a minimal effect on the kinetics of tumor growth (Fig. 1A) or survival (Fig. 1B). In contrast, in mice that received OT-I TCR transduced rather than mock-transduced cell populations, tumor outgrowth was markedly inhibited (Fig. 1A-B; average survival of 24 versus 60 days; $p < 0.005$). Furthermore, in recipients of OT-I transduced cell populations a highly dominant CD8⁺ cell population expressing the V α 2 and V β 5.1 TCR chains of the OT-I TCR quickly became detectable, and this population persisted up to the end of the experiment (average frequency of V α 2⁺V β 5.1⁺ cells ~75% of total CD8⁺ cells at peak, ~40% after 1 month) (Fig. 1C). These data show that a combination of host conditioning plus transfer of TCR-modified T cells that are rendered reactive against a defined self antigen can lead to a prolonged anti-tumor effect in an otherwise self tolerant setting. Furthermore, this combination yields a T cell repertoire that is markedly skewed towards tumor reactivity.

To modify this mouse model to a setting where a possible enhancing effect of further variations in ACT strategies could be apparent, a second cohort of mice was treated with the same combination of irradiation and T cell infusion, but with treatment starting on day 9. Irradiation of mice in combination with transfer of mock-transduced T cells again had no significant effect on tumor growth nor survival as compared to mice that did not receive any form of treatment. In this setting of delayed T cell therapy, host conditioning in combination with ACT of OT-I transduced T cells still resulted in a clear suppression of tumor growth (Fig 1D-E). However, tumors continued to progress, resulting in only a moderate increase in survival (20 days versus 34 days; $p < 0.005$), providing a situation where further improvements in ACT strategies should be detectable. Also in this setting, where T cell infusion was performed at day 10 post tumor inoculation, marked T cell responses of TCR modified T cells were apparent in peripheral blood (Fig 1F).

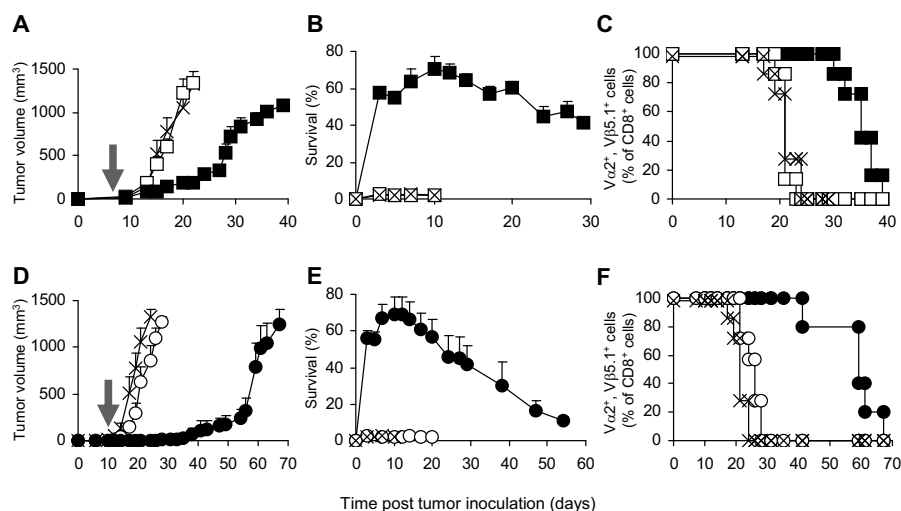


Figure 1. Transfer of TCR transduced T-cells in combination with irradiation-induced host conditioning. RIP-OVA^{hi} mice (N=5-7 per group) were inoculated with 1×10^5 B16-OVA tumor cells subcutaneously, followed by sublethal TBI at day 6 (A-C) or day 9 (D-F) and transfer of 1×10^6 OT-Iwt TCR transduced CD8⁺ T cells (filled symbols) or an equal amount of mock transduced T cells (open symbols) at day 7 (A-C) or day 10 (D-F). Tumor growth was compared to that in control mice (depicted by crosses). (A, D) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. Arrow indicates time point of adoptive transfer. (B, E) Kaplan-Meier survival plot. Mice were sacrificed once tumors reached an average diameter of 10mm or when tumors started bleeding. P-values of B: irradiation vs irradiation + ACT: 0.0014; no treatment vs irradiation + ACT: 0.0014; no treatment vs irradiation 0.016 (Mantel-Cox test); P-values of E: irradiation vs irradiation + ACT: 0.0002; no treatment vs irradiation + ACT: 0.0002; no treatment vs irradiation 0.35 (Mantel-Cox test) (C, F) Analysis of blood cells of irradiated RIP-OVA^{hi} mice at indicated time points post adoptive cell transfer. Bars indicate SD.

Irradiation-induced host conditioning outperforms vaccination as an engraftment regimen for TCR modified T cells. Having established that TCR modified T cells proliferate extensively in a conditioned host, we aimed to compare irradiation-induced host conditioning to active vaccination as strategies to boost the anti-tumor potential of infused TCR modified T cells. To this purpose, T cell responses and tumor outgrowth were compared in three groups. In a first experimental group, OT-I TCR transduced T cells were infused at day 10 in tumor bearing RIP-OVA^{hi} mice, and mice were then vaccinated with a recombinant vaccinia virus expressing the OVA antigen (rVV-OVA). In a second group, OT-I TCR transduced T cells were infused at day 10 in tumor bearing RIP-OVA^{hi} mice that had received sublethal TBI one day prior to ACT. Finally, a third group of mice receiving OT-I modified T cells was treated with a combination of sublethal TBI (one day before ACT) plus rVV-OVA vaccination (day 3 post ACT), to assess whether the combined use of the two engraftment regimens would have an additive or synergistic effect. Because in these experiments T cell responses are compared between groups of mice in which endogenous T cell numbers are either unaffected ('rVV-OVA only' group) or highly reduced ('TBI' and 'TBI → rVV-OVA' groups), both the percentages and absolute numbers of TCR modified T cells were determined.

In vivo activation of OT-I transduced T cells by vaccination with rVV-OVA resulted in a very rapid burst in both the number and frequency of TCR modified T cells, with a peak frequency of TCR modified T cells of 10.3% of CD8⁺ cells on day 5 after transfer. Comparison of TCR modified T cell numbers in these mice that received rVV-OVA with those in mice that had been pretreated by TBI showed that the absolute number of TCR modified T cells early after transfer was indeed significantly higher in mice that received viral vaccination ($p < 0.005$ at day 7). However, within the second week post transfer, numbers of TCR modified T cells significantly declined in rVV-OVA vaccinated mice. Because of this contraction, and because of the continuing homeostatic T cell proliferation in recipients treated by TBI, V α 2⁺V β 5.1⁺ CD8⁺ T cell numbers in TBI-treated mice exceeded those in rVV-OVA vaccinated mice on day 10 post adoptive transfer and onwards ($p < 0.05$ at days 12 and 17).

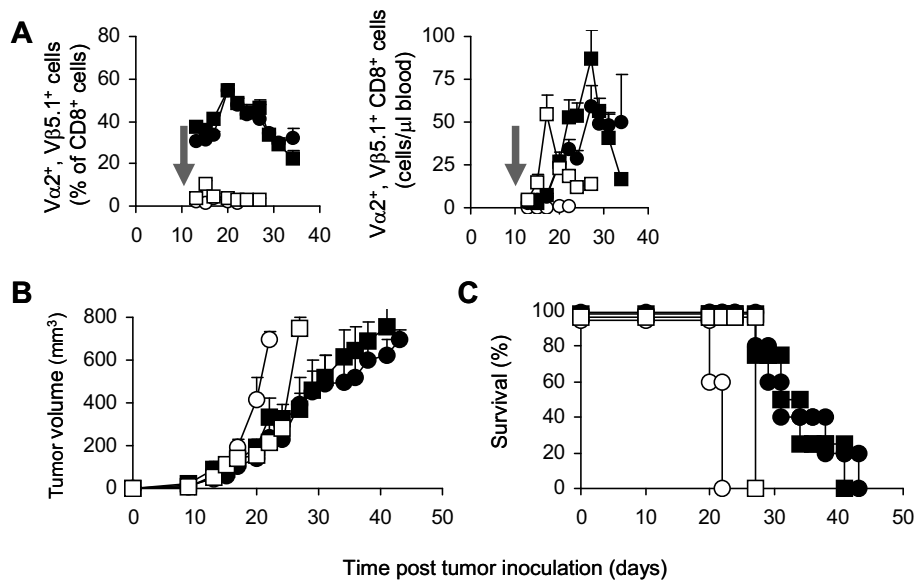


Figure 2: Enhanced persistence and anti-tumor effect of TCR transduced T cells after irradiation-induced host conditioning as compared to active vaccination. RIP-OVA^{hi} mice (N=5-7 per group) were inoculated with 1×10^5 B16-OVA tumor cells subcutaneously, and received an adoptive transfer 1×10^6 OT-I TCR transduced CD8⁺ T cells (filled circles, filled squares, open squares) or an equal amount of mock transduced T cells (open circles) at day 10. Transferred T cells were boosted either by sublethal TBI at day 9 (filled circles, open circles), vaccination with rVV-OVA at day 10 (open squares) or sublethal TBI at day 9, followed by vaccination with rVV-OVA at day 13 (filled squares). (A) Analysis of V α 2⁺V β 5.1⁺ CD8⁺ cells in peripheral blood (percentage in left panel, absolute numbers right) at indicated time points post adoptive transfer. Bars depict SEM. (B) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. (C) Kaplan-Meier survival plot. Mice were sacrificed once tumors reached an average diameter of 10mm or when tumors started bleeding. P-values: irradiation versus ACT + vaccination or ACT + irradiation or ACT + irradiation + vaccination: < 0.005 ; ACT + irradiation vs ACT + vaccination: 0.0055; ACT + irradiation vs ACT + irradiation + vaccination: 0.5; ACT + irradiation + vaccination vs ACT + vaccination: 0.01 (Mantel-Cox test).

As expected, the frequencies of TCR modified T cells in mice that received TBI greatly exceeded those in mice treated with rVV-OVA and this difference was particularly apparent at later time points post transfer (e.g. 55% versus 2.3% at day 10 post transfer). Interestingly, when TBI was combined with viral vaccination, this led to only a modest and transient further increase in both absolute numbers (Fig. 2A right panel) and frequencies (Fig. 2A left panel) of TCR modified T cells, as compared to the values found in mice conditioned by TBI only. Furthermore, there was a trend towards reduced persistence of TCR modified T cells at later time points upon inclusion of vaccination.

The more prolonged nature of TCR modified T cell responses in mice treated by TBI as compared to vaccination was also reflected in the kinetics of tumor outgrowth. The combination of ACT of OT-I TCR modified T cells plus viral vaccination resulted in a transient delay in tumor growth and a small but significant increase in survival (average 22 versus 27 days; $p < 0.005$) (Figure 2B-C). The use of TBI as a pre-conditioning regimen led to a somewhat stronger suppression of tumor outgrowth, also resulting in a more pronounced increase in survival (average 22 versus 31 days; $p < 0.005$). Interestingly, in mice that were treated by TBI, subsequent vaccination with rVV-OVA did not significantly improve tumor control or survival (average 33 days for TBI-rVV-OVA versus 31 days for TBI; $p = 0.5$). Furthermore, also when viral vaccination was given at a later time point (day 10 post ACT), the combination of vaccination and TBI had no benefit over TBI alone with regard to both tumor development and survival (data not shown). From these data we conclude that in this mouse model, irradiation-induced host conditioning outperforms viral vaccination as a regimen to promote persistence of TCR modified T cells. Furthermore, the data suggest that inclusion of a (viral) vaccine does not significantly enhance the anti-tumor effect of the combination of ACT and TBI.

Gene optimization results in a moderate increase in TCR expression but marked increase in anti-tumor efficacy. As described previously, modification of T cell receptor formats such as inclusion of a second interchain disulfide bond, incorporation of the murine constant domains and optimization of gene sequences can all lead to enhanced expression of the introduced T cell receptor. Furthermore, for the latter type of gene optimization this was accompanied by a clear increase in the number of TCR-modified T cells detected upon infusion into recipient mice⁸. To assess whether alterations that enhance the expression of introduced TCR genes also enhance the *in vivo* anti-tumor activity of TCR modified T cells, a gene-optimized variant of the OT-I TCR (termed OT-I_{opt}) was created, and RIP-OVA^{hi} derived splenocytes were retrovirally transduced with either the wild type OT-I TCR or the gene optimized variant (Figure 3A). Gene optimization resulted in a 1.4 fold increase in transduction efficiency as revealed by anti-V α 2 and anti-V β 5 staining (50% versus 69% of CD8⁺ T cells after correction for endogenous V α 2⁺V β 5⁺ cells), and this was accompanied by a 1.3 fold increase in average TCR expression (MFI of 455 versus 603 for the TCR α chain; 37 versus 50 for the TCR β chain).

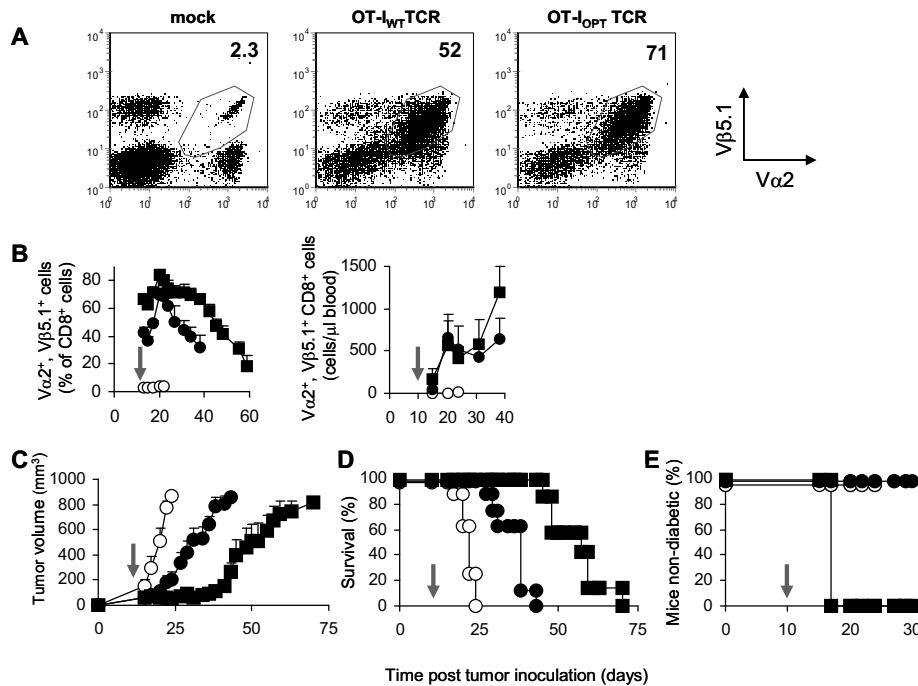


Figure 3: TCR gene optimization increases *in vivo* anti-tumor activity. (A) Flow cytometric analysis of mock (left), OT-I_{wt} (middle) or OT-I_{opt} (right) transduced T cells prior to adoptive transfer. The number in the upper right corner of each dot-plot reflects the percentage of Vα2⁺Vβ5⁺ cells within the CD8⁺ population. (B-E) RIP-OVA^{hi} mice (N=7-8 per group) were inoculated with 1x10⁵ B16-OVA tumor cells subcutaneously, sublethally irradiated at day 9 and received an adoptive transfer of 1x10⁶ OT-I_{wt} TCR transduced CD8⁺ T cells (filled circles), 1x10⁶ OT-I_{opt} TCR transduced CD8⁺ T cells (filled squares), or an equal amount of mock transduced T cells (open circles) at day 10. (B) Analysis of Vα2⁺Vβ5.1⁺ CD8⁺ cells in peripheral blood (percentages in left panel, absolute numbers in right panel) at indicated time points post adoptive transfer. Bars depict SEM. (C) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. (D) Kaplan-Meier survival plot. Mice were sacrificed once tumors reached an average diameter of 10mm or when tumors started bleeding. P-values: irradiation versus irradiation + ACT OT-I_{wt} or irradiation + ACT OT-I_{opt}: <0.005; irradiation + ACT OT-I_{wt} vs irradiation + ACT OT-I_{opt}: <0.005 (Mantel-Cox test) (E) Induction of diabetes. Mice were considered diabetic once blood glucose levels exceeded 20 mmol/l.

To determine the effect of OT-I TCR gene optimization on the anti-tumor activity of OT-I TCR transduced T cells *in vivo*, 1x10⁶ OT-I, OT-I_{opt} or mock transduced CD8⁺ T cells were transferred into tumor bearing, sublethally irradiated RIP-OVA^{hi} mice. Within the first weeks post infusion, the percentage of Vα2⁺Vβ5.1⁺ CD8⁺ T cells was slightly increased in mice that received OT-I_{opt} TCR transduced T cells, as compared to recipients of T cells expressing the parental OT-I TCR (Figure 3B, left panel), likely reflecting the somewhat higher transduction efficiency. However, the increase in absolute numbers of Vα2⁺Vβ5.1⁺ CD8⁺ T cells did not reach significance (day 10-21; p=0.2-0.4) (Figure 3B, right panel). In spite of the fact that the difference in *in vivo* T cell responses between the two groups was modest, the effect on tumor outgrowth was striking. Whereas infusion of T cells

transduced with the wild type OT-I TCR primarily led to a reduction in the kinetics of tumor outgrowth, infusion of OT-I_{opt} TCR modified T cells appeared to halt tumor development for a period of up to 1-2 months (Fig. 3C). This difference resulted in a highly significant increase in survival ($p < 0.0005$, Fig. 3D). As a second parameter of *in vivo* T cell function, 7/7 mice that had received OT-I_{opt} TCR transduced T cells developed diabetes, whereas all mice that had received an equal number of T cells transduced the wild type OT-I TCR stayed normoglycaemic (Fig. 3E). These data show that even for a high affinity TCR that is expressed well without alterations in transgene design, gene optimization has a very significant enhancing effect on the *in vivo* activity of TCR transduced T cells.

Precursor frequency of TCR modified T cells determines anti-tumor effect. While the fraction of T cells that becomes antigen-responsive upon transduction of murine T cells with mouse TCRs such as the OT-I TCR is markedly high, the percentage of antigen-responsive or MHC tetramer-positive T cells that is obtained upon transduction of human T cells with human tumor-specific TCRs generally appears to be substantially lower. Although infusion of large numbers of TCR modified T cells is still feasible with the transduction efficiencies that can be achieved in a clinical setting¹⁸, the resulting cell grafts do contain a higher number of non modified ‘passenger’ cells. To examine whether the presence of a large number of passenger cells in such grafts could affect the *in vivo* potential of the TCR modified T cells, we prepared T cell grafts containing an equal amount (1×10^6) of OT-I_{opt} TCR transduced T cells but with different amounts of ‘passenger cells’. Rather than generating such grafts by transduction with different amounts of retrovirus (in which case the reduced expression of the TCR transgene seen at lower virus doses would be a confounding factor), a single batch of TCR modified T cells was prepared, which was then either used directly, or was mixed with a 9-fold excess of mock-transduced cells (referred to as the ‘low passenger group’ and ‘high passenger group’, respectively; 56% $V\alpha 2^+V\beta 5.1^+$ cells of $CD8^+$ T cells and 5.6 % $V\alpha 2^+V\beta 5.1^+$ cells of $CD8^+$ T cells. Subsequently, the cells were transferred to B16-OVA bearing RIP-OVA^{hi} mice that had been conditioned by TBI, and T cell responses and tumor outgrowth were monitored.

Comparison of peripheral blood samples of recipients of high passenger or low passenger cell grafts revealed that the 10-fold difference in TCR modified T cell frequency prior to ACT was compressed to a difference of less than ~3-fold (79% $V\alpha 2^+V\beta 5.1^+$ of $CD8^+$ T cells in the ‘low passenger group’ versus 33% $V\alpha 2^+V\beta 5.1^+$ of $CD8^+$ T cells in the ‘high passenger group’) (Fig. 4A, left panel). The preferential outgrowth of T cells that express the OT-I TCR that is observed in particular upon infusion of cell grafts with low TCR modified T cell frequencies suggests that part of the *in vivo* proliferation is driven by TCR-specific interactions. This is consistent with the possibility that recognition of the cognate OVA antigen by TCR modified T cells provides an additional stimulus beyond that given by the lymphopenic environment. Alternatively, this preferential outgrowth of TCR modified T cells could reflect recognition of MHC molecules presenting endogenous epitopes, which have previously been shown to contribute to homeostatic proliferation in lymphopenic hosts²⁷.

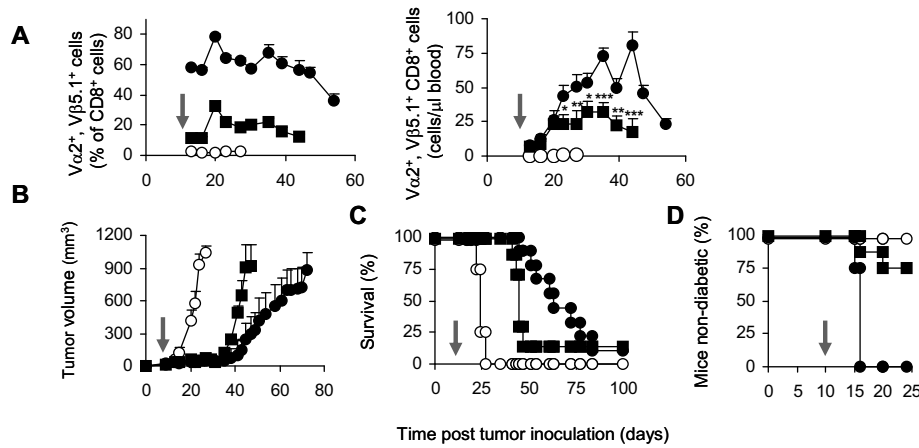


Figure 4: Precursor frequency of TCR transduced T cells affects tumor control. RIP-OVA^{hi} mice (N=7-8 per group) were inoculated with 1×10^5 B16-OVA tumor cells subcutaneously and sublethally irradiated at day 9. To determine the effect of the precursor frequency of TCR transduced T cells, 1×10^6 OT-I_{opt} TCR transduced CD8⁺ T cells were either transferred directly ('low passenger group') (filled circles, transfer of 3.6×10^6 cells in total) or 1×10^6 OT-I_{opt} TCR transduced CD8⁺ T cells were diluted 10 times with mock transduced splenocytes ('high passenger group') (filled squares, transfer of 3.6×10^7 cells in total). Control mice received 3.6×10^6 mock transduced T cells (open circles). (A) Analysis of Vα2⁺Vβ5.1⁺ CD8⁺ cells in peripheral blood (percentage in left panel, absolute numbers right) at indicated time points post adoptive transfer. Bars depict SEM. T-tests were performed to determine differences between low and high passenger groups; * represents P-value <0.05; ** <0.005; *** <0.0005. (B) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. (C) Kaplan-Meier survival plot. Mice were sacrificed once tumors reached an average diameter of 10mm or when tumors started bleeding. P-values: irradiation versus 'low passenger group': <0.0001; irradiation versus 'high passenger group' 0.002; 'low passenger group' group versus 'high passenger group': 0.15 (Mantel-Cox test). (D) Induction of diabetes. Mice were considered diabetic once blood glucose levels exceeded 20 mmol/l.

Although the mice in the 'low passenger' and 'high passenger group' received an equal number of OT-I_{opt} modified T cells, the absolute number of TCR modified T cells in peripheral blood did peak at a lower level in the 'high passenger group' (Fig. 4A, right panel). Thus, in the presence of a substantial number of passenger cells, the *in vivo* expansion of TCR modified T cells was reduced, presumably reflecting competition between the TCR modified T cells and passenger cells for homeostatic cues. Importantly, the reduced numbers of TCR modified T cells obtained *in vivo* upon infusion of grafts with a high number of passenger cells was associated with a substantially reduced capacity to control tumor growth. (Fig. 4B-C). As a second parameter for *in vivo* activity of the TCR modified T cell population, type I diabetes was induced in 25% (2/8) of the mice that received TCR modified T cells amidst a high number of passenger cells, but in 100% (7/7) of the mice that received the same number of TCR modified T cells but in a more homogeneous graft (Fig. 4E).

Immuno-editing by TCR modified T cells. In mice treated with a combination of TBI and infusion of OT-I_{opt} transduced T cells tumor progression was eventually observed in the large majority of mice (Fig. 3 and Fig. 4). Notably, analysis of individual tumor growth curves at this late phase revealed that after varying periods of one to two months in which tumor progression was essentially absent, tumors in individual mice suddenly progressed with kinetics that were comparable to those observed in untreated mice (Fig. 5A). This rapid late outgrowth of tumors in treated mice suggested an acute loss of tumor control, possibly consistent with the selection of escape variants. To address whether tumor outgrowth after prolonged control by TCR modified T cells could be explained by antigen loss, we collected tumors in a series of experiments and analyzed the expression of the CD4 marker that is translated from the same mRNA as the OVA antigen. This analysis revealed that antigen expression was substantially reduced in tumors obtained from mice that had received TCR modified T cells as compared to tumors obtained from control mice (Figure 5B; $p < 1 \times 10^{-6}$). Furthermore, in tumors that escaped immune control after more prolonged periods, evidence for antigen loss became increasingly apparent, consistent with an ongoing process of immune selection.

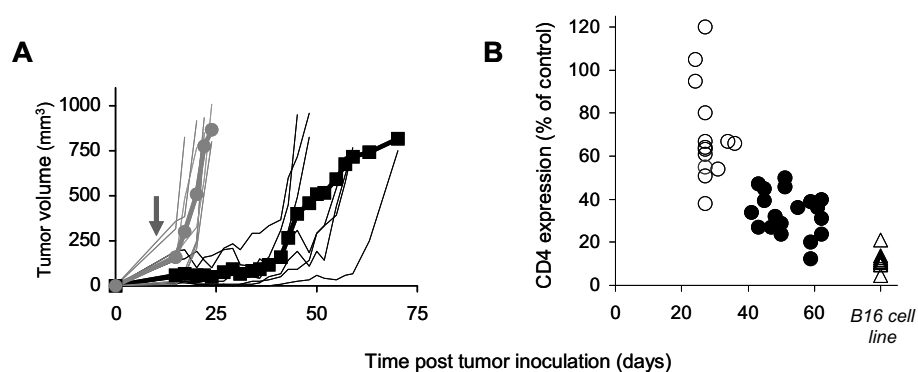


Figure 5: Immuno-editing by TCR modified T cells. (A) Individual tumor growth curves of RIP-OVA^{hi} mice depicted in Fig 3. Thin lines represent individual growth curves, thick lines with symbols represent group averages. Mice challenged with B16-OVA cells either received an adoptive transfer of 1×10^6 OT-I_{opt} TCR transduced CD8⁺ T cells (black squares/lines) or an equal amount of mock transduced T cells (grey circles/lines) at day 10. Arrow indicates timepoint of adoptive transfer. Note that after a variable period of stasis, tumors in mice treated with OT-I transduced T cells ultimately grow out with kinetics that are comparable to those seen in control mice. (B) Antigen expression on a collection of tumors obtained in a series of experiments. Mice were sacrificed when the average tumor diameter exceeded 10 mm. Expression of the CD4 marker gene was used as a surrogate marker for OVA expression and is expressed as a fraction of CD4 surface expression on cultured B16-OVA cells (redetermined at each time point of analysis). Open circles represent tumors derived from mice that received mock transduced T cells, filled circles represent tumors derived from mice that received OT-I_{opt} TCR transduced T cells, open triangles represent *in vitro* cultures of the B16 cell line.

Thus, even though the B16-OVA cell line used was derived from a single cell clone selected for high CD4 expression, the prolonged selection pressure in mice treated by TBI plus TCR modified T cell infusion resulted in the appearance of escape variants with low antigen expression. These data suggest

that -with regard to the possibility of tumor escape- the targeting of tumor associated antigens such as WT-1²⁸ or PRAME²⁹ that contribute to cellular transformation may be preferred. Alternatively, and in analogy with developments in antibody therapeutics, the simultaneous use of two or three TCRs directed against different TAAs will likely suffice to minimize the chance of tumor escape through antigen loss. Clinical implementation of such ‘oligoclonal TCR gene transfer’ will be an interesting future challenge from both a logistic and regulatory point of view.

Discussion

Inspired by the success of recombinant monoclonal antibodies such as trastuzumab (Herceptin) and rituximab (Rituxan)³⁰, much effort has been put into the preclinical testing and clinical implementation of TCR gene therapy, a strategy that can be considered the ‘cellular analogue’ of adoptive antibody therapy. With the feasibility of TCR gene transfer well established, but faced with the suboptimal anti-tumor activity of TCR modified T cells both in preclinical models as within the clinic, we here aimed to determine which factors can positively affect the clinical efficacy of TCR gene therapy. As discussed in the introduction, we consider it likely that substantial improvements can be made in three areas, involving either the host, the cell graft or the TCR itself.

The host environment & engraftment of TCR-modified T cells - We here demonstrate that host conditioning by TBI leads to superior engraftment and anti-tumor efficacy of TCR modified T cells. Whereas viral vaccination resulted in a more pronounced early boost in the number of TCR modified T cells, irradiation-induced host conditioning led to a substantial improvement in the persistence of TCR modified cells. It is noted that because of the rapid growth kinetics of the transplantable tumor model used here, a rapid development of T cell responses is likely to be of greater importance in this model than it will be in the clinical setting, where tumor progression is markedly slower. Thus, the improved tumor control in mice conditioned by TBI as compared to mice receiving viral vaccination seen here may still underestimate the clinical value of chemotherapy- or irradiation-induced host conditioning in TCR gene transfer-based protocols. It seems likely that further improvements can be made in conditioning regimens for ACT. For instance, murine data suggest that myeloablative conditioning plus stem cell support results in an enhanced expansion and function of adoptively transferred TCR transgenic T cells³¹. Alternatively, the selective depletion of the cellular subsets that compete for homeostatic cytokines may yield a more targeted approach to facilitate cell engraftment. Finally, blockade of inhibitory pathways by combination with monoclonal antibody therapy against CTLA-4 or PD-1/ PD-L1 may be considered.

TCR transgene design - Alterations in TCR transgene design fall into two classes, those that aim to change the specificity or affinity of the TCR for its cognate antigen and those that aim to increase the expression of the desired TCR $\alpha\beta$ heterodimer upon T cell modification. Efforts to achieve the latter have stemmed from the observation that non-modified TCR heterodimers are generally expressed at

low levels upon introduction in human peripheral blood T cells. Recent elegant work by Heemskerk and others has shown that this low expression is due to competition of exogenous TCR chains with endogenous TCR chains for assembly with CD3 components, and due to the formation of mixed dimers of endogenous and exogenous TCR chains. Interestingly, the ability of the exogenous and endogenous TCR to compete for surface expression can vary widely between different TCRs, most likely reflecting the efficiency with which the different TCR heterodimers fold³². The OT-I TCR used here can be considered a 'dominant' TCR in that retroviral transduction of mouse T cells with the unmodified TCR leads to TCR transgene expression in a high proportion of cells. Nevertheless, TCR gene optimization still resulted in a modest increase in transduction efficiency and a quite marked effect on the in vivo activity of T cells modified with this TCR. Based on these data it seems plausible that other strategies that have yielded similar increases in TCR expression in vitro (10-12) will also be of significant value to enhance the in vivo function of TCR modified T cells, and a combination of the different strategies may in fact be preferred.

Composition of the cell graft - In a final set of experiments we demonstrated that the frequency of TCR modified T cells within the cell graft determines the efficacy of ACT, even when infused numbers of TCR modified T cells are kept constant. We have considered two non-mutually exclusive explanations for this observation. First, the co-infusion of a large number of unmodified cells may lead to a reduced proliferation and differentiation of the TCR modified T cells by decreasing the availability of cues for homeostatic expansion. Specifically, an increased availability of the IL-7 and IL-15 cytokines has been shown to play an essential role in the enhancement of T cell mediated tumor immunotherapy after lymphodepleting host conditioning and the co-transfer of irrelevant T cells and NK cells may simply limit this effect^{33,34}. Alternatively, regulatory T cells (T-regs) have been shown to suppress immune responses towards B16 melanoma^{35,36}, and the infusion of large numbers of passenger cells may result in a more rapid restoration in regulatory T cell number following host conditioning. In both cases, the development of approaches that would result in a more selective or fully selective infusion of TCR-modified T cells would be desirable. A substantial enrichment of gene modified T cells prior to ACT may be achieved by selection of T cells expressing the V β (or V α) element that is used by the introduced TCR, although this would not select against TCR modified T cells that predominantly express this TCR chain in the form of mixed dimers. A more stringent selection may possibly be achieved by MHC tetramer³⁷ or reversible MHC tetramer-based isolation³⁸ of TCR modified T cells, and in this light the development of a conditional ligand-based platform for the creation of GMP-grade MHC multimers seems worth pursuing³⁹. Finally, while we have here focussed on the frequency of TCR modified T cells within the graft, it seems plausible that alterations in the type of T cells that is used for viral modification may also be beneficial. For instance, the selective modification of T cells with a high capacity for immune reconstitution may potentially be attractive⁴⁰. As a somewhat more futuristic approach, more defined populations of TCR modified T cells for adoptive therapy may conceivably also be generated in systems in which TCR modified T

cells can be obtained *in vitro* from hematopoietic progenitor cells^{41,42}, with the added benefit that endogenous TCR rearrangement is at least partially suppressed⁴¹.

Here we have shown that the effectiveness of TCR gene transfer-based immunotherapy can be substantially enhanced in three ways that each affect a different part of the procedure: 1). Irradiation-induced host conditioning results in the long term persistence of TCR transduced T cells and appears preferable over active vaccination. 2). The use of vectors encoding TCR sequences optimized for expression yields redirected T cells with a substantially increased capacity for *in vivo* tumor control, and this effect may well extend to other alterations in TCR design that result in increased expression. 3). The infusion of grafts in which TCR-modified T cells are present at a high frequency is preferable over infusion of an equal number of TCR modified T cells amidst a higher number of irrelevant cells, and is correlated with an enhanced *in vivo* expansion of the desired tumor-specific T cell population. The combined clinical implementation of these approaches appears warranted.

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