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Adoptive transfer of TCR 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 Ag-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 antitumor 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 antitumor 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 Ag. This suggests TCR gene therapy can achieve a sufficiently strong selective pressure to warrant the simultaneous targeting of multiple Ags. The strategies outlined in this study should be of value to enhance the antitumor activity of TCR-modified T cells in clinical trials. The Journal of Immunology, 2008, 181: 5128–5136.

A doptive 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 Ags 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 cells (1). 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 Ags (2–7). Second, engineering approaches such as optimization of TCR gene sequences (8, 9), inclusion of murine constant domains (10), or inclusion of an engineered disulfide bond (11, 12) can be used to enhance the expression of the introduced TCR. 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 therapy (13). Third, TCR-modified T 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 Ag encounter in vivo (14–16), even when the endogenous T cell repertoire is nonresponsive (17, 18). More recent work has provided first evidence for the clinical potential of TCR gene therapy (19). 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 Ag MART-I. Notably, following T cell infusion, tumor regression was observed in two patients and these clinical responses appeared to correlate with the magnitude of the TCR-modified T cell response upon infusion.

Although 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 loci (19). 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 (20, 21). 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 transplantable (17) and spontaneously developing tumors (18) in otherwise self-tolerant situations, complete remissions are achieved only rarely (17).

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 antitumor activity of TCR-modified T cells in vivo. We reasoned that improvements in TCR gene therapy could involve one of either three factors:
1) alterations within the format of the introduced TCR genes, 2) modification of the cell graft, 3) 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 and Methods

Mice

RIP-OVA<sup>high</sup> mice (22) 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 adoptive transfer

The pMX-OT-Io-ires-OT-Iβ retrovirus encoding the nonmodified OT-I TCR genes (pMX-OT-I<sub>L</sub>) has been described (17). Optimized OT-I TCR genes were produced by GeneArt (GeneArt GmbH) and cloned into the retroviral vector pMX to create pMX-OT-Io-opt-ires-OT-Iβopt (pMX-OT-I<sub>L</sub>). Mouse splenocytes were modified by retroviral transduction as described previously (14).

Mouse received an adoptive transfer of 1 × 10<sup>6</sup> OT-I TCR transduced or mock transduced CD8<sup>+</sup> T cells. Where indicated, TCR-modified T cells were mixed with an excess of mock-transduced cells, either unmanipulated or depleted for CD8<sup>+</sup>, CD4<sup>+</sup>, or CD25<sup>+</sup> cells. For depletion, passenger cells were incubated with PE-labeled anti-CD25, anti-CD4, or anti-CD4 mAb (all from BD Pharmingen) respectively. Subsequently, cells were incubated with anti-PE beads (Miltenyi Biotec), and negative selection was performed by autoMACS (Miltenyi Biotec) according to the manufacturer’s guidelines. Depletion of CD8<sup>+</sup> and CD4<sup>+</sup> cells was performed after T cell activation. Depletion of CD25<sup>+</sup> cells was performed before T cell activation, to avoid removal of T cells that expressed CD25 as a consequence of the in vitro activation procedure.

Tumor experiments

The B16-OVA cell line expressing the C-terminal part of OVA (aa 161–385) and the murine CD4 molecule as a marker gene product (17) was cultured in RPMI 1640 supplemented with 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin. Before inoculation, cells were washed three times with HBSS (Life Technologies) to remove serum components and 1 × 10<sup>6</sup> cells were injected s.c. in the right flank. Tumors were measured with calipers and mice were killed once tumors reached an average diameter of 10 mm.

For ex vivo analysis of Ag expression, sliced tumors were incubated in medium supplemented with collagenase IV (0.2 mg/ml; Worthington) and DNaseI (25 μg/ml; Roche) for 20–30 min at 37°C. Single cell suspensions were generated with the aid of a cell strainer (BD Biosciences). Erythrocytes were removed by NH<sub>4</sub>Cl treatment, and cells were subsequently cultured in RPMI 1640 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 with CD4 expression levels on B16-OVA and B16 cell lines after corresponding times of in vitro culture.

Flow cytometry

Surface TCR expression was measured 24 h after retroviral transduction by flow cytometry. Cells were stained with FITC- or PE-conjugated anti-TCR V<sub>α2</sub> and anti-TCR V<sub>β</sub> 5m Abs (the V<sub>α</sub> and V<sub>β</sub> segments used by the OT-I TCR), and allophycocyanin-conjugated anti-CD8a mAb (BD Pharmingen). Propidium iodide (Sigma-Aldrich) was used to select for live cells. For the measurement of T cell responses, 25 μl of peripheral blood was collected in heparin-coated vials (Mircrovet CD 300 Li-Heparine, Omnilabo) at the indicated days post transfer. Following removal of erythrocytes by NH<sub>4</sub>Cl treatment, the cells were stained with the indicated Abs and analyzed by flow cytometry. Data acquisition and analysis was done on a FacsCalibur (BD Biosciences) with CellQuest and FCS express (De Novo Software) 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), 1 day before adoptive cell transfer. For viral vaccination, mice were infected i.p. at the indicated timepoints with 1 × 10<sup>7</sup> PFU of a recombinant vaccinia strain that expresses OVA (rVV-OVA) (23).

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) measurement. Mice were considered diabetic when glucose levels reached ≥20 mmol/L. To allow long-term follow-up, diabetic mice were treated by s.c. introduction of insulin implants according to the manufacturer’s protocol (LinShin Canada).

Statistics

Survival curves were compared using a log-rank (Mantel-Cox) test. Immune responses were compared using a Student t test. p values <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 Ag 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 Ag-specific vaccination, host conditioning regimens such as nonmyeloablative chemotherapy or irradiation can be used 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 Ag. 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 Ag 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 (24, 25). Specifically, while vaccination results in the rapid emergence of a highly differentiated pool of effector T cells (26), T cell populations induced by host conditioning display properties of memory T cells (24), possibly translating in an enhanced capacity for long-term persistence.

Irradiation- and chemotherapy-induced host conditioning before adoptive T cell transfer has been used to enhance the in vivo expansion and antitumor effect of TCR-transgenic T cells in mouse models (27) and of tumor-infiltrating lymphocytes in melanoma patients (20). 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 (19). 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.

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<sup>high</sup> were injected subcutaneously with B16 tumors expressing OVA. As documented previously, RIP-OVA<sup>high</sup> mice are tolerant toward the self-Ag OVA. As a consequence, the endogenous T cell repertoire is unable to influence the outgrowth of B16-OVA tumors, even if
and no treatment vs irradiation: 0.35 (Mantel-Cox test). 0.0002; no treatment vs irradiation plus ACT: 0.0002; no treatment vs irradiation: 0.016 (Mantel-Cox test).

Average diameter of 10 mm or when tumors started bleeding. Mice were sacrificed once tumors reached an average diameter of 10 mm or when tumors started bleeding. Analysis of blood cells of irradiated RIP-OVAhigh mice (open symbols) at day 7 (A–C) or day 10 (D–F). Tumor growth was compared with that in control mice (depicted by crosses). A and D, Analysis of tumor development. Tumor growth was measured three times a week, bars indicate SEM. Arrow indicates time point of adoptive transfer. B and E, Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 10 mm or when tumors started bleeding. p values of B: irradiation vs irradiation plus ACT: 0.0014; no treatment vs irradiation plus ACT: 0.0014; no treatment vs irradiation: 0.016 (Mantel-Cox test), p values of E: irradiation vs irradiation plus ACT: 0.0002; no treatment vs irradiation plus ACT: 0.0002; no treatment vs irradiation: 0.35 (Mantel-Cox test). C and F, Analysis of blood cells of irradiated RIP-OVAhigh mice at indicated time points post adoptive cell transfer. Bars indicate SD.

Upon vaccination (17) and this model thereby forms a stringent test of the value of different approaches for ACT.

In a first set of experiments, RIP-OVAhigh mice were challenged with B16-OVA tumor cells. Subsequently, mice were either left untreated, or were treated on day 6 × 5 Gy TBI (leading to sublethal lymphodepletion), followed by transfer of 1 × 106 of either OT-I TCR transduced or mock transduced CD8+ T cells the subsequent day. Infusion of mock-transduced cells in mice conditioned by TBI 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. 1, A and B; average survival of 24 vs 60 days; p < 0.005). Furthermore, in recipients of OT-I transduced cell populations a highly dominant CD8+ cell population expressing the Vα2 and Vβ5.1 TCR chains, of the OT-I TCR quickly became detectable, and this population persisted up to the end of the experiment (average frequency of Vα2+ Vβ5.1+ cells ~75% of total CD8+ cells at peak, ~40% after 1 mo) (Fig. 1C). Infusion of OT-I TCR transduced cells into nonconditioned recipients had no substantial effect on tumor growth or survival as compared with untreated mice, and CD8+ cells expressing Vα2 and Vβ5.1 were only detectable for a few days in these mice (data not shown). These data show that a combination of host conditioning plus transfer of TCR-modified T cells that are rendered reactive against a defined self Ag can lead to a prolonged antitumor effect in an otherwise self-tolerant setting. Furthermore, this combination yields a T cell repertoire that is markedly skewed toward 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 with mice that did not receive any form of treatment. Likewise, infusion of OT-I TCR transduced cells into nonconditioned recipients was without substantial effect (data not shown). In contrast, in this setting of delayed T cell therapy, host conditioning in combination with ACT of OT-I transduced T cells resulted in a clear suppression of tumor growth (Fig. 1, D and E). However, tumors continued to progress, resulting in only a moderate increase in survival (20 days vs 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).

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 antitumor 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<sup>high</sup> mice, and mice were then vaccinated with an rVV-OVA. In a second group, OT-I TCR-transduced T cells were infused at day 10 in tumor bearing RIP-OVA<sup>high</sup> mice that had received sublethal TBI 1 day before ACT. Finally, a third group of mice receiving OT-I modified T cells was treated with a combination of sublethal TBI (1 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 10.3% of CD8<sup>+</sup> and frequency of TCR modified T cells, with a peak frequency of vaccination. Because of this contraction, and because of the continuing homeostatic T cell proliferation in recipients treated by TBI, V<sub>51</sub>V<sub>51</sub> 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). 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 vs 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 with the values found in mice conditioned by TBI only. Furthermore, there was a trend toward 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 with 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 vs 27 days; p < 0.005) (Fig. 2, B and C). The use of TBI as a preconditioning regimen led to a somewhat stronger suppression of tumor outgrowth, also resulting in a more pronounced increase in survival (average 22 vs 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 vs 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 antitumor effect of the combination of ACT and TBI.

**FIGURE 2.** Enhanced persistence and antitumor effect of TCR-transduced T cells after irradiation-induced host conditioning as compared with active vaccination. RIP-OVA<sup>high</sup> mice (n = 5–7 per group) were inoculated with 1 × 10<sup>5</sup> B16-OVA tumor cells subcutaneously, and received an adoptive transfer of 1 × 10<sup>6</sup> OT-I<sup>wt</sup> TCR transduced CD8<sup>+</sup> T cells (●, ■, and □, transduction efficiency: 55% of CD8<sup>+</sup> cells) or an equal amount of mock transduced T cells (○) at day 10. Transferred T cells were boosted either by sublethal TBI at day 9 (●, ○), vaccination with rVV-OVA at day 10 (□) or sublethal TBI at day 9, followed by vaccination with rVV-OVA at day 13 (■). A, Analysis of V<sub>51</sub>V<sub>51</sub> CD8<sup>+</sup> cells in peripheral blood (percentage in left panel, absolute numbers in right panel) at indicated time points post adoptive transfer. Bars depict SEM. B, Analysis of tumor development. Tumor growth was measured three times a week. Bars indicate SEM. C, Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 10 mm or when tumors started bleeding. p values: irradiation vs ACT plus vaccination or ACT plus irradiation or ACT plus irradiation plus vaccination: <0.005; ACT plus irradiation vs ACT plus vaccination: 0.0055; ACT plus irradiation vs ACT plus irradiation plus vaccination: 0.005; ACT plus irradiation plus vaccination vs ACT plus vaccination: 0.01 (Mantel-Cox test).

**Gene optimization results in a moderate increase in TCR expression but marked increase in antitumor efficacy**

As described previously, modification of TCR 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 TCR. 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 mice (8). To assess whether alterations that enhance the expression of introduced TCR genes also enhance the in vivo antitumor activity of TCR modified T
cells, a gene-optimized variant of the OT-I TCR (termed OT-Iopt) was created, and RIP-OVAhigh derived splenocytes were retrovirally transduced with either the wild type OT-I TCR or the gene optimized variant (Fig. 3A). Gene optimization resulted in a 1.4-fold increase in transduction efficiency as revealed by anti-V<sub>α</sub>2/V<sub>β</sub>5.1 and anti-V<sub>α</sub>2/V<sub>β</sub>5.2 staining (50 vs 69% of CD8<sup>+</sup> T cells after correction for endogenous V<sub>α</sub>2/V<sub>β</sub>5.1/V<sub>β</sub>5.2 cells), and this was accompanied by a 1.3-fold increase in average TCR expression (MFI of 455 vs 603 for the TCR-<sub>α</sub>-chain; 37 vs 50 for the TCR-<sub>β</sub>-chain).

To determine the effect of OT-I TCR gene optimization on the antitumor activity of OT-I TCR transduced T cells in vivo, 1/10<sup>6</sup> OT-I, OT-Iopt, or mock transduced CD8<sup>+</sup> T cells were transferred into tumor bearing, sublethally irradiated RIP-OVAhigh mice. Within the first weeks post infusion, the percentage of V<sub>α</sub>2/V<sub>β</sub>5.1<sup>+</sup> CD8<sup>+</sup> T cells was slightly increased in mice that received OT-Iopt TCR transduced T cells, as compared with recipients of T cells expressing the parental OT-I TCR (Fig. 3B, left panel), likely reflecting the somewhat higher transduction efficiency. However, the increase in absolute numbers of V<sub>α</sub>2/V<sub>β</sub>5.1<sup>+</sup> CD8<sup>+</sup> T cells did not reach significance (day 10–21; p = 0.2–0.4) (Fig. 3B, right panel). Despite 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-Iopt TCR modified T cells appeared to halt tumor development for a period of up to 1–2 mo (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-Iopt 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 antitumor effect

Although the fraction of T cells that becomes Ag-responsive upon transduction of murine T cells with mouse TCRs such as the OT-I TCR is markedly high, the percentage of Ag-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 setting (19), the resulting cell grafts do contain a higher number of nonmodified “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-Iopt 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% Vα2^+Vβ5.1^+ cells of CD8^+ T cells diluted with mock transduced splenocytes depleted of CD8^+ cells (C), TCR transduced CD8^+ T cells diluted with total mock transduced splenocytes (D), transfer of 1.4 × 10^6 cells in total). To determine the effect of passenger cell subpopulations, mice received 1 × 10^6 OT-Iopt TCR transduced CD8^+ T cells diluted with mock transduced splenocytes depleted of CD8^+ cells (E), transfer of 8.7 × 10^6 cells in total), CD4^+ (F, transfer of 7.8 × 10^6 cells in total), or CD25^+ cells (G, transfer of 1.4 × 10^6 cells in total).

Analysis of Vα2^+Vβ5.1^+ cells in peripheral blood (percentage in left panel; absolute numbers in right panel) at indicated time points post adoptive transfer. Bars depict SEM. T tests were performed to determine differences between low and high passenger groups: *, p < 0.05; **, p < 0.005; ***, p < 0.0005. B, Analysis of tumor development. Tumor growth was measured three times a week. Bars indicate SEM. C, Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 10 mm or when tumors started bleeding. p values: irradiation vs low passenger group: <0.0001; irradiation vs high passenger group 0.002; low passenger group vs high passenger group: 0.15 (Mantel-Cox test). D, Induction of diabetes. Mice were considered diabetic once blood glucose levels exceeded 20 mmol/l. E and F, Tumor bearing and sublethally irradiated mice received 3.6 × 10^6 mock transduced T cells (C). A, Analysis of Vα2^+Vβ5.1^+ CD8^+ cells in peripheral blood (percentage in left panel; absolute numbers in right panel) at indicated time points post adoptive transfer. Bars depict SEM. T tests were performed to determine differences between low and high passenger groups; *, p < 0.05; **, p < 0.005; ***, p < 0.0005. B, Analysis of tumor development. Tumor growth was measured three times a week. Bars indicate SEM. C, Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 10 mm or when tumors started bleeding. The experiment was terminated at day 70.
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 before ACT was compressed to a difference of less than ~3-fold (79% Vα2+Vβ5.1+ of CD8+ T cells in the “low passenger group” vs 33% Vα2+Vβ5.1+ 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 Ag 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 hosts (28).

Although the mice in the low passenger and high passenger group received an equal number of OT-Iopt 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). 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. 4, B and C). As a second parameter for in vivo activity of the TCR-modified T cell population, type 1 diabetes was induced in 25% (2/8) of the mice that received TCR-modified T cells amid a high number of passenger cells, but in 100% (7/7) of the mice that received the same number of TCR modified T cells in a more homogeneous graft (Fig. 4D).

Thus, in the presence of a substantial number of passenger cells, the in vivo expansion and functionality of TCR modified T cells was reduced, presumably reflecting competition between the TCR modified T cells and passenger cells for homeostatic cues. To determine which cellular subset(s) within the passenger cell population would be responsible for this detrimental effect on TCR modified cells, we took defined numbers of passenger cells and then removed different cellular subsets from the passenger cell population before cotransfer with TCR-modified cell populations. As the cell populations obtained after in vitro transduction procedures consist largely of T cells and contain very few NK cells, we focused on the depletion of either CD8+, CD4+, or CD25+ cells. Even though depletion was efficient for all three subsets (only 1.1, 0.8, and 0.3% remaining within the passenger cell population, respectively), removal of either single subset did not abolish the detrimental effect of passenger cells on the capacity of OT-1 transduced cells to control tumor growth (Fig. 4, E and F). This observation that the detrimental effect of passenger cells is not due to a single cell population suggests that the most efficient strategy to avoid the negative effect of cotransferred cells will be the selective purification of the desired TCR-modified T cells.

**Immunoeediting by TCR modified T cells**

In mice treated with a combination of TBI and infusion of OT-Iopt transduced T cells tumor progression was eventually observed in the large majority of mice (Figs. 3 and 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 cells could be explained by Ag 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 Ag. This analysis revealed that CD4 expression was substantially reduced in tumors obtained from mice that had received TCR-modified T cells as compared with tumors obtained from control mice (Fig. 5B; p < 1 × 10^-9). Furthermore, in tumors that escaped immune control after more prolonged periods, evidence for Ag loss became increasingly apparent, consistent with an ongoing process of immune selection.

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 Ag expression. These data suggest that, at least with regard to the possibility of tumor escape, the targeting of tumor associated Ags such as WT-1 (29) or PRAME (30) that contribute to cellular transformation may be preferred. Alternatively, and in analogy with developments in Ab therapeutics, the simultaneous use of two or three TCRs directed against different TAAs will likely suffice to minimize the chance of tumor escape through Ag loss. Clinical implementation of such “oligoclonal TCR gene
transfer” will be an interesting future challenge from both a logistical and regulatory point of view.

**Discussion**

Inspired by the success of recombinant mAbs such as trastuzumab (Herceptin) and rituximab (Rituxan) (31), much effort has been put into the preclinical testing and clinical implementation of TCR gene therapy, a strategy that can be considered the “cellular analog” of adoptive Ab therapy. With the feasibility of TCR gene transfer well established, but faced with the suboptimal antitumor activity of TCR modified T cells both in preclinical models as well as in the clinic, in this study we 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 and engraftment of TCR-modified T cells**

We demonstrate in this study that host conditioning by TBI leads to superior engraftment and antitumor 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 in this study, 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 with 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 myelolat- tivating conditioning plus stem cell support results in an enhanced expansion and function of adoptively transferred TCR transgenic T cells (32). 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 mAb 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 Ag and those that aim to increase the expression of the desired TCR αβ heterodimer upon T cell modification. Efforts to achieve the latter have stemmed from the observation that nonmodified TCR heterodimers are generally expressed at low levels upon introduction in human peripheral blood T cells. Recent elegant work by Heemskerk and others have 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 fold (33).

The OT-I TCR used in this study 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 (8–10) 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 nonmutually exclusive explanations for this observation. First, the coinfection 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 cotransfer of irrelevant T cells and NK cells may simply limit this effect (34, 35). Alternatively, regulatory T cells have been shown to suppress immune responses toward B16 melanoma (36, 37), 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 the experiments shown in this study, neither the removal of CD4+ or CD8+ cells or the removal of CD25+ cells is sufficient to circumvent the negative effect of passenger cells, suggesting that both mechanisms may in fact apply.

Because the negative effect of passenger cells appears to be multifactorial, the development of approaches that can be used to prepare selective grafts of TCR-modified T cells would be desirable. A substantial enrichment of gene modified T cells before ACT may be achieved by selection of T cells expressing the Vβ (or Vα) 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 tetramer (38) or reversible MHC tetramer-based isolation (39) 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 pursuing (40).

Finally, while we have focused in this study 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. In particular, the selective modification of T cells with a high capacity for immune reconstitution may potentially be attractive (41). 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 (42, 43), with the added benefit that endogenous TCR rearrangement is at least partially suppressed (42).

In this study, 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 immunization; 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; and 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 amid 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.

Disclosures

The authors have no financial conflict of interest.

References