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MHC-class I-restricted CD4 T cells: a nanomolar affinity TCR has improved anti-tumor efficacy *in vivo* compared to the micromolar wild type TCR

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Abstract

Clinical studies with immunotherapies for cancer, including adoptive cell transfers of T cells, have shown promising results. It is now widely believed that recruitment of CD4⁺ helper T cells to the tumor would be favorable, as CD4⁺ cells play a pivotal role in cytokine secretion as well as promoting the survival, proliferation and effector functions of tumor-specific CD8⁺ cytotoxic T lymphocytes. Genetically engineered high affinity T cell receptors (TCRs) can be introduced into CD4⁺ helper T cells to redirect them to recognize MHC class I restricted antigens, but it is not clear what affinity of the TCR will be optimal in this approach. Here we show that CD4⁺ T cells expressing a high-affinity TCR (nanomolar K_d value) against a class I tumor antigen mediated more effective tumor treatment than the wild-type affinity TCR (micromolar K_d value). High affinity TCRs in CD4⁺ cells resulted in enhanced survival and long-term persistence of effector memory T cells in a melanoma tumor model. The results suggest that TCRs with nanomolar affinity could be advantageous for tumor targeting when expressed in CD4⁺ T cells.

Keywords

TCR; Adoptive T cell therapy; Tumor targeting; Cancer Immunotherapy; Melanoma

Introduction

It has been suggested that combined recruitment of CD8⁺ and CD4⁺ T cells to tumors can improve responses and generate long-term memory [1–3]. Infiltration of CD4⁺ T cells can provide a cytokine milieu favorable to inducing proliferation, survival and effector function of CD8⁺ T cells within tumors as well as induce the activation of the innate immune system [4–8]. Additionally, CD4⁺ cells are capable of mediating cytotoxic activity, directly eradicating melanoma tumors, and can indirectly elicit tumor inhibition through IFN- γ -dependent effects on host cells [9–12]. CD4⁺ T cell activities at the site of a tumor could

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Conflict of Interest

The authors declare that they have no conflict of interest.

also provide an inflammatory environment that is favorable to the induction of human T cell responses [13,14].

A major problem with the recruitment of CD4⁺ T cells is that most tumors do not express class II MHC, thereby preventing direct recognition and activities associated with CD4⁺ T cells. To overcome this challenge, TCRs have been engineered with higher affinity for a class I tumor antigen, avoiding the requirement for CD8 co-receptors, and thereby mediating CD4⁺ T cell activities [15–20].

Efforts to introduce $\alpha\beta$ TCR genes into T cells activated *ex vivo* for cancer therapy have generated considerable excitement [21–26]. In the well-studied system that targets melanoma antigen MART-1/HLA-A2, two different TCRs have been used clinically, DMF4 and second-generation DMF5 [21,27,22]. Despite promising results with these TCRs, it is not yet clear whether even DMF5 represents an optimal TCR. For example, both DMF4 and DMF5 have relatively low affinities, with K_d values of 170 μ M and 40 μ M, respectively [28], and while DMF5 promotes activity of CD4⁺ T cells it is not clear if the magnitude of this activity is optimal. In addition, it is not known if TCRs with different affinities against class I MHC will mediate lineage commitment of different CD4⁺ subsets.

Experiments *in vitro* suggest that optimal co-receptor independent activation, for directing CD4⁺ T cell activity by a class I pepMHC-specific TCR, occurs at higher affinities, in the range of 0.5–3 μ M [18,20]. However, affinities of most anti-tumor TCRs have not been measured, largely due to problems with expression of soluble TCRs and the normally very low affinity of TCRs for their cognate pepMHC antigens. In addition, recent evidence suggests that the behavior of T cells *in vivo* may not be completely predictable by activities *in vitro* [29,30]. The activity of TCR-transduced CD4⁺ T cells *in vivo*, with well-characterized TCRs requires study.

To examine the role of TCR affinity in adoptive T cell therapies, we took advantage of the well-studied 2C TCR, specific for class I MHC K^b bound to foreign peptide SIYRYYYGL (SIY, K_d = 30 μ M), and its high-affinity TCR variant called m33 (K_d = 30 nM) [31]. Our recent results showed that CD8⁺ T cells with the m33 TCR were deleted *in vivo*, whereas CD4⁺ T cells with the m33 TCR persisted, and were capable of mediating an anti-tumor response [30]. Here we show that the higher affinity TCR m33 was superior to the lower affinity 2C TCR in mediating effective destruction of B16 melanomas by CD4⁺ T cells. While the 2C-transduced CD4⁺ T cells mediated an effect against antigen-bearing tumors, antigen loss variants were responsible for reoccurrence in every case. The m33-transduced CD4⁺ T cells not only mediated a stronger response, as measured by delayed tumor growth and/or elimination of antigen loss variants, but some mice also generated a long-term response. Furthermore, CD4⁺ T cells in these mice persisted and they mediated an antigen-specific memory response against the B16-SIY melanomas. These results indicate that there are benefits to using TCRs with affinities that are higher than current TCRs (e.g. DMF5) in adoptive CD4⁺ T cell therapies.

Results

IFN- γ treatment is required for detectable surface expression of SIY/K^b complexes on B16-SIY melanoma cells

Our recent study showed that the MC57-SIY tumor could be rejected by CD4⁺ T cells transduced with the high-affinity TCR called m33 [30]. We have shown that this tumor expresses constitutively high levels of SIY/K^b (SIYRYYYGL), as measured with a soluble high-affinity TCR called m67 [32]. To explore a tumor system with lower antigen presentation levels, we elected to use the B16 melanoma model, as the SIY-transfected B16

cells did not express SIY/K^b at constitutive levels detectable by the m67 TCR (i.e. B16-SIY expressed less than 1000 SIY/K^b molecules per cell)(Fig. 1a). To determine if detectable levels of SIY/K^b could be induced by treatment with IFN- γ , we cultured B16 F10 (the parental cell line) and B16-SIY cells overnight with 10 ng/ml IFN- γ . Higher surface expression levels of K^b were observed on both cell lines but induction of SIY/K^b expression was observed only with B16-SIY. These findings demonstrate that although B16-SIY cells have low constitutive levels of SIY/K^b, increased levels can be induced by IFN- γ treatment, as might be achieved in an inflammatory setting *in vivo*.

Lysis of IFN- γ treated B16-SIY cells by 2C and m33-transduced CD4⁺ T cells

Recent evidence suggests that CD4⁺ T cells can directly kill tumor cells [11,12]. Accordingly, we evaluated the ability of untreated and IFN- γ -treated B16-SIY cells to be lysed by 2C or m33 TCRs transduced into activated CD4⁺ polyclonal T cells (Fig. 1b). Untreated B16-SIY melanoma cells were killed poorly by both 2C and m33-transduced CD4⁺ T cells, whereas IFN- γ treatment resulted in B16-SIY lysis by both effector cells. Significantly enhanced killing was observed with the high-affinity m33 TCR-transduced T cells at both E:T cell ratios tested. These observations suggest that IFN- γ stimulation at the site of a B16-SIY tumor will be important to raise the surface levels of the SIY/K^b for effective killing. Other studies have shown a critical role for IFN- γ in tumor destruction [33,34]. These results also show that m33-transduced CD4⁺ T cells may provide enhanced recognition and activation, probably due to its higher affinity and ability to mediate signaling in a CD8-independent mode.

Antigen-specific destruction of B16 melanoma tumors is mediated most effectively by CD4⁺ T cells transduced with high affinity m33 TCR

To compare the effectiveness of 2C and m33-transduced T cells *in vivo*, we used the B16-SIY cancer cells (1×10^6) as a transplanted, subcutaneous tumor model in *Rag1*^{-/-} mice. Five days after tumor transplantation, when the tumors were palpable (~ 100 mm³), adoptive T cell transfer was performed. Purified primary T cells from C57BL/6 splenocytes were activated *ex vivo* with anti-CD3/anti-CD28-coated beads and IL-2. The purified CD8⁺ or CD4⁺ T cell populations were mock-transduced, transduced with wild type affinity 2C TCR ($K_d = 30$ μ M) or transduced with the high affinity m33 TCR ($K_d = 30$ nM). The levels of 2C and m33 TCRs on the transduced populations were similar, as judged by staining with an anti-V β 8 antibody (data not shown). The efficiency of T cell transduction in the experiments ranged from 34 to 59%, as judged by the fraction of T cells positive above the mock-transduced controls.

Mice treated with saline or m33-transduced CD8⁺ T cells showed uniformly rapid tumor growth (Fig. 2a) likely due to the disappearance of m33 CD8⁺ T cells [30]. After several days of rapid growth, mock-transduced CD8⁺ T cells were able to delay tumor growth in several mice. This result was SIY-dependent as the parental line B16 F10 was not controlled by CD8⁺ T cells (data not shown). These results are consistent with emergence of some CD8⁺ T cells in the polyclonal population that recognize SIY/K^b [2,35]. However, this tumor control was distinctly less effective than 2C-transduced CD8⁺ T cells (Fig. 2a). The 2C-transduced CD8⁺ T cell population mediated rapid reduction in tumor growth but could not prevent relapse.

Mock-transduced CD4⁺ T cells, unlike mock-transduced CD8⁺ cells, did not show the same ability to delay growth of B16-SIY tumors (Fig. 2b); this is consistent with the notion that CD8⁺ T cells, but not CD4⁺ T cells, express among their native repertoire, TCRs with class I-restricted tumor-antigen specificity. Interestingly, both the 2C and m33-transduced CD4⁺ populations were capable of significant tumor control (Fig. 2b), despite 2C TCR's relatively

low affinity. However, m33 CD4⁺ T cells were more effective at delaying progression of B16-SIY tumors, and two of the mice showed no tumor recurrence even after 60 days. This suggests the importance of TCR affinity in redirected CD4⁺ effector T cells. To directly compare the adoptive T cell populations, cohorts were analyzed for average tumor size at a single time-point (day 26). This analysis (Fig. 2c) highlighted that high-affinity m33 CD4⁺ T cells were the most effective at controlling tumor growth.

Recurrence of B16-SIY tumors is a result of selection of antigen loss variants

Despite the tumor destruction and survival benefit observed with TCR-transduced T cells, most of the B16-SIY tumors ultimately escaped immune attack and recurred. To examine why tumor progression eventually occurred, tumors were excised, cultured, and analyzed for antigen loss variants (ALVs) using the high-affinity TCR m67 as a specific probe. All tumor samples isolated from saline-treated or mock-transduced CD4⁺ T cell-treated mice expressed uniform levels of K^b and SIY/K^b, similar to that of the cultured B16-SIY cell line (Fig. 3). In contrast, all tumor samples isolated from mice with tumor regression, and then progression, after adoptive cell therapies with 2C WT or m33-transduced T cells retained high K^b surface expression but lost SIY/K^b expression. Thus, as was often the case in previous studies (e.g. ref [36]), ALVs accounted for the inability to completely eradicate tumors. Interestingly, given the delayed progression of tumors treated with m33 CD4⁺ populations (and complete eradication in two mice), these T cells appear to have provided enhanced destruction, or delayed growth of ALVs, perhaps through recognition of cross-presented SIY/K^b on stroma [2].

m33 CD4⁺ T cell-treated long-term survivor mice exhibit persistence of SIY-specific effector memory T cells

Adoptive transfer of m33 CD4⁺ T cells, but not 2C CD4⁺ T cells, led to rejection of B16-SIY tumors in some mice. This provided an opportunity to investigate *in vivo* T cell persistence and prolonged immune response against B16 melanoma cells. A long-term survivor mouse completely rejected a B16-SIY cancer cell inoculum after a single transfer of m33 CD4⁺ T cells 65 days earlier (Fig 4a). Thus, even in the absence of additional T cell transfer, tumor growth control was maintained over two weeks compared to rapidly growing melanoma tumors in untreated control mice. Prevention of tumor outgrowth indicates prolonged anti-tumor activity against B16-SIY cells.

To assess whether the immune response against B16-SIY tumor re-challenge was antigen specific, another long-term survivor mouse was bilaterally implanted with B16 F10 and B16-SIY at 49 days post m33 CD4⁺ T cell transfer. This long-term survivor showed uncontrolled B16 F10 tumor growth while selectively rejecting B16-SIY cells. Both B16 F10 and B16-SIY tumors grew rapidly in all untreated control mice (Fig 4b, 4c). Lack of an anti-tumor response against the parental line B16 F10 suggests that SIY-specific immunity was generated against B16-SIY melanoma cells.

Lymph nodes (LNs) and spleens were harvested from long-term survivor mice for phenotypic characterization of T cells by flow cytometry. Analysis of the lymphoid tissue revealed long-term persistence of CD4⁺ T cells over 80 days after m33 CD4⁺ T cell transfer (Fig 5a). These CD4⁺ cells were predominantly CD127^{high} and CD62L^{low}, a phenotype associated with peripheral effector memory T cells [37–39]. Additionally, we confirmed that the transduced m33 TCR was expressed by Vβ8 staining of the CD4⁺ cells (Fig 5b). Thus, persistence of the m33, Vβ8⁺ effector memory T cells correlated with the results observed following tumor re-challenge. Together, these findings demonstrate that primary CD4⁺ T cells transduced with high-affinity TCRs can persist *in vivo* and exert effective anti-tumor

protective immunity against B16-SIY melanoma, despite the presence of ALVs among the original tumor population.

T cell therapy with CD4⁺ cells expressing high affinity m33 TCR lead to enhanced survival of mice with large, established tumors

In our initial studies, tumor-bearing mice were adoptively transferred TCR-transduced T cells 5 days following tumor implant, when tumors measured approximately 100 mm³. We sought to investigate the anti-tumor effectiveness of TCR-transduced cells against more established tumors. Experimental studies with larger and more established tumors provide an improved clinical cancer model in aspects of cellular heterogeneity and tumor vasculature [40,41]. In this tumor model, cell transfer of T cells was performed 10 days post tumor implant, when tumors averaged 500 mm³ in size (1 cm in diameter). T cell therapy with both 2C and m33 CD4⁺ T cell populations resulted in enhanced survival despite increased tumor burden at time of T cell treatment (Fig 6a). Tumors initially continued to grow for several days after adoptive transfer of T cells. However, within 12 days post T cell transfer, tumor size was markedly decreased in both treatment groups compared to mock CD4⁺ treated mice which succumb to their tumor within 18 days post transfer of T cells (Fig 6b).

Importantly, again in the model with more established tumors, high-affinity m33 TCR-transduced cells yielded significantly better survival than wild-type 2C TCR-transduced T cell treatment (Fig 6a) and even led to a dramatic tumor shrinkage in one mouse from close to criteria for euthanasia with a tumor size of 2500 mm³ to no measurable mass lasting a period of 6 days. Although tumor size was greatly reduced, all tumors eventually recurred. *Ex vivo* phenotype of recurring tumors again confirmed that tumor outgrowth was a result of ALVs, which retained expression of K^b but lost surface expression of SIY/K^b complexes (Fig 6c).

Discussion

The experimental design described here, with a model tumor antigen (SIY), addresses the question whether there is an optimal affinity for TCRs that can redirect the activities of CD4⁺ and CD8⁺ T cells against a class I antigen on tumors. Our salient findings are that: 1) lower, wild-type affinity TCR within the range found in normal CD8⁺ T cells (K_d of 30 μ M) was capable of mediating effective CD8⁺ T cell responses in the transduced T cell system, in line with many previous studies that used transgenic 2C CD8⁺ T cells, 2) TCR-transduced CD4⁺ T cells exhibited both direct killing of the class I-positive tumors *in vitro* and effective control of tumors *in vivo*, and 3) an *in vitro* engineered TCR, m33, with high (nanomolar) affinity mediated more effective tumor targeting by CD4⁺ T cells than the wild-type 2C TCR.

Compared to results with CD8⁺ T cells, there was improved tumor targeting efficacy using CD4⁺ T cells transduced with the high-affinity TCR. Accordingly, CD4⁺ cells expressing the m33 TCR (K_d of 30 nM) exhibited significant improvements in tumor destruction and longer survival in comparison to wild-type affinity 2C TCR. Impressively, this included treatment of larger, established tumors of up to 2500 mm³ in one case. By analogy, the second generation MART-1 specific TCR called DMF5 (K_d = 40 μ M) was chosen because it exhibited greater activity than the TCR DMF4 in CD4⁺ T cells in various assays *in vitro* [27,22,42]. The improved efficacy of CD4⁺ T cells may be associated with the production of cytokines such as IFN- γ , which clearly has a dramatic effect on the expression of the SIY/K^b antigen. These results are in line with recent observations by Gajewski and colleagues, where IFN- γ mediated effector function of CD4⁺ cells was associated with B16 melanoma tumor growth control and reduced tumor vasculature [43]. Earlier studies using the B16-OVA system, and CD8⁺ OT-1 TCR transgenic T cells, had shown an effect of IFN- γ on

host cells, in addition to a direct effect of B16-tumors [44]. Thus, the enhanced effect of CD4⁺ T cells in our study could also be due to indirect IFN- γ dependent-mechanisms [9–12].

Most previous studies with tumor-reactive T cells have described their TCRs in the context of high or low “avidity”; this is in part because the actual monomeric binding constants (K_d values) of TCRs are rarely measured. In the context of many studies with T cells, the term “avidity” has generally referred to the notion that one T cell yielded more effective activity (“higher avidity”) against the same antigen compared to another clone (“lower avidity”). It is useful to consider the 2C system in this context. The 2C TCR showed some activity in CD4⁺ T cells, and by this criterion it would be considered “high avidity” in comparison to a SIY-specific TCR that showed no activity in CD4⁺ T cells. However, 2C TCR is clearly less active in CD4⁺ T cells than the m33 TCR. Thus, the term high avidity is a relative term, requiring comparison of multiple different TCRs against the same antigenic peptide. We suggest that TCRs derived from typical TILs or *ex vivo* generated T cells (wild-type TCRs) may not yield the affinities that can mediate more effective activities, as shown here for m33. These affinities may only be achieved by directed evolution *in vitro* (as with m33), or by other strategies that select for TCRs that bind to class I MHC ligands in the absence of the CD8 co-receptor. Nevertheless, it is clear that driving the affinity too high (e.g. picomolar), may also yield self-peptide reactivities even in the CD4⁺ T cell population [18], and thus there will be an optimal range of affinities.

Tumor specific CD4⁺ T cells have been demonstrated to have a direct role in mediating tumor regression. In a clinical study, one patient experienced complete tumor remission following infusion of melanoma antigen-specific CD4⁺ T cells [45]. The possible limitations to the use of TCR-gene modified CD4⁺ cells for ACT include the downregulation of MHC class I levels, and the possible need for CD8 co-receptor to recognize lower levels of antigen. Antigen loss variants (ALVs) are also a major problem associated with adoptive T cell therapies that target a single antigen [36]. In particular with melanoma, T cell therapy exerts a strong selective pressure on tumor cells resulting in the emergence of ALVs in experimental mice as well as clinically [46,47]. To some extent at least, higher affinity TCRs may mitigate each of these issues. However, it remains to be seen if the affinity of TCRs against class I MHC that drive particular CD4⁺ T cell subsets (T_{H1} , T_{H2} , T_{H17} , T_{reg} or memory versus effector) will differ. These studies can now be approached with 2C, m33, and our collection of affinity variants of these TCRs [20].

It is worth considering why some previous studies have described the complete elimination of tumors, including ALVs, in mice that received TCR transgenic T cells, as opposed to TCR-transduced T cells. In the most directly related study with the 2C system, it was shown that large, established MC57-SIY-hi tumors could be completely eliminated by transfer of 2C TCR transgenic CD8⁺ T cells [48]. However, MC57-SIY-lo tumors could not be completely eliminated, due to ALVs and reduced levels of stroma cross-presentation [48,32,49]. Other studies, with MC57-gp33-hi and MC57-gp33-lo tumors, found similar results using P14 TCR transgenic CD8⁺ T cells [50]. Thus, antigen levels clearly are one factor in determining whether ALVs can be destroyed, and in this respect, the B16-SIY tumors express no detectable levels of SIY/K^b unless they are induced with IFN- γ . In contrast, the MC57 tumors constitutively express detectable levels the SIY/K^b antigen [32].

In addition to antigen levels, there are other factors that could contribute to the outgrowth of ALVs as observed in previous reports and in the present study. There is considerable variability in the number of tumor cells used in the different studies, and the timing of adoptive T cell transfers, even in the B16 melanoma models. Other studies have injected 5×10^4 or 10^5 B16 tumor cells [44,50,47], whereas the mice in our study received 10^6 B16-

SIY tumor cells. Other studies transferred T cells one to seven days following tumor injection [44,50,47], while our study injected T cells either five or ten days after B16-SIY injection. Given the rapid growth rate of B16 tumors, clearly the size of the tumor at time of T cell treatment will be related to the likelihood of ALV outgrowth.

Finally, the T cell population(s) used for treatment will also impact the ability to control tumor growth and ALV destruction. A previous study showed that a B16-gp33 tumor was effectively controlled, without ALVs, by P14 TCR-transgenic CD8⁺ T cells, but not by P14 TCR-transduced T cells [50]. These effects could be related to slightly lower transduced TCR levels (compared to transgenic T cells), due to mis-pairing with endogenous TCR chains [51]. Higher affinity TCRs require fewer molecules per T cell in order to mediate activity, and they can do so at lower antigen levels [52], which could in part explain why m33 exhibits improved activity compared to 2C. It should also be noted that in our studies, the recipient T cells are polyclonal CD4⁺ and CD8⁺ T cells from C57Bl/6 mice, not activated TCR transgenic T cells (e.g. OT-1) [50,30]. Furthermore, recipient T cells in our study have undergone *in vitro* activation with anti-CD3/CD28 antibody coated beads, whereas most transgenic T cells studies used T cells activated with the cognate peptide [44,50,47]. It is unclear whether different methods of activation lead to different levels of function (e.g. cytokine release, persistence, activation-induced cell death) that might impact efficacy. Polyclonal T cells may also contain subpopulations of regulatory cells [53] that contribute ultimately to effects on tumor control.

In summary, there are many complex factors that need to be examined to fully understand what the optimal T cell format will be for clinical adoptive T cell therapies. We provide evidence that CD4⁺ T cell therapy with high affinity TCR resulted in tumor rejection; in some cases the rejection resulted in long-term survivor mice exhibiting SIY-specific tumor responses against B16-SIY tumor re-challenge. We also observed CD62L^{low} and CD127^{high} TCR-transduced effector memory T cells. In a well-established tumor model with tumors measuring on average 1 cm in diameter, m33-expressing CD4⁺ cells displayed strong anti-tumor effects. Adoptive transfer of m33 CD4⁺ cells in this model displayed significant tumor destruction and improved survival compared to 2C CD4⁺ treatment. High affinity TCR-transduced CD4⁺ cells not only provide antigen-specific effective immune responses *in vivo* but they also persist long-term after tumor regression. Together, these findings provide evidence of the advantages of using TCRs with high affinity, especially the possibility of redirecting both CD4⁺ and CD8⁺ T cells to target the same MHC class I-restricted tumor antigens. This is especially interesting in the light of the need for having both CD8 and CD4 antigens on the same cell [2].

Materials and Methods

Mice and tumor cell lines

Experiments were performed with C57BL/6 and C57BL/6 *Rag1*^{-/-} mice, 2–5 months of age, purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained as colonies and housed in the animal facilities at the University of Illinois. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Illinois. Murine melanoma B16-F10 cells were purchased from ATCC (Manassas, VA). B16-SIY was derived from B16-F10 cells engineered to express green fluorescent protein (GFP) as a fusion protein with SIYRYYYGL (SIY) [54,55]. Cell lines were cultured in complete RPMI 1640 medium containing 5 mM HEPES, 10% fetal bovine serum (FBS), 1.3 mM L-glutamine, 50 µM 2-ME, penicillin, and streptomycin at 37°C and 5% CO₂.

Interferon gamma treatment and flow cytometry of melanoma cells

Melanoma cells were cultured at a density of 7×10^5 cells per well (6-well plate), with or without 10 ng/mL interferon gamma (IFN- γ , eBioscience) for 24 hours. Cells were stained with anti-K^b (B8.24.3 monoclonal antibody) or soluble biotinylated single-chain TCR m67 to detect SIY peptide bound to H2-K^b on the surface of tumor cells [32]. After 1 hr, cells were washed and streptavidin-allophycocyanin (Invitrogen) was added for 30 min. Cells were analyzed on an Accuri C6 Flow Cytometer.

⁵¹Chromium T cell killing assay

Target cells labeled with ⁵¹Cr (150 μ l of 2 mCi/mL) were incubated with mock, 2C WT or m33 TCR-transduced CD4⁺ T cells at 5:1 and 20:1 effector to target (E:T) ratios for 4 hrs. Supernatants were assayed using a Beckman gamma counter; specific lysis was calculated using the formula: ((sample chromium counts – spontaneous chromium release)/(maximum chromium counts – spontaneous chromium release)) $\times$ 100. Chromium release from B16-SIY melanoma cells incubated with mock CD4⁺ T cells was used to calculate percent specific B16- SIY cell lysis.

Melanoma tumor model and T cell transfer

B16-F10 or B16-SIY melanoma cells were harvested and washed twice with Hanks Balanced Salt Solution (HBSS, Cellgro Mediatech Inc). Shaved mice received $1-1.5 \times 10^6$ tumor cells subcutaneously into the right flank (unilateral tumor) or right and left flank (bilateral tumors) of *Rag1*^{-/-} mice under isoflurane (Baxter) inhalation anesthesia. For adoptive T cell transfer, an average of 7×10^6 mock and TCR-transduced CD4⁺ or CD8⁺ T cells harvested from 24-well plates and washed twice with HBSS, were injected in the tail vein of mice either 5 days or 10 days (established tumor model) following tumor inoculation. Tumor growth was monitored by measuring tumor length and width with a caliper every two days. Tumor volume was estimated as (length $\times$ (width²))/2.

Preparation of melanoma tumors *ex vivo*

For isolation of single-cell suspensions to detect antigen-loss variants, implanted B16-SIY melanoma tumors were harvested, sectioned, and incubated with 300 Collagenase Digestion U/mL (Sigma-Aldrich) and 1 mg/mL Dispase (Roche) for 40 minutes at 37°C. After incubation, 0.002 MU/mL DNase (Calbiochem) was added for further dissociation using gentleMACS Dissociator (Miltenyi Biotec). Cell suspensions were pipetted through 100 μ m filters and centrifuged at 300 $\times$ g for 10 minutes. Single-cell suspensions were cultured for 6–7 days, treated with IFN- γ and stained with TCR m67.

Analysis of lymphocyte cell surface phenotypes

Single-cell suspensions from lymph nodes or spleens were dual-stained with AlexaFluor647- conjugated rat anti mouse CD4 Ab (Clone RM4-5, BD Pharmingen) in combination with either PE-conjugated rat anti-mouse CD62L Ab (Clone MEL-14, BD Pharmingen), AlexaFluor488-conjugated rat anti-mouse CD127 Ab (Clone SB/199, BD Pharmingen), or PE-conjugated mouse anti-mouse V β 8.1/8.2 Ab (Clone MR5-2, BD Pharmingen). For isotype controls, cells were stained with PE-conjugated rat IgG2a, AlexaFluor488-conjugated rat IgG2b, or PE-conjugated mouse IgG2a. After washing, cells were analyzed by flow cytometry (Accuri C6).

TCR gene retroviral transduction of primary murine T cells

Platinum-E (Plat-E) retroviral packaging cells were transfected with 2C TCR or m33 TCR genes cloned into the pMP71 vector (from myeloproliferative sarcoma virus, MPSV) as described [30]. Viral supernatants were harvested 48 hours after transfection, filtered with a

0.45 μm syringe filter, and 50 μl of Lipofectamine 2000 added per 6 mL of viral supernatant. For transductions, CD8⁺ or CD4⁺ T splenocytes from C57BL/6 mice were negatively selected by magnetic sorting using the Mouse CD8 α ⁺ or CD4⁺ T Cell Isolation Kit II (MACS Miltenyi Biotec, Germany), and activated by incubation with anti-CD3/anti-CD28 mouse T-activator Dynabeads (Invitrogen) and 30 U of recombinant mouse IL-2 (Roche, Germany) for 24 hours. Prior to transduction, Dynabeads were magnetically removed from activated CD8⁺ or CD4⁺ T cells and cells were transferred into a 24-well plate coated with Retronectin at 15 $\mu\text{g/mL}$ (Takara, Japan). In each well, 10⁶ T cells in 1 mL of T cell media were mixed with 1 mL sterile viral supernatant and 60U of recombinant murine IL-2. Plates were centrifuged at 2000 rpm at 30°C for 1 h.

Statistical analyses

GraphPad Prism software was used for all statistical analyses. Percent specific lysis was analyzed by t-test and comparison of survival curves by log-rank (Mantel-Cox) test. Tumor volume measurements were compared using a one-way analysis of variance followed by comparisons of individual treatments using the Bonferroni correction for multiple comparisons. Transduced CD4⁺ and CD8⁺ T cells were compared with their respective mock transductants. For tumor volume at 26 days, a planned comparison of wild-type 2C CD4⁺ and m33 CD4⁺ was analyzed by a t-test. Individual p values are given in figure captions.

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Fig. 1. Following interferon gamma (IFN- γ) treatment, the high affinity TCR mediates more efficient killing of B16-SIY melanoma cells than the wild type TCR

(a) Surface expression of MHC class I K^b and SIY/K^b on B16 F10 parental and B16-SIY melanoma cells, with (black line histograms) and without (shaded histograms) IFN- γ treatment *in vitro*, as detected with an anti-K^b antibody and the high-affinity single-chain TCR reagent m67. (b) 2C-transduced and m33-transduced CD4⁺ T cells can effectively kill IFN- γ pre-treated B16-SIY melanoma cells *in vitro*. Chromium release was measured from untreated or IFN- γ pre-treated ⁵¹Chromium-labeled B16-SIY target cells, which had been incubated with 2C TCR or m33 TCR transduced CD4⁺T cells at 5:1 or 20:1 E:T ratios, in triplicates. **p* < 0.01 vs. specific lysis of IFN- γ pre-treated B16-SIY with 2C TCR

Fig. 2. B16-SIY tumor growth delay after treatment with T cells transduced with wild-type affinity 2C TCR or high affinity m33 TCR

(a, b) *Rag1*^{-/-} mice were implanted with unilateral subcutaneous B16-SIY tumors. Five days later, when tumors were approximately 100 mm³, mice were treated with adoptively transferred CD8⁺ (a) or CD4⁺ (b) transduced T cells. Tumors were measured every two days after T cell transfer. Experiments were performed at least twice. Total number of mice for CD8⁺ T cell treatments were: saline = 6, mock = 6, 2C = 4, m33 = 4. Total number of mice for CD4⁺ T cell treatments were: saline = 4, mock = 6, 2C = 4, m33 = 6. (c) Tumor volumes at day 26 post-T cell transfer were averaged and plotted for each T cell condition. Tumors treated with CD4⁺ T cells transduced with m33 were significantly smaller than tumors

treated with CD4⁺ or CD8⁺ T cells with 2C (* $p < 0.05$). Tumor volumes from saline, mock CD8⁺, mock CD4⁺, and m33 CD8⁺ treated mice are included as a comparative reference (*).

Fig. 3. Phenotype of tumors after treatment with transduced T cells

Rag1^{-/-} mice were implanted with unilateral subcutaneous B16-SIY tumors. Five days later, mice were treated with adoptively transferred CD8⁺ or CD4⁺ T cells transduced with wild-type affinity 2C TCR or high affinity m33 TCR. (a) Recurring B16-SIY tumors that were initially rejected by 2C WT or m33 T CD4⁺ T cells were extracted and dissociated, cultured *in vitro*, and after incubating with IFN- γ (colored histograms) or without IFN- γ (shaded histograms), tumor cells were stained for class I MHC K^b and SIY/K^b. Tumors from mice treated with saline or mock transduced T cells, which reached criteria for euthanasia within 2 weeks, were also harvested, dissociated, cultured *in vitro* and incubated with interferon gamma. All of the tumor cells retained K^b surface expression, but only recurring tumors treated with 2C WT or m33 transduced CD4⁺ T cells lost expression of SIY/K^b. Staining is representative of all tumors examined: a total of 25 separate tumors, 16 recurring tumors and 9 primary outgrowth tumors, were analyzed.

Fig. 4. Long-term survivor mice demonstrate persistent antigen-specific immune response against B16-SIY tumor rechallenge

(a) Long-term survivor adoptively transferred with m33 CD4⁺ T cells 65 days earlier and control *Rag1*^{-/-} mice (n=3) were implanted with unilateral subcutaneous B16-SIY tumors and tumor measurements were recorded every two days. The tumor volume of mice without previous adoptive T cell treatment progressively increased while the long-term survivor completely rejected the B16-SIY challenge. (b, c) *Rag1*^{-/-} mice with bilateral subcutaneous B16 F10 parental and B16-SIY tumors were monitored and tumor measurements recorded every two days. The long-term survivor treated with m33 CD4⁺ T cells 49 days prior to tumor re-challenge showed B16 F10 parental tumor growth while selectively rejecting B16-

SIY (b), whereas B16 F10 parental and B16-SIY tumors steadily grew in all mice without previous T cell therapy. (c) Images of *Rag1*^{-/-} mice 12 days post subcutaneous tumor implant of B16 F10 on the left flank and B16-SIY on the right flank. Control mice did not receive adoptive T cell therapy. Both B16 F10 and B16-SIY tumors developed in all mice, except in the long-term survivor mouse previously treated with m33 CD4⁺ T cells (red arrows at point of B16 F10 and B16-SIY tumor injection). A small relapsing tumor was observed rostral to the tumor cell re-challenge site (black arrow); the origin of this is unclear.

Fig. 5. Adoptive cell therapy with m33 CD4⁺ T cells leads to long-term persistence of effector memory cells

Phenotypic characterization of lymph nodes (LNs) and spleens harvested from long-term survivor following tumor B16-SIY tumor rechallenge (87 days post ACT with m33 CD4⁺ T cells) and C57BL/6 untreated control mice. Flow cytometry profiles are gated on viable, CD4⁺ cells. (a) Flow cytometric analysis of CD4⁺ T cells from LNs (*top row*) and spleen (*bottom row*) stained for CD62L and CD127 expression and (b) T cell receptor (TCR) V β 8 surface expression. Shaded histograms represent isotype control staining, solid lines represent C57BL/6 isolated T cells, and dotted lines represent cells isolated from m33 CD4⁺ T cell treated long-term survivor.

Fig. 6. ACT with CD4⁺ T cells transduced with high affinity m33 TCR results in enhanced survival in an established melanoma tumor model

(a) Survival of *Rag1*^{-/-} mice with B16-SIY subcutaneous tumors and ACT with transduced CD4⁺ T cells 10 days post tumor implant, when tumors averaged 500 mm³ in size. Saline and mock CD4⁺ treated mice (solid black and dashed black lines, respectively) reached criteria for euthanasia within 16 days and 2C WT CD4⁺ treated mice (blue line) survived up to 32 days post T cell transfer (median survival: saline 8 days, mock CD4⁺ 11 days, 2C WT CD4⁺ 26 days), while mice treated with high affinity m33 TCR-transduced T cells (red line) survived significantly longer with a median survival of 36 days (**p* < 0.05 compared to all other groups). Total number of mice per experimental group: saline = 5, mock = 2, 2C WT CD4 = 5, and m33 CD4 = 5. (b) Representative images of B16-SIY subcutaneous tumors 12 and 18 days post ACT with TCR-transduced CD4⁺ T cells. (c) *Ex vivo* phenotype of TCR-transduced T cell-treated tumors. Melanoma tumors that recur following ACT with 2C WT CD4⁺ or m33 CD4⁺ T cells, were stained, following incubation with (colored histograms) or without (shaded histograms) IFN- γ , with anti K^b and SIY/K^b reagents. Recurring tumors lost SIY antigen expression while retaining class I MHC K^b surface expression.