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T cell therapy targeting a public neoantigen in microsatellite instable colon cancer reduces *in vivo* tumor growth

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ABSTRACT

T-cell receptor (TCR) transfer is an attractive strategy to increase the number of cancer-specific T cells in adoptive cell therapy. However, recent clinical and pre-clinical findings indicate that careful consideration of the target antigen is required to limit the risk of off-target toxicity. Directing T cells against mutated proteins such as frequently occurring frameshift mutations may thus be a safer alternative to tumor-associated self-antigens. Furthermore, such frameshift mutations result in novel polypeptides allowing selection of TCRs from the non-tolerant T-cell repertoire circumventing the problem of low affinity TCRs due to central tolerance. The transforming growth factor β Receptor II frameshift mutation (TGFβRII^{mut}) is found in Lynch syndrome cancer patients and in approximately 15% of sporadic colorectal and gastric cancers displaying microsatellite instability (MSI). The -1A mutation within a stretch of 10 adenine bases (nucleotides 709–718) of the TGFβRII gene gives rise to immunogenic peptides previously used for vaccination of MSI+ colorectal cancer patients in a Phase I clinical trial. From a clinically responding patient, we isolated a cytotoxic T lymphocyte (CTL) clone showing a restriction for HLA-A2 in complex with TGFβRII^{mut} peptide. Its TCR was identified and shown to redirect T cells against colon carcinoma cell lines harboring the frameshift mutation. Finally, T cells transduced with the HLA-A2-restricted TGFβRII^{mut}-specific TCR were demonstrated to significantly reduce the growth of colorectal cancer and enhance survival in a NOD/SCID xenograft mouse model.

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Adoptive T-cell therapy (ACT); colon cancer; immunotherapy; neoantigen; T cell receptor (TCR)

Introduction

Colorectal carcinoma (CRC) is the third most common cancer in men and the second most common in women worldwide, with the highest rates in the Western world.[1] Virtually all hereditary non-polyposis colorectal cancers (HNPCC), and a subset of sporadic cancers, including colorectal and gastric cancers, display microsatellite instability (MSI). MSI+ colon cancers are considered to be more immunogenic than microsatellite stable (MSS) cancers due to the generation of neoantigens caused by frameshift mutations. Transforming growth factor β Receptor II (TGFβRII) is a common target for inactivating mutations occurring in approximately 90% of MSI,[2,3] and 15% of MSS [4] colon cancers. Interestingly, Lynch syndrome (or HNPCC) and sporadic colon cancers patients with the MSI+ subtype have an improved prognosis compared with other sporadic colon cancer patients. This might be due to the presence of certain frameshift mutations in MSI+ colon cancers, [5] which correlates with the increased density of tumor-infiltrating lymphocytes (TILs).[6,7] Accordingly, an improved survival of MSI+ compared with non-MSI+ colorectal cancer patients is seen.[5,8-11] These observations suggest that some patients with MSI+ CRC may benefit from immunotherapy targeting public neoantigens such as mutated TGFβRII. Recent clinical trials of adoptive transfer of TCR redirected T-cells targeting cancer germline antigens have, however, shown that immunotherapy can be associated with severe toxicity emphasizing the need for careful consideration of the choice of antigen. In one study, three out of nine cancer patients treated with autologous anti-MAGE-A3 TCR-engineered T cells experienced severe neurologic toxicity, being lethal in two cases, due to cross-reactivity of the TCR.[12] A second study targeting MAGE-A3 in myeloma and melanoma patients with a HLA-A*01-restricted TCR demonstrated lethal cross-reactivity with myocardial damage.[13,14] An alternative strategy is to exploit the panel of private neoantigens, isolate the corresponding TCR and reinfuse redirected T cells.[15] Although attractive, this personalized approach will still need further development before being proposed as a realistic therapy. Therefore, true tumor-specific neoantigens that are more frequent may

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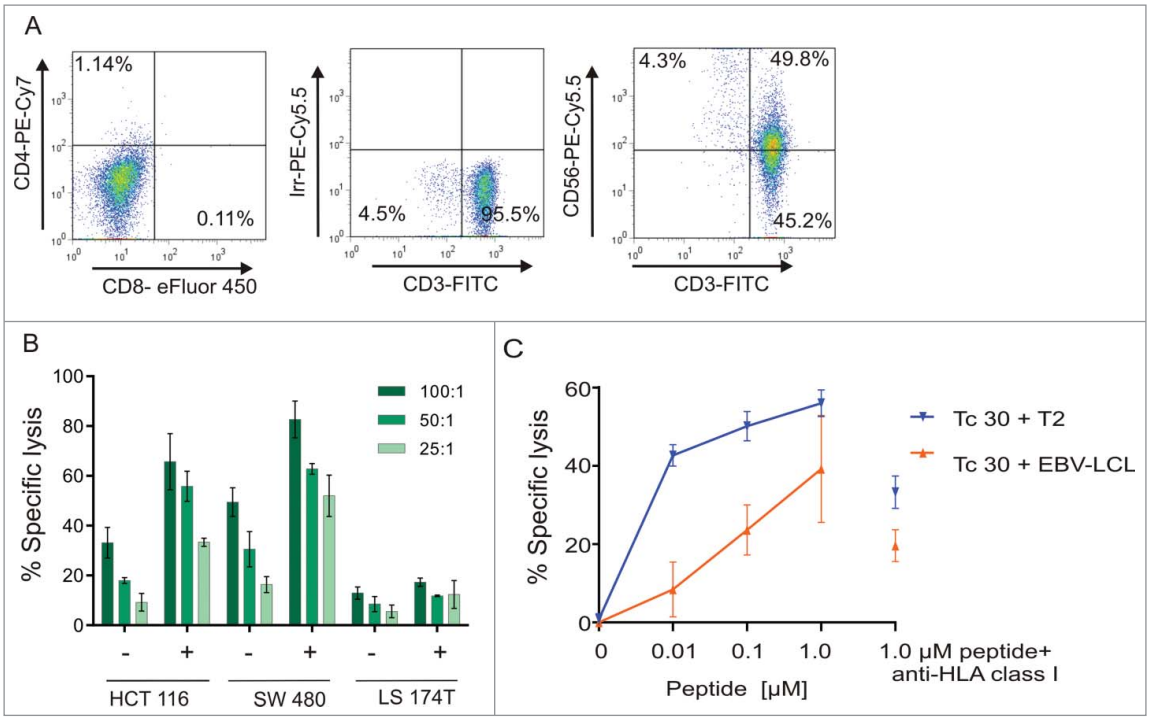

Figure 1. Original T-cell clones are TGFβRII frameshift mutation-specific, $CD8^{-}CD4^{-}CD56^{+}$, and kill target cells in a dose-dependent manner. (A) Flow cytometry analysis showed that the original T-cell clone was $CD8^{-}CD4^{-}$ and $CD56^{+}$. (B) ^{51}Cr-release assays with the specific lysis by T-cell clone TC 30 of colon cancer cell lines, loaded (+) or not (−) with 1 μM p573 at various effector-to-target (E:T) ratios as indicated. (C) ^{51}Cr-release assays with the specific lysis by the TC 30 of autologous EBV-LCL or T2, loaded with titrated concentrations of peptide. Blocking with anti-HLA class I at the highest peptide concentration is also shown. The E:T ratio was 25:1. The results shown are representative of three independent experiments.

represent ideal targets for TCR therapy achieving tumor eradication in the absence of normal tissue destruction.[15,16]

Several T-cell epitopes have been identified within these frameshift peptides,[17,18] including $CD4^{+}$ and $CD8^{+}$ T-cell epitopes in TGFβRII (-1A).[19-21] The present study describes the isolation of an HLA-A2-restricted TGFβRII frameshift mutation-specific T-cell clone. We demonstrate that its TCR is efficiently and functionally expressed in redirected $CD8^{+}$ and $CD4^{+}$ T cells, resulting in production of IFNγ and TNF-α upon specific stimulation. TCR-transfected $CD8^{+}$ T cells were also capable of target cell lysis. Finally, a murine model of colon cancer used to test the *in vivo* potency of the TCR-redirected T cells showed a significant reduction in tumor growth and an enhanced survival of the study animals. We conclude that this TCR is a potential candidate for immunotherapy. The present study might pave the way for the exploitation of TCRs isolated from successfully vaccinated patients in development of clinical cancer therapy.

Results

Isolation of a TGFβRII frameshift mutation-specific T-cell clone

An MSI+ colon cancer patient had been vaccinated with a 23-mer TGFβRII frameshift peptide and showed a long survival (> 10 y, *manuscript in preparation*). We therefore detected an immune response against the vaccine. A TGFβRII frameshift mutation-reactive HLA-A2-restricted CTL was previously isolated from his blood. The CTL was isolated and shown to be $CD8^{-}CD4^{-}$, in addition about 50% of the cells were $CD56^{+}$, suggesting it may be Natural Killer (NK) cell-like (Fig. 1A). The CTL expressed TCR Vβ3 (or TRBV 28, IMGT nomenclature) chain.[22] The clone (TC 30) showed specific lysis of the colon cancer cell lines HCT 116 and SW 480 (HLA-A2+, frameshift+). As expected, the effector:target (E:T) ratio required for lysis of cell lines with endogenous peptide was higher than if cell lines were loaded exogenously with TGFβRII frameshift peptide (p573). As a control, another colon cancer cell line, LS174T (HLA-A2-, frameshift+) was not killed (Fig. 1B). Importantly, despite the expression of CD56 on the T-cell clone (Fig. 1A); the HLA-A2 negative LS174T cell line was not killed, indicating that the killing was not mediated by NK-cell like activity, but by specific recognition of MHC molecules loaded with the correct peptide.

To test the relative avidity of the T cell clone, TAP-deficient T2 cells were loaded with titrated amounts of peptide (0.01–1.0 μM). We observed that the killing activity was following the peptide concentration and that the addition of HLA-blocking antibodies specifically reduced the killing (Fig. 1C). Similar observations were made when autologous Epstein–Barr virus-transformed lymphoblastoid cell lines (EBV-LCLs) were used as APC. Taken together, our data show that, although the TGFβRII^{mut}-specific T-cell clone was co-receptor negative, the clone was still peptide-specific and HLA class I restricted.

Radium-1 TCR redirects both $CD4^{+}$ and $CD8^{+}$ T cells

The TCR α and β chains from the TGFβRII^{mut}- reactive T-cell clone were identified and referred to here after as Radium-1 TCR. A 2A construct was designed and sub-cloned into an

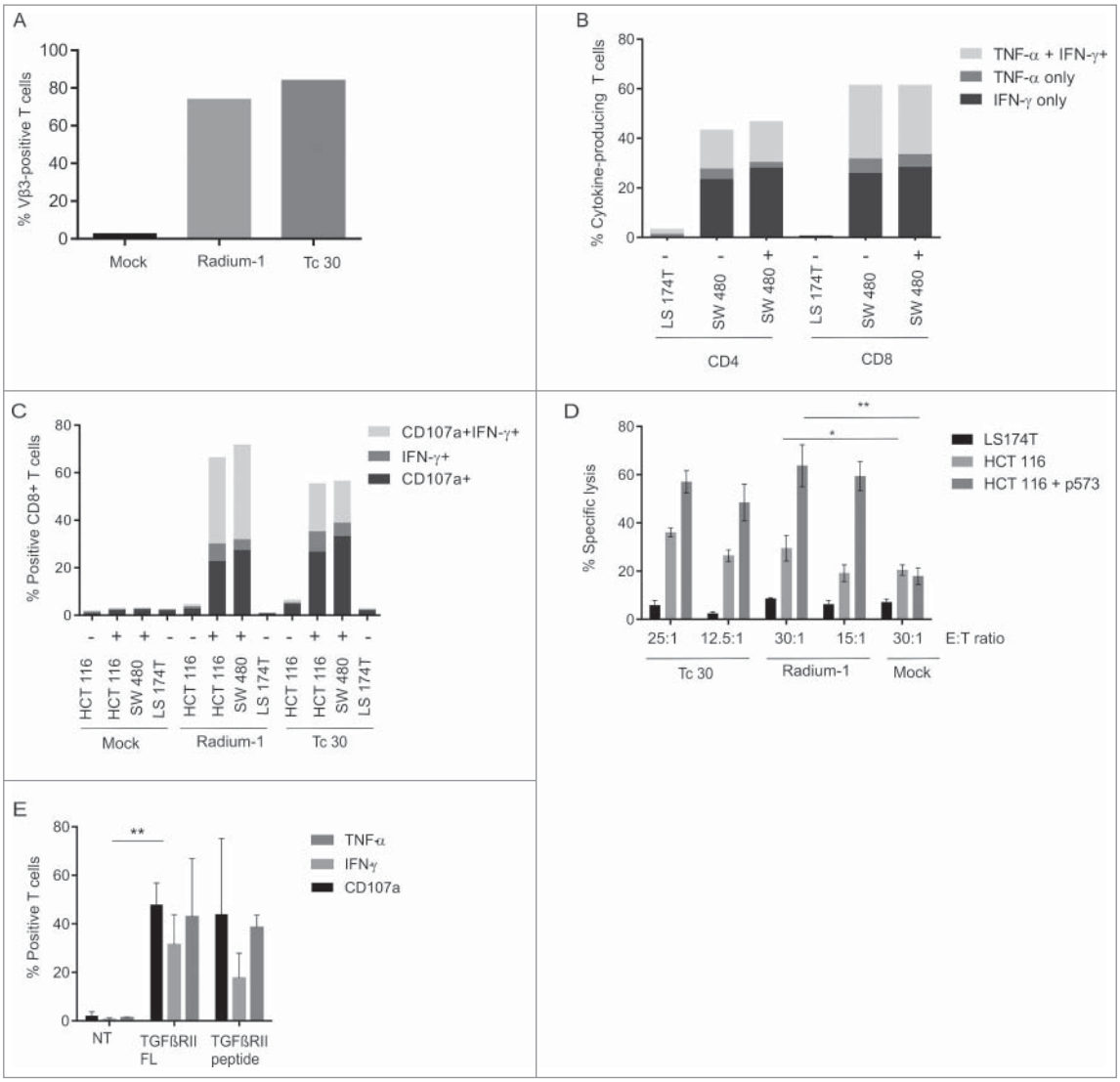

Figure 2. Radium-1 TCR expression and function in transfected *in vitro* expanded T cells. (A) Vβ3 staining of mock-transfected T cells, Radium-1-transfected T cells and the patient T-cell clone (TC 30). (B, C) T cells transfected with Radium-1, mock transfected or the patient T-cell clone (TC 30) were co-incubated with colon cancer cell lines LS174T, SW 480 and/or HCT 116 expressing mutated TGFβRII. LS174T (HLA-A2 neg), SW 480 (HLA-A2 pos) and HCT 116 (HLA-A2 pos) were loaded (+) or not (−) with frameshift peptide (p573) as indicated. Intracellular cytokine staining was performed after overnight incubation (B), or after 6 h (C). (D) The same cells as above were used for 6-h ^{51}Cr-release assays at E:T ratios as indicated. The results shown are representative of two or three independent experiments (Mock Tc vs. TCR-transfected Tc target HCT 116 $p = 0.0021$, Mock Tc vs. TCR transfected Tc target HCT116+p573 $p = 0.0011$). E, Radium-1 TCR-transfected T cells were tested against HLA-A2+ target cells (EBV-LCLs) presenting either TGFβRII frameshift peptide (positive control), non-transfected EBV-LCL (NT, negative control) or transfected with mRNA encoding the full-length frameshift mutated TGFβRII. The cells were co-incubated for 6h before degranulation (CD107a) and intracellular cytokine staining were evaluated. Data from two different experiments using two different donors were pooled and mean (+/− SD) was plotted. Statistical significance was tested with unpaired, two-tailed *t*-tests ($p = 0.0013$ for NT vs. TGFβRII FL, $p = 0.48$ for TGFβRII FL vs. TGFβRII peptide).

mRNA expression vector for equimolar expression of the TCR α and -β chains (see Materials and Methods). T cells expanded from PBMCs were electroporated with Radium-1 encoding mRNA to assess their ability to specifically recognize their targets. Transferred TCR expression was measured by anti-Vβ3 antibody surface staining and showed that between 60 and 70% of transfected T cells expressed the Vβ3 chain in both CD4$^+$ and CD8$^+$ cells, whereas less than 5% of the cells naturally expressed Vβ3 (Fig. 2A and Fig. S3).

We then monitored the activity of Radium-1-transfected T cells by intracellular cytokine staining upon co-incubation with the colon cancer cell lines SW 480 and LS174T for 15 h. We chose a long incubation to get maximal activation of CD4$^+$ as well as CD8$^+$ T cells. SW 480 cells were recognized by both CD8$^+$ and CD4$^+$ T cells in the absence and presence of exogenously loaded peptide. The T cells produced TNF-α and IFNγ (Fig. 2B). As expected the colon cancer cell line LS174T was not recognized. These data confirmed the HLA-peptide restriction of Radium-1; furthermore, it suggests that this TCR is very potent as it was able to efficiently redirect both CD4$^+$ and CD8$^+$ T cells. (Gating strategy for intracellular staining is shown in Fig. S5).

In vitro *cytotoxicity of Radium-1 redirected T cells*

To determine the cytotoxic potential of Radium-1 TCR-transfected CD8$^+$ T cells, mRNA-electroporated T cells were co-incubated with colon cancer cell lines for only 6 h and stained with antibodies against the degranulation marker CD107a and IFNγ (Fig. 2C). Surprisingly, very low levels of IFNγ and

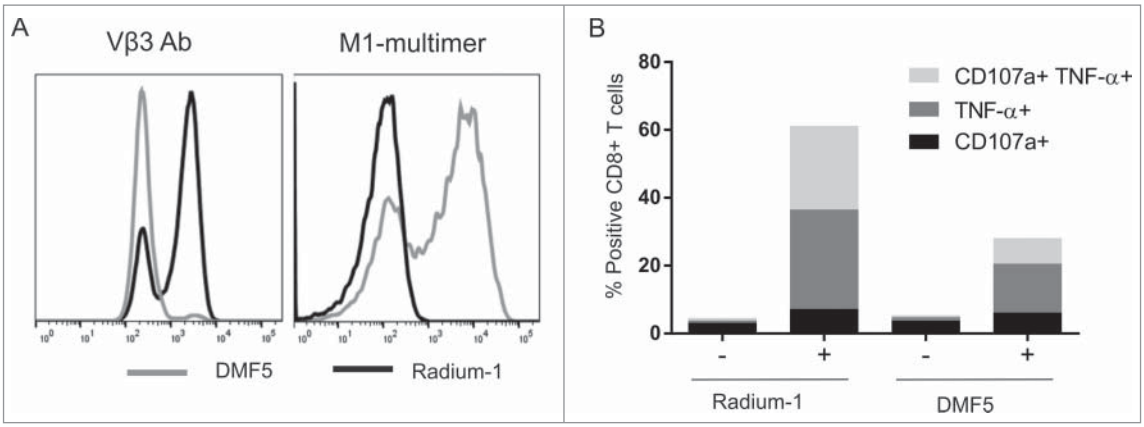

Figure 3. TCR-transduced T cells are functional *in vitro*. (A) Donor T cells were transduced with Radium-1 or DMF5 TCR. Transduction efficiency was found to be around 60% for each of the TCRs when T cells were stained with either Vβ3 antibody (Radium-1) or MART-1 multimer (DMF5). (B) Transduced T cells were tested for reactivity against their cognate antigen before infusion. HLA-A2+ EBV-LCLs were loaded (+) with either a long TGFβRII frameshift mutation peptide covering the CTL epitope, p621, or the native MART-1 26–35 peptide or not (−). T cells were co-incubated with EBV-LCLs for 5 h and stained for degranulation (CD107a) and TNF-α.

CD107a were detected in the absence of exogenously loaded peptide. Importantly, this was also the case for the original clone, suggesting that although co-receptor independent, Radium-1TCR signal strength in response to cell lines presenting endogenous TGFβRII frameshift peptide may be due to low stability of pMHC complexes. The increased T-cell activation seen upon longer incubation times could be a result of increased level of cumulative TCR stimulation over several hours. Upon the addition of peptide (p573), both Radium-1-redirected T cells and the original T-cell clone were strongly activated after 6-h incubation. Interestingly, TCR-transfected T cells were more efficient IFNγ producers and also displayed higher levels of degranulation than the original T-cell clone. Since mock-transfected T cells were not activated, this result suggests that the $CD8^+$ co-receptor, although not present in the original clone, could still improve Radium-1 signaling. We further tested the influence of the co-receptor by using HEK 293 cells (HLA-A2 negative) transfected with either HLA-A2 wild type (wt) or a mutant defective in $CD8^+$ binding [23] and loaded with peptide to stimulate Radium-1-transfected T cells. The fraction of stimulated redirected T cells was slightly but reproducibly decreased when the mutant was presenting (CD107a+ 36% in the WT versus 26% in the mutant, Fig. S1). Since the original clone was double negative (DN), we investigated if Radium-1-transfected T cells were capable of target cell lysis. We tested Radium-1 reactivity against colon cancer cell lines in 6-h Cr-release assays (Fig. 2D). Radium-1-transfected T cells lysed HCT 116 cells at levels comparable to the original patient T-cell clone. As expected, lysis was further increased when exogenous p573 was added. The lysis of the HLA-A2 negative cell line LS174T was similar to that of mock-transfected T cells demonstrating low background lysis of HCT 116 is likely due to TRAIL-R expression on the target cells (Fig. S2).[24] To validate that the frameshift-mutated TGFβRII T-cell epitope is processed and presented by HLA- A2+ target cells, we also tested Radium-1 TCR reactivity against non-transfected target cells and target cells transfected with mRNA encoding full-length frameshift-mutated TGFβRII (Fig. 2E). As anticipated, the Radium-1 TCR-transfected T cells recognized only the target cells transfected with FL mRNA and not the non-transfected cells (NT). Taken together these data suggest that the Radium-1 is unique in the sense that it is a TCR with a strong co-receptor independency and recognizes a processed and presented T-cell epitope. The TCR signal strength in response to peptide endogenously processed and presented may require cumulative TCR stimulation as longer incubation times are required for full activation of TCR-bearing T cells. One may speculate that this is due to low stability of the pMHC complex. However, due to the ability of Radium-1 to transfer both cytotoxic activity and cytokine release in redirected T cells this prompted us to test its efficacy *in vivo*.

Radium-1 TCR-transduced T cells are effective in vitro *and* in vivo

We established a xenograft mouse model of colon cancer by intraperitoneal (i.p.) injection of HCT 116 cells modified to express luciferase. Radium-1 2A construct was transferred into a retroviral construct (see Materials and Methods). T cells were transduced with Radium-1 and DMF5 TCR (negative control,[25]) and TCR expression was shown to be around 60% for both constructs (Fig. 3A). Prior to injection, T cells were confirmed to be functional against HLA-A2+ EBV-LCLs loaded or not with specific peptides (Fig. 3B). NSG mice were injected i.p. with 10^6 HCT 116 cells on day 0 (d0) and on d2, d5 and d10 mice were injected with 8×10^6 and 20×10^6 redirected T cells, respectively (Fig. 4A). Control mice were treated with T cells from the same donor expressing DMF5. *In vivo* live imaging of the mice showed that the tumor load was significantly lower ($p = 0.043$) in mice that received the treatment with Radium-1 T cells compared with the MART-1-specific control T cells (Fig. 4B and Fig. S4). The mice receiving Radium-1 T cells also had enhanced survival compared with control mice ($p = 0.0035$, Fig. 4C). Tumors from mice that had to be killed due to high tumor load were dissected and single-cell suspension stained with anti-human CD3 and anti-Vβ3 or MART-1 multimer. This revealed that the percentage of TCR expressing T cells in the tumor was significantly higher in Radium-1-treated mice despite a similar transduction efficiency ($p = 0.0038$, Fig. 4D) indicating that the Radium-1 T cells were either recruited to the tumor more efficiently and/or proliferated *in*

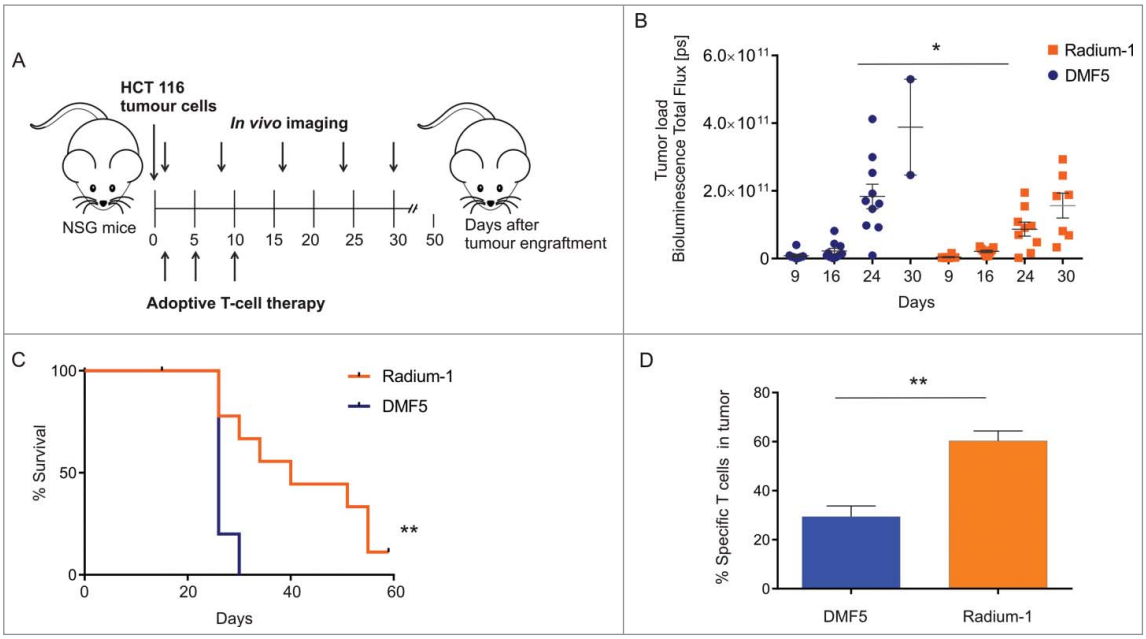

Figure 4. Radium-1 TCR-transduced T cells are effective *in vivo*. (A) NSG mice (Radium-1, n = 10; DMF5, n = 10) were injected i.p. with 10^6 HCT 116 ff-Luc 2 d before the i.p. injection of 8×10^6 TCR-transduced T cells on day 2. Treatment was repeated on days 5 and 10 with 20×10^6 TCR+ T cells. Tumor load was evaluated by bioluminescence imaging on day 2, 9, 16, 24, 30. (B) Bioluminescence signals (photons/sec) for all mice are shown in the scatter plot with mean indicated (+/− SD). (C) Kaplan–Meier analysis showed that Radium-1-treated mice had a significantly prolonged survival compared with control DMF5 mice ($p = 0.0035$; Unpaired *t*-test). *In vivo* experiments were repeated three times and one representative experiment is shown. (D) Tumors were dissected from killed mice 20 d after the last T-cell infusion; single-cell suspension was made and stained for the presence of transduced T cells using anti-CD3 and either Vβ3 antibody (Radium-1) or MART-1 multimer (DMF5). The percentage of MART-1-specific T cells in the control group tumors was significantly lower than the percentage of TGFβRII^{mut}-specific T cells in tumors of mice treated with Radium-1 TCR ($p = 0.0038$).

vivo due to antigenic stimulation. Taken together, these data demonstrate the pre-clinical potency of Radium-1 TCR *in vivo*.

Discussion

From a long-term surviving patient with MSI+ colorectal cancer vaccinated with a long synthetic TGFβRII(-1A) peptide, we isolated a $CD4^-$ $CD8^-$ T-cell clone specific for a CTL epitope of the peptide presented on HLA-A2. The clone was able to lyse HLA-A2+ colon cancer cell lines harboring the specific TGFβRII frameshift mutation in a co-receptor-independent and dose-dependent manner. Since CD8-independence is generally seen as a feature associated with TCRs of high affinity,[26] we therefore hypothesized that the TCR from this clone was a promising candidate for TCR transfer in adoptive cell therapy. We isolated and sub-cloned Radium-1 and used an mRNA transfection protocol to redirect T cells *in vitro*.[27] Our results demonstrated that the redirected T cells were poly-functional cytokine producers and after overnight stimulation, TNF-α and IFNγ production was observed in both $CD8^+$+ and $CD4^+$ T cells. However, after shorter incubation (6 h) with target cells, cytokine production in both $CD4^+$ and $CD8^+$ T cells and the accumulation of CD107a in $CD8^+$ T cells could be detected only in the presence of exogenously loaded peptide. The cognate peptide for Radium-1 is predicted to be a good HLA-A2 binder (IEDB analysis resource Consensus tool), but our preliminary data suggest that the stability of the pMHC complex may be low. It is therefore tempting to speculate that this peptide has a fast K_{OFF} rate and therefore does not stay bound to the HLA-A2 molecule for very long. Supporting this, we were never able to obtain pMHC multimers despite the attempted production by several companies. Cumulative TCR stimulation with increased co-incubation times may compensate for this and could also be important *in vivo* (reviewed in Ref.[28]). The difference in activation seen in $CD4^+$ vs. $CD8^+$ TCR-transfected T cells could be due to different activation thresholds, differential requirements for co-stimulatory molecules, and distinct mechanisms for effector cell development which exist in $CD4^+$ and $CD8^+$ T cells.[29] The requirement for CD28 co-stimulation in $CD4^+$ T cells can be compensated for by higher concentrations of antigen or longer durations of exposure to antigen, [30] which explains the reduced difference between $CD8^+$ and $CD4^+$ T-cell activation seen after overnight stimulation compared with 6 h. In T cells transduced with a CD8-independent tyrosinase-specific TCR, an important difference in $CD8^+$ and $CD4^+$ T-cell tumor reactivity was observed *in vitro*.[31] Although $CD56^+$, the same kinetics were observed in the original clones, suggesting that the TCR was *per se* the only killing force. This was finally supported by *in vivo* experiments: Our xenograft mouse model of colon cancer showed that redirected T cells reduced tumor growth and enhanced survival compared with control T cells expressing a MART-1-specific TCR (DMF5). There are several limitations with the use of such xenograft models. The HCT 116 used is a very fast growing cancer cell line and we therefore have to start treatment as soon as we can detect the tumor engraftment (2 d later). The i.p. injection of this cell line provided a more physiologic model replicating human disease. The disadvantage of this in a therapeutic model is that the tumor cell line tends to grow faster than in subcutaneous models. Infusing human T cells into immunodeficient mice where T-cell xenoreactivity is reported

to be seen from day 30–40 limits the possibility for observation of long lived T-cell responses. We chose not to use systemic dosing of exogenous human IL-2 in this model based on results from other xenograft studies of adoptive T-cell therapy,[32-34] but still observed live TCR-transduced T cells in excised tumors 20 d post-infusion. In summary, we have demonstrated the ability to confer reactivity against frameshift-mutated TGFβRII to human T cells. Interestingly, the level of peptide-MHC (pMHC) complexes did not make a difference in cytokine production after 24 h. It is well established that both the MHC class I and antigen expression levels influence CTL recognition.[35,36] An important difference in these assays was the E:T cell ratio which was 1:2 for intracellular cytokine staining assays, but was titrated down from 100:1 down to 12.5:1 for the ^{51}Cr-release assays. Although the specific lysis was clearly dose-dependent, the pMHC density did not strongly influence the lysis efficacy at high E:T ratios, whereas there is a striking difference between the specific target-cell lysis in the absence and presence of exogenously loaded peptide at lower E:T ratios.

The Radium-1 target antigen, a particular TGFβRII frameshift mutation (-1A), has been reported to be present in 76% of MSI+ colorectal cancer.[18] Being strictly tumor-specific, redirecting T-cells against this antigen in adoptive cell therapy would reduce the risk of on-target off-tumor toxicity, which was observed in clinical trials using TCRs and CARs targeting overexpressed antigens.[12] Furthermore, several studies have shown that metastatic MSI+ colon cancers have reduced sensitivity to chemotherapy,[37-39] leaving these patients with few treatment options. Radium-1 may therefore have potential for adoptive T-cell therapy of patients with MSI+ colon cancers, and, in particular, HNPCC. Recent clinical results have shown that anti-PD-1 treatment is effective in patients with MSI+ colon cancers.[40] Our observation that HCT 116 cells express high levels of PD-L1 (data not shown) indicate that more robust clinical responses may be obtained by combining anti-PD1/anti-PDL-1 treatment with adoptive T cell therapy [41] using Radium-1 retargeted T cells.

Clinical studies reporting severe toxicities when using engineered T cells targeting MAGE-A3 used high affinity TCRs.[12,13] While enhancing tumor antigen recognition, such modifications may also cause the receptors to recognize additional and unrelated peptides expressed by normal tissues. Reports have demonstrated that T-cell function reaches a plateau which cannot be further enhanced above a certain TCR-pMHC affinity threshold.[42] Whereas affinity matured TCRs have been shown to enhance the speed of T-cell activation, they may require higher densities of pMHC complexes for the initiation of T-cell responses.[43] The close relationship between T-cell antitumor activity and autoimmunity requires tight regulation of high avidity T-cell responses and therefore TCRs with affinities above the natural range may be inhibited by negative feedback mechanisms.[44] We speculated that since Radium-1 might have been part of the patient beneficial response *as is*, we would use it as a non-modified TCR. Furthermore, any modification of the protein sequence could result in unpredictable effects for the receiver. This was supported by our assays with a modified version of Radium-1 where extra cysteines were added to the constant part [45] of the TCR and although this improved activity *in vitro*, it brought conflicting results *in vivo* (data not shown).

Many tumor antigens targeted with T-cell therapy are non-modified self-antigens. The affinity of TCRs specific for these antigens is limited by central tolerance during T-cell development in the thymus, resulting in decreased antitumor efficacy. Common approaches to overcome this are to generate high-affinity TCRs through mutation of TCR genes from naturally occurring T cells or to generate these TCRs by vaccination of HLA transgenic mice. The first report on TCR therapy in colon cancer was targeting carcinoembryonic antigen (CEA) where some evidence of clinical response was seen, but the T-cell function may have been inhibited due to the necessity to resolve the severe colitis which occurred.[46] This demonstrates not only the feasibility of T-cell therapy in metastatic colon cancer, but also the limitations of targeting CEA as an antigen. Another approach to circumvent central tolerance is to isolate self-antigen-specific TCRs from allogeneic HLA-mismatched TCR repertoires.[47] This could generate TCRs unable to distinguish between cancer cells expressing high levels of the self-antigen and healthy cells expressing low levels of the antigen. TCRs generated in an allogenic setting can also have a high potential for cross-reactivity as shown by Arber *et al.*[48]

Recent pre-clinical and clinical results support the use of private neoantigens as targets, fitting well with the use of TCRs to target frequent frameshift mutations.[49]

Materials and methods

Ethical approval

The study was approved by the Regional Committee for Medical Research Ethics (Oslo, Norway). All mouse experiments were conducted under an Institutional Animal Care and Use Committee-approved protocol.

Cell lines, media and reagents

A TGFβRII frameshift mutation-reactive, HLA-A2-restricted CTL clone was isolated from the blood of a MSI+ colon cancer patient and cloned by limiting dilution. The patient had been vaccinated with a 23-mer TGFβRII (-1A) frameshift peptide. The clinical trial was approved by the Norwegian Medicines Agency, the Committee for Medical Research Ethics Region South and the Hospital Review Board. The treatment was performed in compliance with the World Medical Association Declaration of Helsinki. Informed consent was obtained from the patient. The autologous Epstein–Barr–Virus-transformed lymphoblastoid cell line (EBV-LCLs) was generated by transformation of B cells from the donor. The antigen processing-deficient T2 cell line was used as a T-cell target in flow cytometry and cytotoxicity assays. Colon cancer cell lines HCT 116, SW 480 and LS174T as well as Human Embryonic Kidney (HEK) 293 cells were obtained from ATCC (Rockville, MD, USA). All cell lines were passaged for fewer than 6 mo after their purchase. Human cell line identities were verified using short tandem repeat profiling. Cell lines were tested for mycoplasma contamination using a PCR-based detection kit (Venor®GeM, Minerva Biolabs).

Hek-Phoenix (Hek-P, our collection) were grown in DMEM (PAA Laboratories) supplemented with 10% HyClone FCS (GE

Healthcare) and 1% antibiotic-antimicotic (penicillin/streptomycin, p/s, PAA). Where nothing else is indicated, cells were cultured in RPMI-1640 (PAA Laboratories) supplemented with gentamicin, 10% heat-inactivated FCS (PAA Laboratories). Colon cancer cell lines were treated with 500 U/mL IFNγ (PeproTech) overnight before use as target cells.

All T cells were grown in CellGro DC medium (CellGenix GmbH) supplemented with 5% heat-inactivated human serum (Trina Bioreactives AG), 10 mM N-acetylcysteine (Mucomyst 200 mg/mL, AstraZeneca AS), 0.01 M HEPES (Life Technologies, Norway) gentamycin 0.05 mg/mL (Garamycin, Schering-Plough Europe), denoted complete medium hereafter, unless otherwise stated.

Generation of T-cell lines and clones specific for TGFβRII frameshift peptides

PBMCs collected pre- and post-vaccination were available for analysis. The PBMCs had been isolated and frozen as described previously.[50] Thawed PBMCs were stimulated one round *in vitro* with peptide for 10–12 days and then tested in triplicates in T-cell proliferation assays (^{3}H-Thymidine) using autologous PBMCs as APCs. PBMCs from various time points were stimulated with TGFβRII frameshift peptides. This included peptide 573 (p573), RLSSCVPVA (amino acid sequence 131–139) and 621 (p621), KSLVRLSSCVPVALMSAMT (amino acid sequence 127–145) from a TGFβRII frameshift protein resulting from a 1 bp-deletion (-1A) in an adenosine stretch (A10) from base number 709–718 of *TGFBRII*. (The GenBank sequence for wild-type human *TGFBRII*: NM 003242). hTERT peptide I540 (ILAKFLHWL) was used as a negative control. Both peptides were provided by Norsk Hydro ASA).

The MART-1 peptide EAAGIGILTV (amino acid sequence 26–35) was manufactured by ProImmune Ltd, UK. The stimulated T cells were then tested in proliferation assays against peptide-loaded APCs, either autologous PBMC or EBV-LCL. The stimulation Index (SI) was defined as proliferation with peptide divided by proliferation without peptide and an SI $\geq$ 2 was considered a positive response. T-cell clones from responding T-cell lines were generated as described previously.[20]

TcR, HLA-A2 and TGFβRII frameshift cloning

Frameshift-specific T-cell clones (26 and 30) were grown and total RNA was prepared. The cloning was performed using a modified 5′-RACE method. Briefly, cDNA was synthesized using an oligo-dT primer and was tailed at the 5′-end with a stretch of cytosines. A polyguanosine primer together with a constant domain-specific primer was used to amplify TCR chains. The amplicon was cloned and sequenced. The expression construct was prepared by amplifying TCR-α and -β chains separately with specific primers and a second PCR was performed to fuse the TCR chains as a TCR-2A construct. The TCR-2A reading frame was cloned into pENTR (Invitrogen) and subsequently recombined into other expression vectors. For RNA synthesis, we sub-cloned the insert into a Gateway modified version of pCIpA$_{102}$.[51] A detailed method as well as the primer sequences can be found in Ref.[52] For retroviral transduction the insert was sub-cloned into the pM71 vector. The HLA-A*0201pCIp$_{102}$ was cloned as described previously.[53] This construct was used as a template to generate a CD8$^+$ binding-deficient mutant by targeting the residues D227 and T228 and replacing them with K and A, respectively, as described by Xu *et al.*[24] A standard site-direct mutagenesis was performed using the following primers:

5′-GAGGACCAGACCCAGAAGGCGGAGCTCGTGGAGAC-3′ and 5′-GTCTCCACGAGCTCCGCCTTCTGGGTCTGGTCCTC-3′. HEK 293 cells were transfected with these constructs using FuGENE-6 (Roche) following the manufacturer's protocol.

The sequence of TGFβRII frameshift mutant was ordered as codon optimized to Eurofins MWG Operon (Ebersberg, Germany) and subcloned into pCIpA102 for mRNA production (see next section).

In vitro *mRNA transcription*

The *in vitro* mRNA synthesis was performed essentially as described previously.[28] Anti-Reverse Cap Analog (Trilink Biotechnologies Inc.) was used to cap the RNA. The mRNA was assessed by agarose gel electrophoresis and Nanodrop (Thermo Fisher Scientific).

In vitro *expansion of human T cells*

T cells from healthy donors were expanded using a protocol adapted for GMP production of T cells using Dynabeads CD3/CD28 essentially as described previously.[28] In brief, PBMCs were isolated from buffy coats by density gradient centrifugation and cultured with Dynabeads (Dynabeads® *ClinExVivo*™ CD3/CD28, kindly provided by Thermo Fisher Scientific) at a 3:1 ratio in complete CellGro DC Medium with 100 U/mL recombinant human interleukin-2 (IL-2) (Proleukin, Novartis Healthcare) for 10 days. The cells were frozen and aliquots were thawed and rested in complete medium before transfection.

Electroporation of expanded T cells

Expanded T cells were washed twice and resuspended in CellGro DC medium (CellGenix GmbH) and resuspended to 70 $\times$ 10^6 cells/mL. The mRNA was mixed with the cell suspension at 100 μg/mL, and electroporated in a 4-mm gap cuvette at 500 V and 2 ms using a BTX 830 Square Wave Electroporator (BTX Technologies Inc., Hawthorne, NY, USA). Immediately after transfection, T cells were transferred to complete culture medium at 37°C in 5% CO_2 overnight to allow TCR expression.

Antibodies and flow cytometry

T cells were washed in staining buffer (SB) consisting of phosphate-buffered saline (PBS) containing 0.1% human serum albumin (HSA) and 0.1% sodium azide before staining for 20 min at RT. The cells were then washed in SB and fixed in SB containing 1% paraformaldehyd. For intracellular staining, T cells were stimulated for 6 h or overnight with APCs, loaded or not with p573, at a T-cell to target ratio of 1:2 and in the presence of BD GolgiPlug and BD Golgistop at a 1/1,000 dilution.

Cells were stained both extracellular and intracellular using the PerFix-nc kit according to the manufacturer's instructions (Beckman Coulter Inc., USA). The following antibodies were used: Vβ3- FITC (Beckman Coulter-Immunotech SAS, France), CD3-eFluor450, CD4-eFluor 450, CD4-PE-Cy7, CD8-APC, CD8-eFluor 450, CD8-PE-Cy7, CD56-PE-Cy5.5 (BD Biosciences, USA) and CD107a-PE-Cy5 (BD Biosciences, USA), CXCR2-PE, IFNγ-FITC, IL-2-APC, TNF-α-PE (BD Biosciences, USA), CD261/TRAIL-R4-PE (BD Biosciences, USA). MART-1 (aa 26–35)-specific TCR was detected with dextramer staining (Immudex) following the manufacturer's recommendations. All antibodies were purchased from eBioscience, except where noted. Cells were acquired on a BD LSR II flow cytometer and the data analyzed using FlowJo software (Treestar Inc.).

^{51}Cr-relase assays

^{51}Cr-release cytotoxicity assay was performed by labeling of 2×10^6 target cells in 0.5 mL FCS with $Na_2{}^{51}CrO_4$ (7.5 MBq) (Perkin Elmer), for 1 h with gentle mixing every 15 min. Cells were washed three times in cold RPMI-1640 and seeded at 2×10^3 target cells in 96-well, U-bottomed microtitre plates. Autologous EBV-LCL, T2 target cells or colon cancer cell lines HCT 116, SW 480, and LS174T, were pulsed with 10 μM p573 or pI540 for 1 h at 37°C. The original T-cell clone, TCR-transfected T cells, or mock-transfected T cells were added at the effector-to-target (E:T) ratios indicated and the plate was left for 4–6 h at 37°C as indicated. The maximum and spontaneous ^{51}Cr release of target cells was measured after incubation with 5% Triton X-100 (Sigma-Aldrich) or medium, respectively. Supernatants were harvested onto Luma Plates (Packard, Meriden, CT) and ^{51}Cr released from lysed cells was measured using a TopCount microplate scintillation counter (Packard Instrument Company, Meriden, USA). The percentage of specific chromium release was calculated by the formula: [(experimental release − spontaneous release)/(maximum release spontaneous release)] × 100.

Retroviral transduction

PBMCs isolated from healthy donors were cultured and activated in CellGro DC medium (CellGenix GmbH) supplemented with 5% human serum (HS) and 100 U/mL IL2 (Proleukin, Novartis Healthcare) for 48 h in a 24-well plate pre-coated with anti-CD3 (OKT-3) and anti-CD28 antibodies (BD Biosciences). After 2 d of culture PBMCs were harvested and transduced twice with retroviral supernatant. Spinoculation of PBMC was performed with 1 Volume of retroviral supernatant in a 12-well culture non-treated plate (Nunc A/S) pre-coated with retronectin (20 μg/mL, Takara Bio. Inc.). After 2 days, cells were harvested with PBS-EDTA (0.5 mM). Transduced T cells were further expanded using Dynabeads CD3/CD28 as described above.

Mouse xenograft studies

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were bred in-house under an approved institutional animal care protocol and maintained under pathogen-free conditions. 6–8 week old mice were injected in intra-peritoneally (i.p.) with 1–1.5$\times10^6$ HCT 116 tumor cells. The HCT 116 cells were engineered with a retroviral vector (provided by Dr Rainer Löw, EUFETS AG) to express firefly luciferase and EGFP. Tumor growth was monitored by bioluminescent imaging using the Xenogen Spectrum system and Living Image v3.2 software. Anaesthetized mice were injected i.p. with 150 mg/kg body weight of D-luciferin (Caliper Life Sciences). Animals were imaged 10 min after luciferin injection.

Statistical analysis

Continuous data were described with median, mean and range. The Mann–Whitney test was used for analysis of tumor load, while survival was calculated using the Kaplan–Meier method with the unpaired t-test used for comparison of survival between groups. All *p*-values given are 2-tailed values. A *p*-value below 0.05 was considered significant. All statistical analyses were performed using *GraphPad Prism*® (GraphPad Software, Inc.).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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