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Immunotherapy of melanoma : toward clinical application

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Voor mijn ouders,
voor Rutger & Doeke

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The cover photo was taken at the National Pharmaceutical Museum in Gouda, the Netherlands, by Rutger Smit.

Contents

Chapter 1

Introduction

Introduction

Melanoma

Melanoma is a type of skin cancer that arises from melanocytes, cells producing pigments (melanin) responsible for skin and hair color that are found mostly in the skin and eyes. In the skin, melanocytes reside in the basal layer of the epidermis, where they produce melanin in response to ultraviolet (UV) radiation. This way, UV radiation results in tanning of the skin, thereby preventing skin cancer. On the other hand, high doses of UV radiation can disrupt growth control of melanocytes, initially leading to the formation of a naevus or common mole, which are benign. Eventually, these can progress into local invasive lesions or melanoma cells with metastatic potential[1-3].

Melanoma has an increasing incidence: in the past 20 years, the number of cases worldwide has doubled (160,000 in 2002)[4], which is partly accounted for by an increase in sun exposure and severe sunburns[5]. Standard treatment of melanoma is surgical resection, sometimes followed by radiotherapy, and this can cure patients that are diagnosed early. However, progressive metastatic melanoma can not be treated this way, and has a very poor prognosis: median survival of these patients is less than one year, and the 5-year survival rate is less than 10%[6,7]. Hence, new treatment strategies are urgently needed. This thesis will focus on immunotherapy of cancer in general, and of melanoma in particular.

T cells and antigen recognition

T cells play an important role in immune responses against pathogens but also against certain types of cancer, as illustrated by an increased incidence of Karposi sarcoma, non-Hodgkin lymphoma and cervical cancer in immunosuppressed individuals[8]. Recognition of cancer cells is mediated via the T cell receptor (TCR), which consists of a heterodimer of an alpha and beta chain expressed on the surface of the T cell. The TCR of a $CD8^+$ T cell can specifically recognize its cognate antigen when this is bound in the groove of a class I MHC molecule expressed on an antigen presenting cell (APC). When sufficient costimulation is provided by the APC, binding of the peptide-MHC complex leads to activation of naïve T cells, which will then induce cell proliferation and differentiation, generating a large pool of effector cells. Eventually, these effector $CD8^+$ T cells can induce lysis of infected or transformed targets by the release of cytotoxic proteins. Once the source of antigens is cleared, T cells no longer receive the stimulus required for their activation, causing cell death and contraction of the antigen-specific T cell pool. A small population of antigen-specific cells will remain present in the circulation as memory T cells, thereby enabling the organism to respond very efficiently to future encounters with the same antigen.

T cell precursors are generated in the bone marrow and then migrate to the thymus where they develop into mature T lymphocytes. During development, TCRs are generated via rearrangement of alpha and beta chain gene segments, resulting in receptors with an almost infinite range of specificities. Only cells that are able to recognize the body's own MHC molecules receive a signal that allows them to survive (positive selection). On the other hand, cells that bind to self-peptide MHC complexes with high avidity are deleted before they become fully mature (negative selection), thereby preventing self-reactive cells to enter the periphery and cause autoimmunity. Besides this process of central tolerance, T cells can also undergo tolerization in the periphery, ensuring inactivation of cells that have escaped negative

selection in the thymus. If a TCR binds to self antigens in the absence of co-stimulation, this will lead to T cell anergy, a state in which the T cell becomes refractory to activation by the specific antigen. Together, these processes shape a T cell repertoire that is largely unreactive towards self-antigens (self tolerant), but can efficiently respond to foreign antigens.

Melanoma antigens and tumor-specific T cell responses

The basis for antigen-specific immunotherapy was provided by the identification of the first melanoma antigen, MAGE[9]. Soon, the molecular characterization of other antigens followed, leading to a large collection of human tumor antigens recognized by T cells[10]. Melanoma antigens can be divided in four different categories, based on their origin:

- Cancer/testis antigens, such as MAGE[11] and NY-ESO[12], are expressed in male germline cells and in many tumors. Since germline cells do not express HLA molecules, presentation of these antigens is completely restricted to tumor cells, but some form of thymic tolerance might still exist[13,14].
- Unique tumor antigens are strictly tumor specific, and result from somatic point mutations caused for example by chemical agents or radiation[15]. These point mutations will be different for every tumor and every patient, which limits the use of unique tumor antigens for immunotherapy.
- Overexpressed antigens such as PRAME[16], p53[17] and TERT[18] originate from self proteins that are produced in higher levels by the tumor than by normal tissue. These antigens are shared between many tumors, a characteristic that makes them suitable targets for immunotherapy. They are however not tumor specific, so T cells that bind these antigens with high affinity will be deleted in the thymus. More importantly, targeting of these ubiquitously expressed antigens could potentially lead to destruction of vital healthy tissues.
- Melanocyte differentiation antigens are derived from genes that are also expressed in normal melanocytes, such as tyrosinase[19], gp100[20], MART-1 [21,22], TRP-1[23], and TRP-2[24]. Targeting of these antigens can lead to skin depigmentation (vitiligo) and inflammation of the eye (uveitis) due to destruction of melanin producing cells[25], these side effects are however relatively mild and easy to control. Since T cells recognizing melanocyte differentiation antigens regularly escape central tolerance, as will be discussed below, these antigens are often selected as targets in melanoma immunotherapy.

Most melanoma antigens suitable for immunotherapy are non-mutated self antigens, so T cells specific for these antigens are susceptible to negative selection in the thymus. However, this process is incomplete since T cells that bind with low affinity to self peptide/MHC complexes can escape deletion and enter the circulation as mature cells[26-28]. Especially in the case of the differentiation antigen MART-1, high frequencies of specific T cells can be regularly found in the peripheral blood of both healthy individuals (0.07% of $CD8^+$ cells) and melanoma patients (0.1% of $CD8^+$ cells). These frequencies are markedly higher than the estimated frequency of naïve single epitope-specific cells (≤ 0.00001% of $CD8^+$ cells). In tumor-infiltrated lymph nodes, frequencies of MART-1-specific T cells even reached up to 15% of $CD8^+$ cells. Interestingly, a significant fraction of MART-1-specific cells in patients has a memory effector phenotype, as compared to mainly naive cells in

healthy individuals[29-31], indicating that these antigen-specific cells can be activated *in vivo* by the tumor. Spontaneous T cell responses against other melanoma antigens, such as tyrosinase and NY-ESO, also occur but are much less common[32,33].

The presence of melanoma-specific lymphocytes at the site of the tumor has been associated with better prognosis[34-37], although this correlation could not be found for circulating melanoma-specific cells in advanced-stage melanoma patients[38]. Also, spontaneous vitiligo and spontaneous tumor regression occasionally occur in melanoma. Although these data indicate that tolerance towards melanoma self-antigens is not absolute, the vast majority of metastatic melanoma patients have progressive disease despite these responses. Therefore, immunotherapeutic strategies that can enhance anti-melanoma T cell responses seem worthwhile to pursue. This can be achieved by either active immunization, where the patients own naïve T cells are activated via vaccination, or by passive immunization via the infusion of melanoma-specific T cells.

Active vaccination

In addition to the existence of spontaneous (i.e. tumor-induced) responses towards melanoma antigens, the success of vaccines in the prevention and even eradication of viral diseases also provided a rational base for the development of anti-melanoma vaccines[39]. Nowadays, there are many different vaccination strategies, but they are all based on the concept of antigen administration within an immunostimulatory context. The first anti-cancer vaccines consisted of preparations of irradiated tumor cells, which enables the induction of responses with a broad range of specificities. However, the production of these vaccines requires isolation of tumor cells from each individual patient, making broad application difficult. The identification of melanoma antigens led to the development of vaccines consisting of peptide or protein, either directly administered or loaded onto dendritic cells, or consisting of DNA encoding these antigens, either in the form of "naked" DNA or as viral vectors. Peptide, protein and DNA vaccines are relatively easy to produce and administer, enabling production of an "off the shelf" vaccine.

In mouse models, active immunization against tumor self antigens could elicit effective anti-tumor T cell responses, resulting in tumor protection or rejection, in some cases accompanied by autoimmune destruction of melanocytes[40-44]. In humans, vaccination elicited anti-melanoma T cell responses, but frequencies of antigen-specific cells were generally low[45,46]. In line with this, active immunization of melanoma patients seldom resulted in objective tumor regression[47].

Apparently, vaccination can induce expansion of melanoma-specific cells, but these cells are not capable of efficient tumor kill. It seems that rather than becoming fully activated, these T cells become anergic upon encountering self-antigen due to peripheral tolerance mechanisms[33,48-50]. A successful vaccination strategy for the targeting of self antigens would need to break these peripheral tolerance mechanisms in order to result in an effective anti-tumor response.

Chapter 1 describes a new intradermal vaccination method that uses a tattoo device to deliver DNA into the skin, a preferred site for the induction of immune responses due to the abundant presence of antigen presenting cells. Tattoo vaccination results in a faster induction of antigen specific T cell responses compared to conventional intramuscular DNA vaccination. Moreover, this vaccination strategy could efficiently target established tumors in

mice and provide protection against infection with influenza A. In chapter 2, DNA tattoo vaccination is combined with sublethal irradiation of recipients and adoptive transfer, a strategy that results in marked skewing of the T cell repertoire towards tumor recognition, and in a more pronounced anti-tumor effect compared to DNA vaccination alone. In both chapters a viral epitope is used as model antigen, whereas the feasibility of targeting self antigens via this vaccination strategy has not yet been assessed. Since T cells with a high avidity for self antigens are deleted from the endogenous repertoire, active immunization is likely to be a suboptimal strategy when targeting these antigens.

Passive immunization

In cases where the available T cell repertoire is strongly affected by tolerance mechanisms, passive immunization by adoptive transfer of tumor-specific cells enables the infusion of T cells that are not affected by tolerance. Furthermore, these cells can be injected in high numbers, and in an activated state.

The feasibility of immunotherapy using antigen-specific T cell populations was first demonstrated by the adoptive transfer of allogeneic T cells to immunocompromised patients. In these patients, infusion of CMV-specific clones or EBV-specific T cell lines could restore immunity against CMV and EBV, and prevent the development of virus-related disease[51,52]. In melanoma, the infusion of MART-1 or gp100-specific T cell clones demonstrated that it was feasible to isolate tumor-specific T cells from peripheral blood or tumor biopsies, but clinical effects were low or absent[53-56].

Great progress was made when the infusion of a polyclonal population of autologous tumor infiltrating lymphocytes (TIL) was combined with lymphodepleting chemotherapy and administration of high dose IL-2. In this strategy, the removal of regulatory T cells and endogenous cells competing for proliferative cytokines enabled repopulation of the patient's immune system with tumor-specific T cells. Treatment of melanoma patients with metastasized disease resulted in objective tumor regression in more than 50% of patients. Destruction of normal melanocytes was also detected in most of these patients, as demonstrated by the development of vitiligo and uveitis[25,57].

Although this was the first study to demonstrate the potency of adoptive immunotherapy in advanced-stage melanoma patients, it is not without limitations. One problem is the ex vivo expansion of T-cells to very large numbers (10^9-10^{10} T cells), which is limited by the maximal replicative life span of cells, especially in the case of T cell clones. Chapter 3 describes a strategy that extends the life span of human T cells by introducing the human telomerase reverse transcriptase (hTERT) gene. These hTERT-transduced cells can be cultured far beyond the number of population doublings observed for wild type cells, while maintaining their anti-tumor functionality in vivo.

A more fundamental constraint of the adoptive transfer of antitumor lymphocytes is caused by the difficulty of isolating suitable T cells. In fact, in more than 50% of the melanoma patients, sufficient tumor reactive T cells can not be generated at all[58], reducing the overall success rate of the TIL trial. Furthermore, tumor reactive cells are totally absent in many other cancer types, limiting general application of this treatment strategy.

Instead of transferring T cells upon isolation and ex vivo expansion, the transfer of T cell receptor (TCR) genes into autologous T cells can be used to generate large numbers of

tumor reactive lymphocytes in a relatively simple manner. Dembic et al. were the first to show that it is indeed possible to generate antigen-specific cells by the transfer of alpha and beta TCR genes[59]. A number of in vitro studies subsequently demonstrated the transfer of virus or tumor specificity into human T cells lines or primary lymphocytes[60-64]. In vivo, the adoptive transfer of TCR-modified cells demonstrated that these cells could expand upon viral vaccination and were capable of inducing regression of a tumor expressing a viral antigen[65]. Furthermore, TCR modified cells could also induce a substantial anti-tumor effect in a setting where they were targeted to a self antigen, indicating that TCR gene transfer can circumvent tolerance[66]. Recently, the first clinical trial of TCR gene transfer demonstrated that T cells modified with a MART-1-specific T cell receptor persisted in the peripheral blood of melanoma patients for more that 2 months[67]. However the clinical response rate of this trial was disappointingly low (2/18), suggesting that there is room for improvement.

In chapter 4 we use the RIP-OVA mouse model to elucidate the requirements for efficient targeting of self antigens by TCR modified cells. Factors such as host conditioning regimen, the format of the introduced TCR genes, and the properties of the infused T cell graft have a marked effect on the anti-tumor efficacy of TCR modified cells, indicating that these factors should be taken into consideration when designing clinical TCR gene therapy trials. Chapter 5 shows that gene-modification of melanoma-reactive TCRs can markedly enhance TCR expression and in vivo functionality. This chapter also describes a procedure for the selection of a human TCR that is well expressed and highly affine, and that is therefore likely to enable more efficient targeting of human melanoma. The approaches developed in chapter 4 and the TCR identified and optimized in chapter 5 can improve the anti-tumor activity of TCR modified cells. Implementation of these factors in the design of clinical trials is therefore likely to positively affect the clinical efficacy of TCR gene therapy.

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Chapter 2

A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression

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A rapid and potent DNA vaccination strategy defined by *in vivo* monitoring of antigen expression

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Induction of immunity after DNA vaccination is generally considered a slow process. Here we show that DNA delivery to the skin results in a highly transient pulse of antigen expression. Based on this information, we developed a new rapid and potent intradermal DNA vaccination method. By short-interval intradermal DNA delivery, robust T-cell responses, of a magnitude sufficient to reject established subcutaneous tumors, are generated within 12 d. Moreover, this vaccination strategy confers protecting humoral immunity against influenza A infection within 2 weeks after the start of vaccination. The strength and speed of this newly developed strategy will be beneficial in situations in which immunity is required in the shortest possible time.

Over the past decade, DNA vaccines have emerged as a promising approach for the induction of immune responses. Generally, current DNA vaccination strategies use a regimen of multiple intramuscular or intradermal administrations, at intervals of 2 weeks or more, and require at least 1 month to achieve immunity[1–6]. The slow development of T-cell responses after DNA vaccination contrasts sharply with immune responses induced by a physiological antigen encounter, such as viral infection, that build up rapidly and often peak within 10 d[7]. The reason for this slow development of immune responses has remained unclear. It has been postulated that DNA vaccination leads to transfection of few cells and to the expression of relatively small amounts of antigen[8,9], therefore requiring a time-consuming prime-boost strategy. But direct evidence for the proposed low antigen expression is scarce and its role in immune induction has not been addressed. To examine whether the slow induction of immune responses could be causally related to the kinetics of antigen expression induced by DNA vaccines, we analyzed *in vivo* levels of antigen expression after intramuscular and intradermal DNA delivery, and correlated these with the induction of T-cell immunity. Based on these data, we developed a short-interval DNA vaccination method that generates functional T- and B-cell responses within a minimal time frame.

RESULTS

Intradermal DNA vaccination by skin tattooing

To be able to administer DNA to the skin in a controlled manner, over a large surface and only in the upper nonvascularized layers, we made use of a simple tattoo device[10] for intradermal DNA delivery. Histochemical analysis of 'DNA-tattooed' skin showed that transfected cells were distributed over the upper layers of the dermis and the epidermis. (**Fig. 1a**,**b**). To test the immunogenicity of this method, we vaccinated two groups of mice with a DNA vaccine encoding the influenza A nucleoprotein epitope (amino acids 366–374; NP_{366}) fused to the carboxy terminus of a tetanus toxin fragment (d1TTFC-NP)[11], either by intramuscular injection or by skin tattoo, following the conventional DNA vaccination regimen of three administrations at 2-week intervals. This comparison shows that the intradermal delivery of a DNA vaccine using a tattoo device is an efficient strategy for the induction of T-cell immunity (**Fig. 1c**).

Imaging of *in vivo* antigen expression

To monitor antigen expression upon DNA vaccination, we constructed a plasmid encoding the NP_{366} epitope fused to the carboxy terminus of firefly luciferase (Luc-NP). The Luc-NP vaccine elicits potent NP_{366}-specific T-cell responses (**Fig. 1d**). After tattoo or intramuscular administration of the Luc-NP vaccine, we used a light-sensitive camera to determine longitudinal *in vivo* antigen expression. Notably, a single intramuscular injection of DNA resulted in high levels of luciferase activity, peaking after 1 week and remaining detectable up to 1 month after injection. The antigen expression kinetics induced by DNA tattooing were markedly different. First, peak values of antigen expression were at least ten times lower. Second, luciferase activity in the skin peaked after 6 h (data not shown) and disappeared over the next 4 d (**Fig. 1e**).

We subsequently determined the capacity of both methods to present the vaccine-encoded NP_{366} epitope to naive, lymph node–resident T cells. For this purpose, 5 million carboxy-fluorescein diacetate succinimidyl ester (CSFE)-labeled splenocytes from F5 TCR transgenic mice[12] were injected into mice at different time points after a single intramuscular DNA injection or DNA intradermal tattoo. Luciferase

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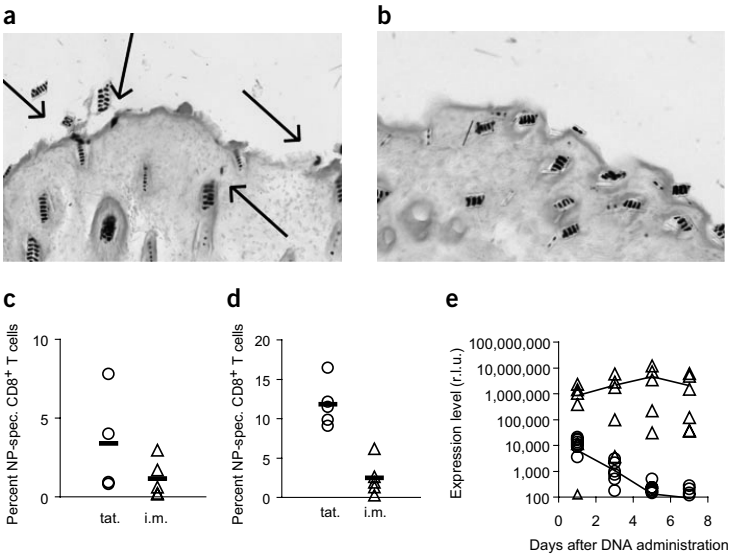

Figure 1 DNA tattooing results in transfection of epidermal and dermal cells and leads to efficient T-cell induction. (**a**) The abdominal skin of a mouse was tattooed with a β-galactosidase (*lacZ*)-encoding construct. Six hours after treatment, transfected cells were shown in a skin biopsy, using the X-gal substrate to generate a blue precipitate in cells expressing the transgene (arrows). (**b**) The abdominal skin of a mouse tattooed with empty vector and processed as in **a**. (**c**) NP_{366}-specific $CD8^+$ T-cell responses induced by tattoo (tat., circles, $n = 5$) or intramuscular (i.m., triangles, $n = 5$) DNA vaccination. The d1TTFC-NP DNA vaccine was administered three times at 2-week intervals. T-cell responses were measured 7 d after the last DNA administration, by staining peripheral blood lymphocytes with H-$2D^b$/$NP_{366–374}$ tetramers. Horizontal bars depict averages. (**d**) NP_{366}-specific T-cell responses induced by tattoo DNA vaccination (circles, $n = 5$) and i.m. DNA vaccination (triangles, $n = 5$) with the Luc-NP DNA vaccine. (**e**) Kinetics of Luc-NP antigen expression after a single intradermal DNA tattoo (circle) and after a single intramuscular DNA injection (triangle).

activity was measured on the day of cell transfer, and 3 d later the animals were killed, lymphoid organs excised and the CFSE signal of the F5 cells assessed by flow cytometry. These experiments showed that although antigen production is markedly greater upon intramuscular delivery, presentation of this antigen to naive T cells is markedly more efficient upon intradermal DNA delivery. Specifically, in spite of the high antigen expression in the muscle after intramuscular injection, the fraction of F5 cells in the draining lymph node that had undergone proliferation was marginal in all of the three tested time windows (days 1–4, days 8–11 and days 22–25; **Fig. 2a–c**). In marked contrast, the priming of F5 T cells after tattooing was very efficient (**Fig. 2a**). Independent of the number of F5 T cells that were infused (5 million or 1 million; latter not shown), over 80% of the draining lymph node–resident F5 cells had lost CFSE signal on day 4, despite the low antigen level expressed in skin (**Fig. 2d**). At later time points, NP_{366}-specific T-cell activation became substantially less (**Fig. 2b**,**c**), consistent with the loss of luciferase signal observed by *in vivo* imaging. Similar results were obtained with spleen-resident F5 cells. No F5 T-cell division could be detected in nondraining lymph nodes (data not shown).

Induction of T-cell immunity by short-interval DNA tattooing

Based on the transient antigen expression and presentation induced by DNA tattooing as compared to intramuscular injection, we speculated that in the case of dermal DNA vaccination, shortening of the conventional 2-week interval could result in faster T-cell induction. To test this hypothesis, we reduced the interval between consecutive vaccinations to 3 d, resulting in a day 0, 3 and 6 regimen. Notably, this compact vaccination protocol induced profound T-cell responses of 4–8% of total $CD8^+$ T cells within 12 d after the start of vaccination (**Fig. 3a**). This regimen

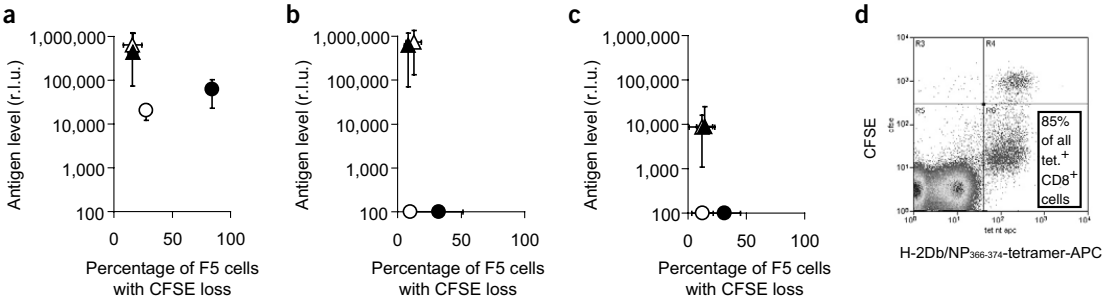

Figure 2 Analysis of antigen production and presentation after intradermal and intramuscular DNA vaccination. (**a**–**c**) Cohorts of mice ($n = 7$) were vaccinated once with the Luc-NP vaccine, either by intradermal DNA tattooing (filled circles) or by intramuscular DNA injection (filled triangles). Control groups ($n = 3$) received a plasmid encoding luciferase only (intradermal, open circles; intramuscular, open triangles). CFSE-labeled naive F5 T cells were transferred on day 1 (**a**), day 8 (**b**) or day 22 (**c**) after DNA administration, and were recovered from the draining lymph nodes 3 d after transfer. Proliferation was assessed by analysis of CFSE loss of H-$2D^b$/$NP_{366–374}$tetramer–positive $CD8^+$ cells. (**d**) Representative draining lymph node sample from the tattooed cohort on day 4.

of short-interval vaccination does not lead to detectable T-cell responses when the vaccine is administered intramuscularly, consistent with the observation that the duration of antigen expression is not a limiting factor in intramuscular DNA vaccination.

To test the value of this new vaccination regimen for other methods of intradermal DNA delivery, we compared T-cell induction by short-interval gene-gun and short-interval tattoo vaccination, using the previously described Hsp70-HPV-E7 DNA vaccine[13]. Using the short-interval regimen, both methods generated strong T-cell responses within 12 d against the immunodominant human papillomavirus (HPV) $E7_{49–57}$ cytotoxic T lymphocyte (CTL) epitope ($E7_{49}$) (**Fig. 3b**). Comparison of T-cell responses induced by short-interval DNA tattooing with other previously established vaccination strategies showed that short-interval DNA tattooing yields markedly higher T-cell responses (tenfold or more) than peptide–incomplete Freund adjuvant–CD40-specific monoclonal antibody[14] or peptide-synthetic CpG oligodeoxynucleotide[15] vaccines (**Fig. 3c**). Furthermore, T-cell induction is efficient for both internal epitopes and those fused at the carboxy terminus (**Supplementary Fig. 1** online). The resulting T cells are capable of direct effector function, as indicated by antigen-induced interferon (IFN)-γ production (**Supplementary Fig. 2** online).

Repetitive application of DNA may conceivably boost T-cell responses by enhancing the absolute amount of antigen expression, or by prolonging the duration of antigen expression. To address this issue, we compared antigen expression levels and T-cell responses induced by three consecutive 4 s tattoos with those induced by one single 16 s tattoo. The cumulative antigen produced by one 16 s application exceeds that of three 4 s applications (**Fig. 3d**). Whereas three consecutive 4 s applications induce a T-cell response that is readily detectable, a single 16 s application is essentially without effect (**Fig. 3e**). To further assess whether the observed requirement for repetition reflects a need for continued antigen presence, or is related to the prolongation of non-specific inflammatory signals, groups of mice were vaccinated at day 0, 3 and 6 with different combinations of a mock vaccine and the Luc-NP vaccine. Replacing either the first or the last Luc-NP vaccine with a mock vaccine lead to a significant drop in the size of the ensuing T-cell response (Student *t*-test, $P = 0.002$ and $P = 0.003$, respectively; **Fig. 3f**). Together these results show that for the induction of primary T-cell responses by intradermal DNA vaccination, prolonged antigen expression is crucial.

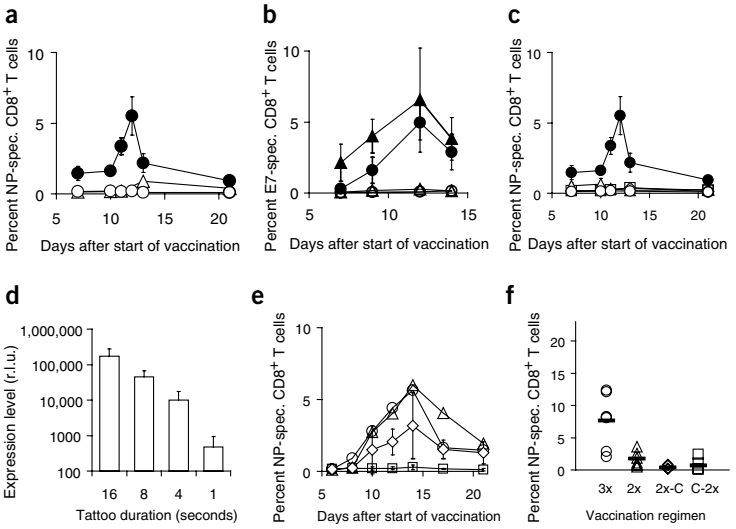

Figure 3 Features of tattoo DNA vaccination. (**a**) NP_{366}-specific T-cell responses in cohorts of mice ($n = 5$) upon vaccination with d1TTFC-NP on day 0, 3 and 6 either by tattoo (filled circles) or intramuscular injection (open triangles). Control mice (open circles) were tattooed with a TTFC mock vaccine. NP_{366}-specific T-cell responses were determined at indicated time points by tetramer staining of peripheral blood lymphocytes ($n = 5$). (**b**) The $E7_{49}$-specific T-cell response after intradermal application of the sigE7hsp DNA vaccine at day 0, 3 and 6 in cohorts of mice ($n = 6$), either by DNA tattooing on the left leg (filled circles) or by gene gun on both flanks of the abdomen (filled triangles). Control groups of mice ($n = 3$) were vaccinated with empty vector (open triangles and open circles). (**c**) NP_{366}-specific T-cell responses in cohorts of mice ($n = 5$) upon subcutaneous vaccination with the NP_{366} peptide in incomplete Freund adjuvant at day 0 (open triangles), subcutaneous injection of NP_{366} peptide with CpG in PBS at day 0 (open circles) or at day 0, 3 and 6 (open squares), NP_{366} peptide with CpG in PBS tattoo vaccination at day 0, 3 and 6 (open diamonds) and DNA tattoo vaccination with d1TTFC-NP on day 0, 3 and 6 (filled circles). (**d**) Antigen expression level after single tattoos of 16, 8, 4 and 1 s, as determined by a light-sensitive camera 1 d after tattooing with the Luc-NP vaccine. (**e**) NP_{366}-specific T-cell responses ($n = 5$) induced by 16 s (open circles), 8 s (open triangles) and 4 s (open diamonds) tattoos on day 0, 3 and 6, or a single 16 s tattoo on day 0 (open squares). (**f**) In mice vaccinated with the Luc-NP DNA vaccine at day 0, 3 and 6, the replacement of Luc-NP with a mock DNA vaccine on day 0 (open squares; C-2x) or day 6 (open diamonds; 2x-C) reduces T-cell responses from levels induced by three consecutive tattoos (open circles; 3x), to levels induced by two consecutive tattoos (open triangles; 2x).

Functional immunity induced by short-interval DNA tattooing

To assess the ability of short-interval tattoo DNA vaccination to induce therapeutic amounts of tumor-specific T cells, we used the transplantable HPV E6/E7-transformed TC-1 tumor cell model[16]. Three days after subcutaneous injection of 10^5 TC-1 cells, we vaccinated B6 mice using the short-interval tattoo method with a plasmid encoding $E7_{49}$ attached to the carboxy terminus of green fluorescent protein (GFP-E7; **Fig. 4a**). Control mice were either given an intramuscular injection in the same regimen with the same plasmid (**Fig. 4b**), or were tattooed with a control plasmid encoding green fluorescent protein (GFP) only (**Fig. 4c**). In both control groups, no T-cell responses could be detected after vaccination. In contrast, mice that had been tattooed with GFP-E7 mounted a sizeable T-cell response, up to 15% of the circulating $CD8^+$ T-cell pool. Notably, the onset of $E7_{49}$-specific T-cell responses coincided with rejection of established subcutaneous tumors, whereas in both control groups tumors grew out rapidly (**Fig. 4d**). Furthermore, median survival of GFP-E7–tattooed animals was 50 d compared to 17 d in control animals (**Fig. 4e**).

To assess the value of short-interval DNA vaccination in providing antibody-mediated protection against acute infections, we evaluated its ability to confer protection against influenza A virus infection. Given that protection against reinfection with homotypic influenza A strains is primarily mediated by antibodies[17–20], we generated a plasmid that

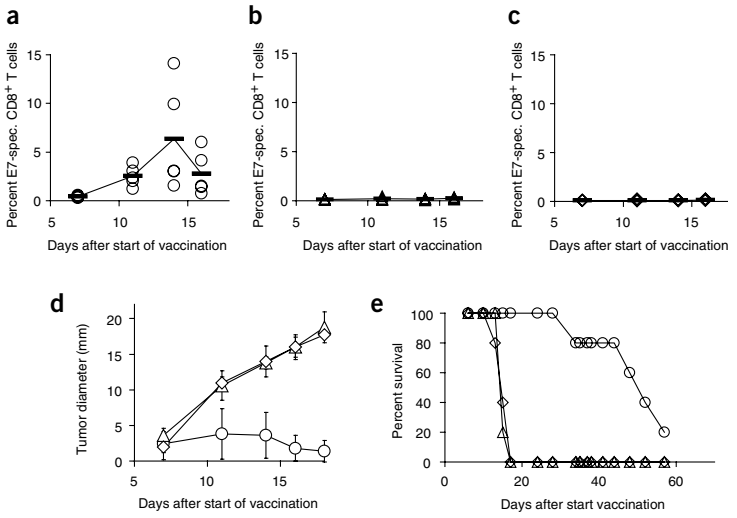

Figure 4 Tattoo DNA vaccination leads to rapid induction of functional tumor-specific T cells. (**a**–**c**) Mice were injected subcutaneously with 10^5 TC-1 cells. Three days later, mice were vaccinated three times at 3-d intervals either by GFP-E7 tattooing (open circles; **a**), by intramuscular injection (open triangles; **b**), or by tattooing with a mock vaccine (open diamonds; **c**). $E7_{49}$-specific $CD8^+$ T-cell responses were determined by major histocompatibility complex tetramer staining of peripheral blood cells at indicated time points. Horizontal bars depict averages. (**d**) Tumor outgrowth in the cohorts of mice depicted in **a**–**c**; symbols are as in **a**–**c**. (**e**) Long-term survival of the mice depicted in **a**–**c**; symbols are as in **a**–**c**. Values represent mean ± s.d.

Fig. 3 online). Collectively, these experiments indicate that short-interval intradermal DNA vaccination leads to the rapid and sustained development of both T- and B-cell responses and that such responses can mediate the regression of established tumors and can prevent virus-induced morbidity.

encodes the gene for hemagglutinin of influenza A/HK/2/68, a protein that forms a major target for neutralizing antibodies. Using this DNA vaccine, mice were vaccinated by short-interval DNA tattooing, whereas control groups were either tattooed with a GFP-encoding plasmid, or given intramuscular injection with the hemagglutinin-encoding DNA vaccine. Two weeks after vaccination, we intranasally infected mice with a sublethal dose of influenza A, and determined virus-induced morbidity by measuring body weight loss[1]. In the week after infection, mice that had previously been exposed to influenza A virus showed a minimal weight loss (1% at day 4 after infection). In contrast, mice given tattoo vaccination with the control plasmid or given an intramuscular injection of the hemagglutinin construct showed a sizable (10% and 14%, respectively) drop in body weight. Notably, mice that had received a short-interval hemagglutinin tattoo were largely protected from influenza A–induced morbidity (maximal weight loss of 4%; Student *t*-test, $P = 0.039$ versus control vaccine; **Fig. 5a**). The protection correlated with the induction of neutralizing antibodies (**Fig. 5b**). Furthermore, intradermal DNA tattooing also conferred long-term (4 months) protection (**Supplementary**

DISCUSSION

In this study we established a short-interval regimen that induces T- and B-cell immunity within 12 d. Analysis of antigen expression levels showed that after intradermal tattoo, 1/10 to 1/100 of the amount of antigen is produced than after intramuscular injection, and that compared to intramuscular administration, tattoo-induced antigen production occurs over a limited timespan. In spite of this, the presentation of the vaccine-encoded epitope is markedly better upon intradermal tattoo vaccination. The efficiency with which dermally expressed antigens are presented to T cells probably results from the high numbers of antigen-presenting cells (APCs) present in this tissue. Also, the infliction of thousands of perforations could conceivably serve as a potent adjuvant.

Contrary to murine skin dendritic cells (DCs), human skin DCs do not express Toll-like receptor 9 (TLR9), and this may partly explain why DNA vaccines have performed poorly in human trials as compared to mouse model systems[21,22]. Encouragingly, a comparison of the immunogenicity of tattoo DNA vaccination in wild-type and

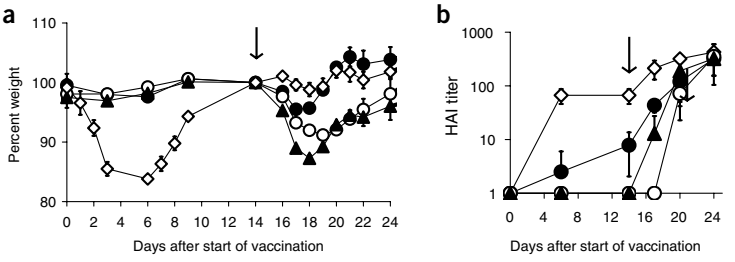

Figure 5 Tattoo DNA vaccination induces B-cell immunity against influenza A within 2 weeks. (**a**) Cohorts of Balb/c mice ($n = 6$) were vaccinated at day 0, 3 and 6 either by intramuscular injection (filled triangles) or by tattooing (filled circles) with a construct encoding hemagglutinin. A positive control group was infected intranasally with influenza (open diamonds) and a negative control group tattooed using a mock vaccine (open circles). All mice were challenged with a sublethal dose of the influenza A virus at day 14 (indicated by the arrow), and weighed daily. The hemagglutinin-tattooed mice showed a significantly smaller drop in weight than the mock-tattooed mice (Student *t*-test, $P = 0.039$). Both in the mock-vaccinated and the intramuscularly vaccinated group, one mouse died from influenza-induced pneumonia. These animals were not included in the analysis. Values represent the weight relative to the weight at time of challenge. Error bars, s.e.m. (**b**) Influenza A/HK/2/68-specific antibodies in the sera of the mice depicted in **a**, at various time points after the start of vaccination and the influenza A challenge (symbols as in **a**). The presence of antibodies was assessed in an HAI assay. Values represent mean ± s.d.

Tlr9$^{-/-}$ mice showed that the absence of TLR9 does not measurably affect the immunogenicity of this method (**Supplementary Fig. 4** online).

The short-interval repetitive schedule developed here leads to rapid induction of T-cell responses upon either intradermal tattoo or gene-gun vaccination, and may well work for all intradermal vaccination techniques. Because of its simplicity and because the required equipment is orders of magnitude less expensive, DNA tattooing may become the preferred method, in particular in countries with a less developed healthcare system.

In the past years, outbreaks of Ebola virus, severe acute respiratory syndrome and influenza A have been causes for concern. To contain such outbreaks conventional DNA vaccination strategies appear too slow at inducing immunity[23,24]. The vaccination regimen described here retains the rapid production and the safety of DNA vaccines, but gains the speed of immune induction that is the characteristic of physiological antigen encounters.

METHODS

Animals. We obtained C57BL/6 mice and Balb/c mice from the experimental animal department of The Netherlands Cancer Institute. The *Tlr9*$^{-/-}$ mice were a gift of H. Wagner ((with permission of S. Akira) Institute for Microbiology, Immunology and Hygiene, University of Munich, Germany). We purchased from the US National Cancer Institute the C57BL/6 mice used for the experiments involving a gene gun and kept them in the oncology animal facility of the Johns Hopkins Hospital. We performed all animal procedures according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the Dutch Animal Research Committee.

DNA vaccines. We generated DNA vaccines by the introduction of fusion genes in pcDNA3.1. The GFP-E7 fusion gene has been described previously[25], all other constructs were generated following a similar design. We included the four naturally flanking amino acid residues of $NP_{366–374}$ (amino acids GVQI) as a linker at the amino terminus of each epitope in all constructs. We constructed the d1TTFC-NP DNA vaccine with optimized codon usage, in a template-free PCR using overlapping oligonucleotides of 100 bp, spanning the entire first domain of the TTFC gene and the preceding p2 epitope (amino acids 831–1,315; ref. 26). We generated the Luc-NP vaccine, encoding influenza A $NP_{366–374}$ epitope fused to the carboxy terminus of firefly luciferase, by genetic linkage of the $NP_{366–374}$ fragment to the carboxy terminus of the gene encoding full-length firefly luciferase. The gene encoding influenza A/HK/2/68 hemagglutinin was obtained by RT-PCR of RNA isolated from virus particles, and inserted in pcDNA3.1. to generate the hemagglutinin DNA vaccine. We purchased the construct used for histochemical detection of transfected cells (pVAX:LacZ) from Invitrogen. Replacement of the *lacZ* gene with chicken ovalbumin generated the construct encoding whole ovalbumin. The generation of the sigE7hsp DNA vaccine was described previously[13]. Sequences were confirmed by sequence analysis. All DNA batches were purified using EndoFree Plasmid kit (Qiagen).

DNA vaccination. For intramuscular DNA vaccination, we shaved the hind leg of the mouse and injected it with 100 μg of DNA in 50 μl HBSS (Life Technologies). For intradermal DNA vaccination, we shaved the left hind leg of the mouse, applied a droplet of 20 μg DNA in 10 μl HBSS to the skin and used a sterile disposable 11-needle bar (Radical Clean Magnum11, Eurl Toupera) mounted on a rotary tattoo device (Cold skin, B&A Trading) to apply the vaccine. Needle depth was adjusted to 0.5 mm, and the needle bar oscillated at 100 Hz. DNA vaccines were applied to the skin by a 16 s tattoo. Gene-gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad) according to the manufacturer's protocol. At various time points after immunization, we drew approximately 20 μl of peripheral blood for analysis of T-cell responses.

Detection of NP- and HPV-specific T cells in peripheral blood. Peripheral blood lymphocytes were stained with phycoerythrin-conjugated antibody to CD8β (BD Pharmingen) plus allophycocyanin-conjugated H-$2D^b$/$NP_{366–374}$-tetramers or allophycocyanin-conjugated H-$2D^b$/$E7_{49–47}$ tetramers, at 20 °C for 15 min in FACS buffer (1× PBS, 0.5% BSA and 0.02% sodium azide). We washed cells three times in PBA and analyzed them using flow cytometry. Live cells were selected based on 7-AAD exclusion. We performed IFN-γ assays using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. Peripheral blood lymphocytes were stimulated for 4 h at a 100 nM peptide concentration.

Intravital imaging. We anesthetized mice with isofluorane (Abbott Laboratories). We intraperitoneally injected an aqueous solution of the substrate luciferin (150 mg/kg, Xenogen) and 18 min later the luminescence produced by active luciferase was acquired during 30 s in an IVIS system100 CCD camera (Xenogen). Signal intensity was quantified as the sum of all detected light within the region of interest, after subtraction of background luminescence.

Influenza A infection and detection of influenza A–specific antibodies. Purified influenza A/HK/2/68 virus was provided by G. Rimmelzwaan (Department of Virology, Erasmus University). We infected mice intranasally with 20 hemagglutinating units (HAU) of virus in PBS, under general anesthesia. Influenza A/HK/2/68-specific antibodies were detected in a hemagglutination inhibition (HAI) assay. Serum samples (50 μl) were serially 1:2 diluted with PBS (1% BSA) in round-bottomed polystyrene microtiter plates, in duplicate, and 50 μl of A/HK/2/68 influenza virus corresponding to 4 HAU was added to each well. After 45 min incubation at room temperature, we added 50 μl of 0.5% chicken red blood cells to each well. After 1 h, we determined the maximal serum dilution that fully inhibited the hemagglutination caused by the virus in the duplicate wells. The HAI titer was expressed as the reciprocal of this serum dilution.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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Chapter 3

Skewing the T-cell repertoire by combined DNA vaccination, host conditioning, and adoptive transfer

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Skewing the T-Cell Repertoire by Combined DNA Vaccination, Host Conditioning, and Adoptive Transfer

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Abstract

Approaches for T-cell–based immunotherapy that have shown substantial effects in clinical trials are generally based on the adoptive transfer of high numbers of antigen-specific cells, and the success of these approaches is thought to rely on the high magnitude of the tumor-specific T-cell responses that are induced. In this study, we aimed to develop strategies that also yield a T-cell repertoire that is highly skewed toward tumor recognition but do not rely on *ex vivo* generation of tumor-specific T cells. To this end, the tumor-specific T-cell repertoire was first expanded by DNA vaccination and then infused into irradiated recipients. Subsequent vaccination of the recipient mice with the same antigen resulted in peak $CD8^+$ T-cell responses of ~50%. These high T-cell responses required the presence of antigen-experienced tumor-specific T cells within the graft because only mice that received cells of previously vaccinated donor mice developed effective responses. Tumor-bearing mice treated with this combined therapy showed a significant delay in tumor outgrowth, compared with mice treated by irradiation or vaccination alone. Furthermore, this antitumor effect was accompanied by an increased accumulation of activated and antigen-specific T cells within the tumor. In summary, the combination of DNA vaccination with host conditioning and adoptive transfer generates a marked, but transient, skewing of the T-cell repertoire toward tumor recognition. This strategy does not require *ex vivo* expansion of cells to generate effective antitumor immunity and may therefore easily be translated to clinical application. [Cancer Res 2008;68(7):2455–62]

Introduction

Virus-induced tumors, such as human papillomavirus (HPV)-induced cervical carcinoma, express foreign antigens that are potential targets for immunotherapy. Unfortunately, clinical trials that assess the efficacy of therapeutic vaccination against HPV oncoproteins E6 and E7 have shown only a limited therapeutic benefit for cervical cancer patients to date (1–5). Furthermore, although vaccine-induced T-cell responses against E6 and E7 are often observed, these responses generally are of limited magnitude, and it may be argued that a substantial enhancement of vaccine-induced T-cell responses is required to improve clinical efficacy.

In line with the notion that the development of marked tumor-specific T-cell responses may be essential, a number of successful T-cell–based immunotherapy trials have been performed that involved the adoptive transfer of high numbers of virus- or tumor-specific T cells. Specifically, infusion of virus-specific T-cell clones has been used for the prophylaxis and treatment of cytomegalovirus- and EBV-associated diseases after hematopoietic stem cell transplantation (6–8). Furthermore, objective cancer regression in patients with metastatic melanoma was accomplished after nonmyeloablative chemotherapy and adoptive transfer of *ex vivo* expanded tumor-infiltrating lymphocytes (TIL; refs. 9, 10). In these latter studies, T-cell infusion is performed subsequent to the administration of lymphodepleting chemotherapy, and this host conditioning regimen is thought to be essential to allow efficient engraftment of the infused cells. Importantly, the general application of these strategies for the treatment of cancer patients is hampered by the difficulty of expanding sufficient numbers of tumor-reactive T cells *ex vivo*. Our aim in this study was therefore to develop an immunotherapeutic strategy that results in a marked skewing of the T-cell repertoire toward tumor reactivity and that is solely based on a combination of vaccination and host conditioning.

We recently described a vaccination method that uses a high frequency tattoo device to deliver DNA vaccines to the epidermis, a preferred site for the induction of immune responses due to the abundant presence of antigen-presenting cells. In mice, this vaccination strategy generates robust T-cell responses within 2 weeks, eliciting effective immunity toward established HPV-transformed tumors (11). Also, in a nonhuman primate model, DNA tattoo vaccination is highly effective and induces $CD8^+$ T-cell responses that are superior to those obtained upon i.m. DNA vaccination.[1]

To test whether an effective DNA vaccination strategy can be used to achieve a substantial skewing of the T-cell repertoire toward tumor recognition without a requirement for *ex vivo* T-cell expansion, we combined DNA tattoo vaccination with irradiation-induced host conditioning and adoptive transfer. Prior studies that combine host conditioning regimens and tumor cell vaccination have shown that such a combined strategy could protect mice against subsequent tumor challenge and induce regression of established metastases (12, 13). However, as these studies did not assess the effect of host conditioning on vaccine-induced T-cell responses, it is difficult to ascertain whether a substantial skewing of the T-cell repertoire was achieved and if this was responsible for the induction of antitumor immunity.

In the current study, we address this issue and show that the combination of DNA vaccination, host conditioning, and adoptive

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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[1] Verstrepen B, Bins A, Rollier C, Mooij P, Koopman G, Sheppard N, Sattentau Q, Wagner R, Wolf H, Schumacher T, Heeney J, Haanen J. Improved HIV-1 specific T-cell responses by DNA tattooing as compared to intramuscular immunization in non-human primates. Submitted for publication.

transfer results in a strong skewing of the T cell repertoire toward tumor recognition. This combined therapy resulted in a significant growth delay of established tumors and was associated with a markedly increased accumulation of tumor-specific and IFNγ-producing T cells at the site of the tumor. This strategy may easily be translated to a clinical setting and is therefore an attractive method for the enhancement of vaccine-induced immune responses.

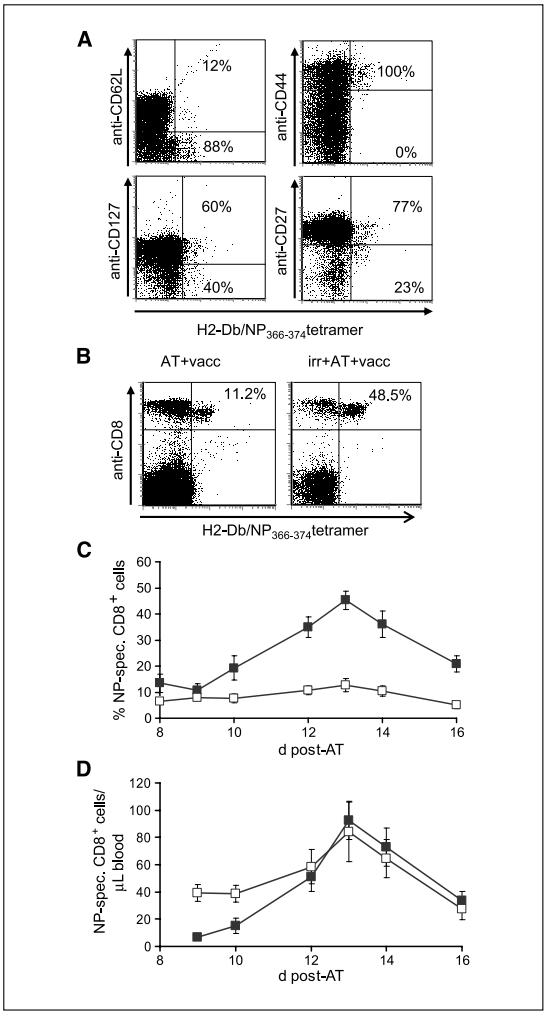

Figure 1. Lymphodepletion strongly enhances vaccine-induced T-cell responses. *A,* donor mice were vaccinated with a DNA vaccine encoding the NP_{366} epitope on day -14, -11, and -8. On day 0, splenocytes were isolated and analyzed by flow cytometry. Numbers in the top and bottom right corners represent the percentage of marker$^+$ cells and marker$^-$ cells of D^b-NP_{366}-tetramer$^+$ CD8$^+$ cells, respectively. *B* to *D,* on day 0, recipient mice were either irradiated or left untreated, and, subsequently, the mice received an adoptive transfer (*AT*) of 3×10^7 splenocytes from previously vaccinated donors. On day 0, 3 and 6, the mice were vaccinated with a DNA vaccine encoding the NP_{366} epitope. *B,* representative flow cytometry plots of peripheral blood samples of nonirradiated (*left*) or irradiated (*right*) mice at day 13 postadoptive transfer. *C* and *D,* peripheral blood of irradiated (■) or nonirradiated (□) mice was collected at different time points after adoptive transfer, and the percentage (*C*) and absolute number (*D*) of D^b-NP_{366}-tetramer$^+$ CD8$^+$ cells were determined by flow cytometry analysis. *Points,* mean (n = 5); *bars,* SE. *NP-spec. CD8$^+$ cells,* NP-specific CD8$^+$ cells.

Materials and Methods

Mice and cell lines. Female C57Bl/6 mice were obtained from the experimental animal department of The Netherlands Cancer Institute. Mice were housed under specific pathogen-free conditions and used at ages 6 to 12 wk. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the local Dutch Animal Research Committee.

B16 (H-2^b haplotype) is a spontaneous murine melanoma obtained from the National Cancer Institute tumor repository. The $B16^{NP}$ tumor cell line was obtained by transduction of B16 cells with a retrovirus encoding the influenza A $NP_{366-374}$ epitope as a COOH-terminal fusion with the enhanced green fluorescent gene product (14). The $B16^{NP}$ tumor cell line was maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum (PAA Laboratories GmbH), 100 μg/mL streptomycin, and 100 μg/mL penicillin.

DNA vaccination. The luc-NP DNA vaccine was generated as described previously (11). In brief, the influenza A $NP_{366-374}$ epitope was genetically fused to the carboxy terminus of the firefly luciferase gene and cloned into pcDNA3.1. All DNA batches were purified using EndoFree Plasmid kit (Qiagen).

For intradermal DNA vaccination, a droplet of 20 μg DNA in 10 μL endotoxin-free TE buffer (Qiagen) was applied on the shaven hind leg of a mouse. A sterile disposable 11-needle bar (Radical Clean Magnum11; Earl Toupera) mounted on a rotary tattoo device (Cold skin; B&A trading) was then used to administer the vaccine into the skin. Needle depth was adjusted to 0.5 mm, and the needle bar was oscillated at 100 Hz. The DNA vaccine was applied to the skin by a 16s tattoo.

Detection of NP_{366}-specific CD8$^+$ cells in peripheral blood. For analysis of T-cell responses, peripheral blood was drawn at the indicated time points. Erythrocytes were removed by incubation in erylysis buffer [155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA (pH 7.4)] at −20°C for 4 min. Cells were stained with phycoerythrin-conjugated antibody to CD8β (BD PharMingen) plus allophycocyanin-conjugated H-$2D^b/_{NP366-374}$-tetramers for 15 min at room temperature in fluorescence-activated cell sorting buffer (1× PBS, 0.5% bovine serum albumin, and 0.02% sodium azide). Before analysis, propidium iodide (1 μg/mL; Sigma Aldrich) was added to enable selection for propidium iodide–negative (living) cells. Data acquisition and analysis were performed on a FACSCalibur flow cytometer (BD Biosciences) using FCS Express software.

Combined immunotherapy. Donor mice were vaccinated with NP_{366}-encoding plasmid DNA 2 wk before isolation of splenocytes or at the time points indicated. At day 0, recipient mice were sublethally irradiated with 5 Gy or left untreated, and received an i.v. adoptive transfer of 3×10^7 freshly isolated donor splenocytes 4 to 6 h later. Mice were then vaccinated at day 0, 3, and 6 with the luc-NP DNA vaccine. Peripheral blood samples were collected at the indicated time points by tail-bleed.

In tumor rejection experiments, mice received a s.c. injection of 5×10^4 $B16^{NP}$ tumor cells, 3 d before start of treatment. Tumors were measured with calipers and the products of perpendicular diameters were recorded. In each tumor experiment, tumor-bearing mice were pooled and randomly divided into treatment or control groups.

Analysis of TIL. Tumor-bearing mice were sacrificed at day 14 postadoptive transfer, and s.c. tumors were isolated. To prepare single-cell suspensions, tumors were digested in a mixture of 0.1% collagenase type IV (Worthington) and 0.01% DNase I (Roche) in RPMI. Tumors were then disrupted over a cell strainer, and viable lymphocytes were collected after separation over a Ficoll gradient.

Lymphocytes were stained with H-$2D^b/_{NP366-374}$-tetramers, anti-CD8, anti-CD62L, anti-CD127, anti-CD44, anti-CD27, anti-CD4, or anti-CD25 antibody (BD PharMingen). Alternatively, lymphocytes were incubated for 4 h in the presence of recombinant human interleukin (IL)-2 (40 units/mL;

Chiron) and Brefeldin A (1 μL/mL; BD Biosciences). Subsequently, cells were stained with anti-CD8 antibody and analyzed for IFNγ production by intracellular cytokine staining with anti-IFNγ antibody (BD PharMingen).

Results

Host conditioning strongly enhances vaccine-induced T-cell responses. To generate high levels of tumor-specific T cells without a need for *ex vivo* expansion, we evaluated the following strategy. First, *in vivo* expansion of antigen-specific precursor cells in donor mice was induced via tattoo vaccination with a DNA vaccine encoding a model viral antigen (the immunodominant epitope of influenza A virus nucleoprotein; $NP_{366\text{-}374}$). Using this strategy, an NP-tetramer$^+$ cell population of on average 0.5% to 1% of CD8$^+$ splenocytes was induced 2 weeks postvaccination. Consistent with the previously described phenotype of pathogen- or vaccine-induced CD8+ T cells at the peak of an antigen-induced response, these cells predominantly expressed an effector/effector-memory T-cell phenotype (CD62L$^-$CD27$^+$CD44$^+$ but with heterogeneous CD127 expression; Fig. 1*A*).After this *in vivo* expansion step, splenocytes of donor mice were isolated and transferred to sublethally irradiated recipients or control mice. After transfer, recipient mice were vaccinated with the identical DNA vaccine to assess to what extent the T-cell repertoire could be skewed toward the NP antigens. In nonirradiated recipients, DNA tattoo vaccination induced responses of on average 10% NP-tetramer$^+$ CD8$^+$ cells at day 13. Notably, vaccination-induced responses were strongly enhanced in irradiated mice, amounting to 50% NP-tetramer$^+$ CD8$^+$ cells (Fig. 1*B* and *C*). These data show that a vaccination–conditioning–adoptive cell transfer (ACT)–vaccination (VCAV) protocol can be used to generate T-cell responses that are substantially more pronounced than those induced by vaccination alone.

The use of host conditioning regimens in T-cell–based immunotherapy has largely been based on the observation that T cells display a "homeostatic proliferation" when infused into lymphopenic hosts, and this proliferation is independent of the presence of their cognate antigen. It is however less clear to what extent such homeostatic expansion of antigen-specific T cells still contributes to T-cell accumulation when cognate antigen is offered in parallel. To examine this issue, we determined the absolute number of NP_{366}-specific CD8$^+$ cells that was induced by DNA vaccination of recipients that had or had not been conditioned by irradiation. No significant difference was detected in the absolute number of NP-tetramer$^+$ CD8$^+$ cells in irradiated or nonirradiated recipients (Fig. 1*D*), suggesting that when cognate antigen is offered, homeostatic expansion of antigen-specific cells no longer substantially contributes to the *in vivo* accumulation of transferred T cells. To further test the notion that the expansion of the NP_{366}-specific T-cell pool was unaltered by host conditioning, we specifically analyzed the number of donor-derived NP-tetramer$^+$ CD8$^+$ cells in irradiated or nonirradiated recipients using an Ly5 congenic marker. As expected, the NP_{366}-specific CD8$^+$ cell population in irradiated mice consisted solely of donor cells (Fig. 2*A*). However, also in nonirradiated recipients, 70% to 75% of NP_{366}-specific CD8$^+$ cells were Ly5.1$^+$ and, thus, donor derived (Fig. 2*B*). These data show that the combination of DNA vaccination with host conditioning and adoptive transfer results in a marked skewing of the T-cell repertoire toward the vaccine antigen. Notably, the skewing of the repertoire in irradiated mice is not so much caused by an increased proliferation of the donor-derived NP_{366}-specific CD8$^+$ cells but rather by the fact that a similar proliferative burst now occurs in the absence of other cells.

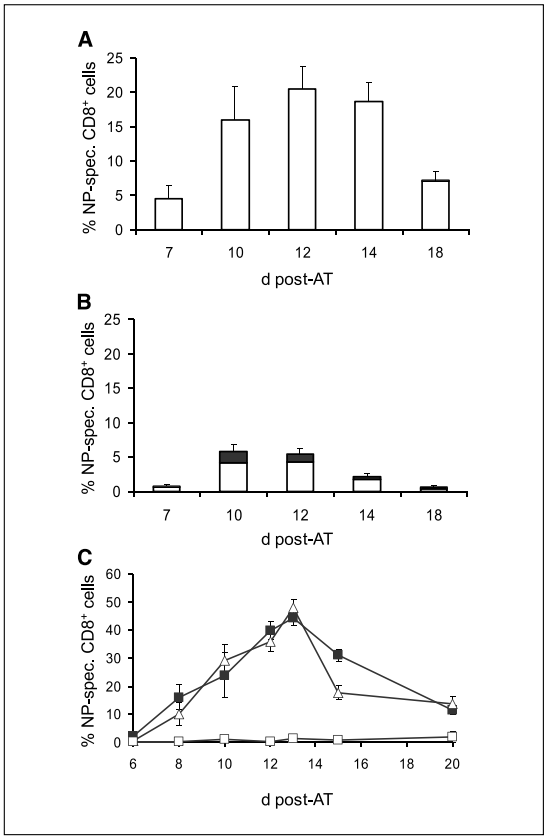

Figure 2. Antigen-specific CD8$^+$ T-cell responses in lymphodepleted mice are mainly donor-derived and require the presence of antigen-experienced T cells within the graft. *A* and *B*, irradiated (*A*) and untreated (*B*) recipients received donor cells from vaccinated Ly5.1$^+$ donors. Peripheral blood was collected at different time points after adoptive transfer and vaccination, and the percentage of Ly5.1$^+$ (□) and Ly5.1$^-$ (■) D^b-NP_{366}-tetramer–binding cells was determined by flow cytometry analysis. *Columns,* mean (n = 5); *bars,* SE. *C,* mice treated by irradiation received an adoptive transfer of 3×10^7 cells from naïve donors (□) or from donors that were vaccinated 2 (■) or 4 (△) wk before isolation of splenocytes. Recipient mice were subsequently vaccinated on day 0, 3, and 6. The percentage of D^b-NP_{366}-tetramer$^+$ CD8$^+$ cells in peripheral blood was determined at the indicated time points. *Points,* mean (n = 5); *bars,* SE.

To assess whether the transfer of grafts that contain CD8$^+$ T cells that have prior antigen experience is required for the induction of the profound vaccination-induced skewing in irradiated recipients, recipient mice were given an adoptive transfer of cells from either naive donor mice or from donors that had previously been vaccinated. After adoptive transfer, recipients were vaccinated with the luc-NP DNA vaccine and vaccine-induced T-cell responses were analyzed. Irradiated recipient mice that received donor cells from naive mice did not develop any NP_{366}-specific responses over background (<1.9%). In contrast, recipients that received donor cells from mice that had been vaccinated showed NP_{366}-specific CD8$^+$ responses of 40% to 50% of total CD8$^+$ cells (Fig. 2*C*). These responses were indistinguishable when grafts were prepared 2 or 4 weeks after primary vaccination (the peak of the CD8$^+$ T-cell response and the start of the memory phase, respectively),

indicating that there is no stringent requirement with respect to the timing of vaccination and ACT (Fig. 2*C*).

VCAV treatment improves immunotherapy of established s.c. tumors. To assess whether skewing of the T-cell repertoire toward tumor recognition could enhance antitumor efficacy, the combined treatment regimen, consisting of DNA vaccination, adoptive transfer in conditioned hosts, and subsequent vaccination was evaluated in a stringent therapeutic tumor setting using the $B16^{NP}$ tumor cell line (14, 15).

Mice were inoculated with tumor cells 3 days before start of immunotherapy, and therapy-induced T-cell responses were monitored. As a control, $CD8^+$ T-cell responses were compared with responses induced by the same regimen in tumor-free mice. Consistent with the data shown above, $CD8^+$ T-cell responses in tumor-free mice were significantly higher when ACT was performed in mice pretreated by irradiation compared with untreated mice (55.4% versus 6.3%, respectively). In tumor-bearing mice, therapy-induced $CD8^+$ cell responses were substantially reduced, consistent with an inhibitory effect of the $B16^{NP}$ tumor, possibly through transforming growth factor (TGF)-β and IL-10 (16–18). Importantly, however, T-cell responses in tumor-bearing mice treated with the combined treatment remained significantly higher than those observed in mice in which host conditioning was omitted (25.5% versus 4.9%). As expected, there were no detectable antigen-specific responses in nontreated mice or mice treated with irradiation and adoptive transfer only (data not shown).

In nontreated mice, tumors grew out rapidly and all mice had to be sacrificed within 18 days. Treatment with irradiation and adoptive transfer only, or vaccination and adoptive transfer only resulted in a slight delay in tumor growth. Combined VCAV treatment resulted in an increased antitumor effect, with a significant difference in tumor size between days 18 and 31 compared with control groups. Combined treatment prolonged mean survival of tumor-bearing mice with 10 days compared with nontreated mice (Fig. 3*B* and *C*). This eventual tumor outgrowth could not be explained by the emergence of antigen-loss variants because analysis of persistent $B16^{NP}$ tumors showed maintainance of NP epitope expression (data not shown).

When tumor-bearing rather than tumor-free mice were used as recipients for the primary vaccination, T-cell responses obtained in secondary recipients were reduced detectably (with peak $CD8^+$ T-cell levels reaching ~60% of those observed when cell grafts are obtained from tumor-free mice). In line with a reduced potency of cell grafts obtained from tumor-bearing mice, there was a (nonsignificant) trend toward reduced tumor control in this group (Supplementary Fig. S1*A* and *B*; see Discussion).

Although the therapy-induced T-cell responses are marked in this regimen, high levels of antigen-specific cells are only maintained for a short period; vaccine-induced responses peak around day 14 but then rapidly decline to baseline level. Because the persistence of tumor-specific $CD8^+$ T cells is associated with tumor regression (19), the rapid contraction of the population of tumor-specific $CD8^+$ cells seen here seems a plausible causal factor in the subsequent outgrowth of $B16^{NP}$ tumors. To test whether prolonged presence of the antigen could maintain high levels of antigen-specific $CD8^+$ T cells for a longer period of time, recipient mice were treated with a continuous vaccination regimen after ACT and host conditioning.

When adoptive transfer of donor cells in irradiated recipient mice was followed by continuous vaccination, a peak $CD8^+$ T-cell response was observed that was comparable with that observed in recipients that received the standard vaccination regimen. However, responses declined to baseline levels more slowly upon continuous vaccination, resulting in a significant higher level of antigen-specific cells on day 20 (Fig. 4*A*). Furthermore, absolute numbers of NP-specific $CD8^+$ cells were slightly enhanced in mice treated with continuous vaccination (Fig. 4*B*). Tumor growth in mice treated with continuous vaccination was delayed compared with mice treated with the

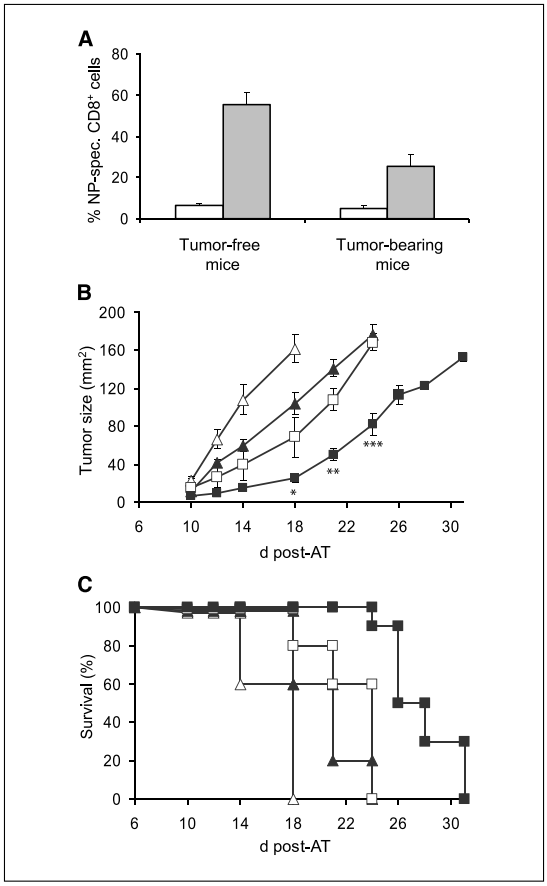

Figure 3. VCAV treatment improves immunotherapy of established s.c. tumors. *A,* mice were inoculated with 5×10^4 $B16^{NP}$ cells s.c. in the right flank on day 3 (*right*) or were left tumor free (*left*). On day 0, mice were either irradiated (▨) or not irradiated (□). On the same day, all mice received an adoptive transfer of 3×10^7 cells from previously vaccinated donors and were subsequently vaccinated on day 0, 3, and 6. The percentage of D^b-NP_{366}-tetramer$^+$ $CD8^+$ cells at the peak of the response is depicted. *Columns,* mean (n = 4); *bars,* SE. *B* and *C,* mice were inoculated with 5×10^4 B16-NP cells s.c in the right flank on day 3. Subsequently, mice were left untreated (△); treated by irradiation and adoptive transfer (▲); adoptive transfer and vaccination (□); or by the combination of irradiation, adoptive transfer, and vaccination (■). *B,* analysis of tumor development. Tumor size was measured thrice per week. *Points,* mean (n = 5); *bars,* SE. Student's t tests based on a one-tailed distribution were performed to determine differences between the VCAV treatment group and the adoptive transfer and vaccination control group; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. *C,* Kaplan-Meier survival plot. Mice were sacrificed when the longest diameter was >15 mm.

standard vaccination regimen, although the effect on survival was marginal (Fig. 4*C* and *D*).

These data show that a slight increase in the persistence of vaccine-induced CD8$^+$ cells led to a small enhancement of the antitumor effect of this treatment strategy. This suggests that it may be worthwhile to analyze strategies that can result in a more pronounced enhancement of T-cell persistence in ACT protocols (see Discussion).

Functional characterization of TIL. Delayed tumor growth in mice treated with the combined VCAV treatment is associated with a marked presence of tumor-specific CD8$^+$ cells in peripheral blood. However, peripheral blood T-cell responses do not necessarily reflect the quantity or quality of T cells present at the site of the tumor. To characterize CD8$^+$ cell infiltrates at the effector site, TIL were isolated from B16^{NP} tumors at day 14 postadoptive transfer, at which time peripheral blood T-cell responses are maximal. Infiltrating lymphocytes were analyzed for CD8 expression, NP-tetramer binding, and spontaneous IFNγ production, as well as for several phenotypic markers such as CD62L and CD127.

NP-specific TIL of both the VCAV treatment group and the adoptive transfer plus vaccination group displayed an effector phenotype of CD62L$^-$, CD127$^+$, CD44$^+$, and CD27$^+$ cells (Fig. 5*A*). However, whereas absolute numbers of NP-specific CD8$^+$ T cells in peripheral blood were not increased upon combined treatment, both the percentage (31%) and absolute number (200,000 cells) of tumor-infiltrating CD8$^+$ T cells were enhanced in mice that received VCAV treatment, compared with control mice (20% and 60,000 cells in mice treated with adoptive transfer and vaccination only). Furthermore, a higher percentage (24%) and a higher absolute number (60,000 cells) of CD8$^+$ cells from mice treated with the combined treatment bound NP-tetramers, compared with mice treated with adoptive transfer and vaccination (12% and 7,000 cells, respectively). Thus, inclusion of the conditioning regimen led to a >8-fold increase in the number of tumor resident NP-specific CD8$^+$ cells (Fig. 5*C* and *D*). More importantly, combined treatment resulted in a marked increase in TIL that displayed intratumoral effector activity, as assessed by direct *ex vivo* INFγ production in the absence of further antigenic stimulation (45.3% versus 6.5% of CD8$^+$ cells, corresponding to a more than 10-fold increase in absolute numbers of IFNγ-producing cells; Fig. 5*B, C*, and *D*). These results suggest that the improved antitumor effect of a combined vaccination-irradiation-adoptive transfer-vaccination strategy is associated with a strongly enhanced accumulation of tumor-specific CD8$^+$ cells at the site of the tumor, and that these cells display an enhanced capacity for effector function at this site.

Although recent data have suggested that the ratio of intratumoral cytotoxic T cells to regulatory T cells predicts capacity for immune control (20–22), the percentage of CD4+CD25^{high} T cells was not affected by combined VCAV treatment, and neither was the ratio of CD4+CD25^{high} T cells versus total CD8$^+$ or antigen-specific CD8$^+$ T cells (Supplementary Fig. S2*A* and *B*). These data suggest that the enhanced antitumor reactivity of TIL in VCAV-treated mice may not be accounted for by an effect of VCAV treatment on regulatory T cells.

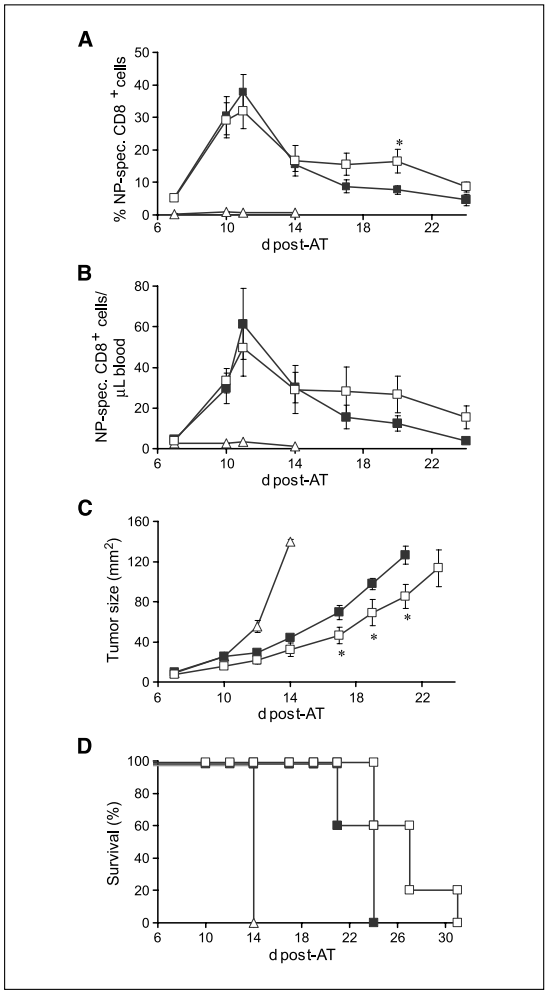

Figure 4. Effect of continued vaccination. *A* and *B,* mice were inoculated with 5×10^4 B16^{NP} cells s.c. in the right flank on day 3. On day 0, mice were irradiated and subsequently received an adoptive transfer of 3×10^7 donor cells. Vaccination was performed on day 0, 3, and 6 (■), or every 3 d up to day 21 (□). Control mice were left untreated (△). Peripheral blood was collected at the indicated time points after adoptive transfer, and the percentage (*A*) and absolute number (*B*) of D^b-NP$_{366}$-tetramer$^+$ CD8$^+$ cells were determined by flow cytometry analysis. *Points,* mean (n = 5); *bars,* SE. *C,* analysis of tumor development. Tumor size of mice in the treatment groups as indicated in *A* and *B* was measured thrice per week. *Points,* mean (n = 5); *bars,* SE. Student's t tests based on a one-tailed distribution were performed to determine differences between the standard and continuous vaccination groups; *, $P < 0.05$. *D,* Kaplan-Meier survival plot. Mice were sacrificed when the longest diameter was >15 mm.

Discussion

The more successful approaches for T-cell–based immunotherapy of cancer used to date have required *ex vivo* expansion of tumor-specific T cells to generate sufficiently high numbers for subsequent adoptive transfer. As the immune system has an intrinsic capacity to support massive expansion of antigen-specific T-cell populations, it seems plausible that a similar skewing of the T-cell repertoire may, in theory, also be achieved *in vivo*. Here, we describe our first efforts toward this goal. A strategy was developed that combines *in vivo* T-cell expansion with host conditioning and adoptive transfer, thereby circumventing the difficulties associated with current adoptive cell therapy.

This combination of vaccination, host conditioning, and ACT resulted in a marked skewing of the T-cell repertoire toward the vaccine antigen: T-cell responses in mice that were treated with this strategy reached maximal levels of close to 50%. Furthermore, when analyzing TIL during the peak of the T-cell response, an increased accumulation of both MHC-tetramer$^+$ CD8$^+$ cells and CD8$^+$ cells capable of spontaneous IFNγ production was apparent. These data are in agreement with other studies showing increased accumulation (23) or improved effector function (24) of T cells at the tumor site of irradiated mice receiving *in vitro* activated or T-cell antigen receptor (TCR) transgenic cells. Notably, irradiation led to a specific increase in the number (and activity) of tumor-specific T cells at the tumor site but not in peripheral blood. This indicates that the beneficial effect of conditioning regimens may not always be reflected by a change in the level of tumor-specific immunity detected in blood samples.

The higher levels of activated, IFNγ-producing cells may indicate a more effective differentiation of the transferred cells upon host conditioning, for instance due to the removal of endogenous cells competing for cytokines, such as IL-7 and IL-15 (24–26), or by a direct effect of host conditioning on the local tumor environment, inducing tumor necrosis and apoptosis, and thereby enhancing the presentation of tumor antigens (27–29). Although host conditioning is also known to remove regulatory T cells (30, 31), the combined treatment regimen in this study did not affect the frequency of regulatory T cells within TIL. Regardless of the mechanism, the 10-fold increase in TIL that display *in vivo* effector activity is striking.

When donor T cells were derived from tumor-bearing hosts, both vaccine-induced T-cell responses and antitumor efficacy seemed to be impaired to some extent (Supplementary Fig. S1*A* and *B*). It is somewhat difficult to generalize this finding, as the effect of tumor growth on immune status will likely be variable. However, the data do suggest that at least for some tumor types, the ability of VCAV to induce strong tumor-specific T-cell responses may be reduced through the action of tumor-derived factors such as TGF-β. It will therefore be interesting to determine whether the immunosuppressive effect of established tumors can be counteracted *in vivo*, for example, via *in vivo* blockade of TGF-β (32).

Based on prior data by other groups (33, 34), it seems likely that only those T cells in the primary T-cell graft that retain the capacity to migrate to secondary lymphoid organs contribute to the vaccine-induced T-cell response in secondary recipients. In line with this, T-cell responses in secondary recipients were identical when T-cell grafts were obtained from primary recipients at the peak of the T-cell response or when the majority of effector T-cells had disappeared. Conceivably, the magnitude of secondary T-cell responses could therefore be further enhanced with primary vaccines that would selectively induce antigen-specific T-cell populations with a central memory phenotype. Although such selective induction of T_{cm} cells by manipulation of culture conditions is feasible *in vitro* (35), it is still unclear whether robust

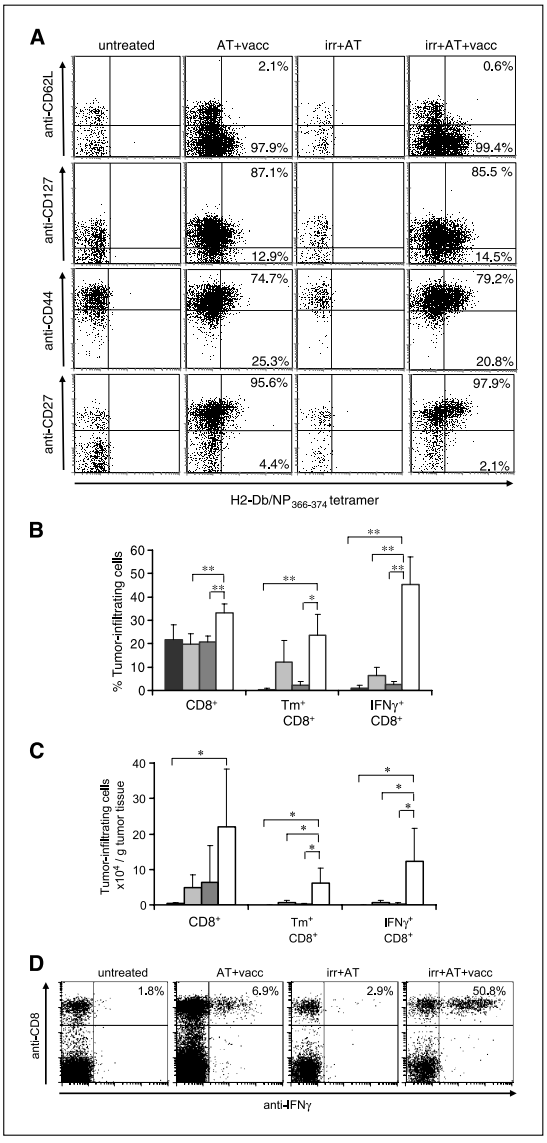

Figure 5. Functional characterization of tumor-infiltrating lymphocytes. Mice were inoculated with 5 × 10^4 B16^{NP} cells s.c. in the right flank on day 3. On day 0, mice were irradiated and subsequently received an adoptive transfer of 3 × 10^7 donor cells. Vaccination was performed on day 0, 3, and 6. Control mice were left untreated, were treated by irradiation and adoptive transfer only, or by adoptive transfer and vaccination only. At day 14 after adoptive transfer, TIL were isolated from the tumors and analyzed by flow cytometry. *A,* phenotypic analysis of TIL. Flow cytometry plots are gated on CD8$^+$ cells and show D^b-NP$_{366}$-tetramer staining in combination with anti-CD62L (*first row*), anti-CD127 (*second row*), anti-CD44 (*third row*), and anti-CD27 staining (*fourth row*). Numbers in the top and bottom right corners represent the percentage of marker$^+$ cells and marker$^-$ cells of D^b-NP$_{366}$-tetramer$^+$ CD8$^+$ cells, respectively. *B* and *C,* analysis of TIL from untreated mice (*black bars*), mice treated by irradiation and adoptive transfer (*light gray bars*), adoptive transfer and vaccination (*dark gray bars*), or VCAV combined treatment (*white bars*). The percentage (*B*) and absolute number (*C*) of total CD8$^+$ cells, D^b-NP$_{366}$-tetramer$^+$ CD8$^+$ cells, and spontaneous IFNγ-producing CD8$^+$ cells were determined by flow cytometry analysis. *Columns,* mean (n = 4); *bars,* SD. Student's t tests based on a one-tailed distribution were performed to determine differences between the VCAV treatment group and control groups; *, $P < 0.05$; **, $P < 0.005$. *D,* spontaneous IFNγ release by TIL. Flow cytometry shows anti-CD8 staining in combination with intracellular anti-IFNγ staining. Plots are representative for four mice per group, one of three experiments.

central memory T-cell responses can be induced *in vivo* by manipulation of vaccination conditions.

In addition to potential changes in the way by which primary T-cell responses are induced, we consider it likely that substantial further improvements can also be made downstream. Specifically, therapy-induced T-cell responses in secondary recipients were relatively transient with a peak around day 14 followed by a rapid decrease to levels below 10%. The kinetics of these T-cell responses are reminiscent of those seen during classic pathogen-induced T-cell responses, in which the majority of T-cell output is predestined to die by apoptosis (36, 37). In line with the notion that the drop in tumor-specific T-cell frequency at later time points is caused by contraction rather than an increased abundance of other T-cell specificities, absolute numbers of antigen-specific $CD8^+$ T cells also go down. As a possible correlate of the transient nature of the skewing of the T-cell repertoire toward tumor recognition upon VCAV, the antitumor effect of combined therapy was also temporary, with tumors growing out in all mice eventually. Notably, when the presence of antigen is prolonged, by continuous vaccination, this results in only a somewhat slower decline in levels of antigen-specific cells.

These data suggest that vaccination may form a suboptimal way to steer the T-cell repertoire after ACT when long-term persistence is required. In the current setting, vaccination post-ACT was required to boost tumor-specific T-cell frequencies from the low frequencies present in the original graft (0.5–1% of total donor splenocytes). It therefore seems attractive to prepare grafts that are already highly enriched for tumor reactivity, thereby potentially obviating the need for subsequent vaccination. Based on prior data using TCR transgenic T cells, it seems plausible that the high level of tumor reactivity present in such selective cell grafts would be maintained during homeostatic expansion, thereby resulting in a long-term dominance of the tumor reactive T-cell repertoire. Selective T-cell grafts may be prepared by MHC multimer–based sorting, using either classic MHC tetramers (38) or reversible MHC tetramers that may result in a higher viability of the resulting cell product (39). Furthermore, the production of clinical-grade MHC multimers for a large collection of T-cell epitopes seems a realistic option with the development of more efficient production methods (40).

It should be relatively straightforward to translate the strategy described here, or further modifications that use selective T-cell grafts, to a clinical setting. As is the case for all immunotherapeutic strategies that are based on a mobilization of the endogenous T-cell repertoire, VCAV may in particular be suitable for tumor types for which a high avidity tumor-specific T-cell repertoire is present. For instance, patients with cervix carcinoma may be vaccinated with vaccines encoding the HPV E6 and E7 oncoproteins. Such a vaccination may either involve the type of DNA vaccines used here or one of the approaches that have previously been shown to yield $CD8^+$ T-cell reactivity against HPV E6 and E7 in clinical trials (3–5). After isolation of peripheral blood lymphocytes and administration of lymphodepleting chemotherapy, reinfusion of the autologous cells followed by a second round of vaccination could be used to induce skewing of the T-cell repertoire toward the HPV oncogenes. This type of study would be valuable to determine whether this approach for skewing the antigen-specific T-cell repertoire can be effective in a clinical setting.

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Chapter 4

Human telomerase reverse transcriptase-transduced human cytotoxic T cells suppress the growth of human melanoma in immunodeficient mice

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(*these authors contributed equally to this work)

Human Telomerase Reverse Transcriptase-Transduced Human Cytotoxic T Cells Suppress the Growth of Human Melanoma in Immunodeficient Mice

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ABSTRACT

Immunotherapy of melanoma by adoptive transfer of tumor-reactive T lymphocytes aims at increasing the number of activated effectors at the tumor site that can mediate tumor regression. The limited life span of human T lymphocytes, however, hampers obtaining sufficient cells for adoptive transfer therapy. We have shown previously that the life span of human T cells can be greatly extended by transduction with the human telomerase reverse transcriptase (hTERT) gene, without altering antigen specificity or effector function. We developed a murine model to evaluate the efficacy of hTERT-transduced human CTLs with antitumor reactivity to eradicate autologous tumor cells *in vivo*. We transplanted the human melanoma cell line melAKR or melAKR-Flu, transduced with a retrovirus encoding the influenza virus/HLA-A2 epitope, in RAG-2$^{-/-}$ IL-2Rγ $^{-/-}$ double knockout mice. Adoptive transfer of the hTERT-transduced influenza virus-specific CTL clone INFA24 or clone INFA13 inhibited the growth of melAKR-Flu tumors *in vivo* and not of the parental melAKR melanoma cells. Furthermore, the hTERT-transduced CTL clone INFA13 inhibited tumor growth to the same extent *in vivo* as the untransduced CTL clone, as determined by *in vivo* imaging of luciferase gene-transduced melAKR-Flu tumors, indicating that hTERT did not affect the *in vivo* function of CTL. These results demonstrate that hTERT-transduced human CTLs are capable of mediating antitumor activity *in vivo* in an antigen-specific manner. hTERT-transduced MART-1-specific CTL clones AKR4D8 and AKR103 inhibited the growth of syngeneic melAKR tumors *in vivo*. Strikingly, melAKR-Flu cells were equally killed by the MART-1-specific CTL clones and influenza virus-specific CTL clones *in vitro*, but only influenza-specific CTLs were able to mediate tumor regression *in vivo*. The influenza-specific CTL clones were found to produce higher levels of IFNγ on tumor cell recognition than the MART-1-specific CTL clones, which may result from the higher functional avidity of the influenza virus-specific CTL clones. Also, melAKR-Flu tumors were growing faster than melAKR tumors, which may have surpassed the relatively modest antitumor effect of the MART-1-specific CTL, as compared with the influenza virus-specific CTL. Taken together, the adoptive transfer model described here shows that hTERT-transduced T cells are functional *in vivo*, and allows us to evaluate the balance between functional activity of the CTL and tumor growth rate *in vivo*, which determines the efficacy of CTLs to eradicate tumors in adoptive transfer therapy.

INTRODUCTION

The goal of immunotherapy is to bolster the immune system of the patient in such a way that it eradicates an established tumor. One way to achieve this goal is to increase the number of activated effector cells at the tumor site by adoptive transfer of *in vitro* generated and expanded CTLs. Indeed, infusion of human T cells has been shown to cause a delayed tumor growth in human xenograft mouse models (1–7). More importantly, adoptive transfer therapy has been successful in clinical settings as well. Transfer of virus-specific T cells has been shown to be effective in preventing reactivation of latent cytomegalovirus infections in patients after organ transplantation (8, 9) and in the treatment of lymphoproliferative disorders caused by EBV infection (10, 11). Adoptive transfer therapy of cancer requires the isolation of T cells with tumor reactivity. Tumor-infiltrating lymphocytes may be enriched for such T cells and were shown to be effective in mediating tumor regression in metastatic renal cell carcinoma patients after nephrectomy (12). Recently, the infusion of polyclonal T lymphocytes that were expanded from the tumor-infiltrating lymphocytes, combined with high doses of IL-2, has been shown to induce substantial tumor regression in metastatic melanoma patients (13). In addition, Yee *et al.* (14) reported regression of individual tumor metastases by adoptive transfer of CD8+ T-cell clones that recognize the melanoma antigens MART-1 or gp100 in combination with low doses of IL-2 in metastatic melanoma patients. These clinical studies show that adoptive T-cell therapy is feasible to treat cancer patients, has low toxicity, and can be effective in mediating tumor regression.

Adoptive transfer of tumor-specific CTLs has required large doses of at least 10^9-10^{10} T cells. This means that, after isolation from peripheral blood or tumor-infiltrating lymphocytes, CTLs need to be expanded *in vitro*. Whereas it is in general not a problem to expand freshly isolated polyclonal T cells to very large numbers, this is not the case with well-defined tumor antigen-specific T-cell clones. The application of adoptive transfer therapy of well-defined antigen-specific T-cell clones is, therefore, limited by the relatively low success rate of isolating sufficient numbers of specific T cells from individual patients. CTLs with tumor reactivity that are found in cancer patients are derived frequently from the memory T-cell pool. Human CD8+CD28− memory T cells have a replicative life span of maximally 40 population doublings (PDs) *in vitro* but most often much less (15), which limits large-scale expansion of these cells. For initial screening approximately 10^5-10^6 cells are needed, which amounts to 17–20 PD when starting from one cell. Therefore, the isolation and cloning of tumor-reactive T cells selects for the relatively young T cells, or rare T cells with an exceptionally long life span, which may not be found in all cancer patients. Moreover, the limited life span of human T cells may also have contributed to the fact that the most prominent antitumor responses seen to date were obtained with relatively young cultures of tumor-infiltrating lymphocytes (16).

We have described previously that ectopic expression of the enzyme complex, telomerase reverse transcriptase (hTERT) greatly extends the life span of both human CD8+ and CD4+ T cells (17–19). Ectopic hTERT expression prevents telomere shortening in the cells, which occurs at each cell division or by oxidative DNA damage. Telomeres are DNA repeats at the distal ends of the chromosomes, which protect against chromosome end-to-end fusions (20). Critically short telomeres have an impaired function and may lead to cell cycle

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arrest. Since murine T cells have longer telomeres, resulting in a longer life span than human T cells, T-cell life span generally does not limit adoptive transfer experiments of murine T cells. Interestingly, human T cells express hTERT upon activation, allowing repair of short telomeres during activation and proliferation (21). We have shown previously that during prolonged proliferation *in vitro*, T cells loose the ability to up-regulate hTERT expression, and the level of telomerase activity becomes insufficient to repair the telomere erosion (19). Moreover, we observed lower levels of hTERT expression in activated memory cells, as compared with activated naive cells of the same donor (19). This indicates that the loss of hTERT expression also occurs upon proliferation *in vivo*, which reduces the proliferative capacity of memory T cells, as compared with naive T cells. Because tumor-reactive T cells may be more frequently found in the memory T-cell pool, transduction of memory T cells with hTERT provides a tool to overcome the limitation of a reduced proliferative capacity. We have observed that hTERT-transduced T cells retain their antigen specificity and effector function upon activation *in vitro* (17, 18). Furthermore, we observed that proliferation of hTERT-immortalized T cells *in vitro* remained dependent on activating signals and cytokines, which underlines the notion that ectopic hTERT expression allows the continuation of proliferation (17, 18), but does not promote entry into cell cycle by itself, nor does it cause growth deregulation (22). Ectopic hTERT expression in combination with stimulation of T cells therefore enables large-scale cultures and serial cloning to isolate human T cells of desired specificity in sufficient numbers for adoptive transfer (17, 18, 23, 24). Moreover, ectopic hTERT expression allows large-scale expansion of those tumor-specific T-cell clones, which would otherwise not expand to sufficient numbers due to telomere erosion. Thus, hTERT transduction will enlarge the repertoire of CTLs that can be used for adoptive transfer. Having solved the problem of the low success rate in obtaining high numbers of cloned CTLs, it was important to show that these hTERT-transduced T cells were effective *in vivo*. We developed an *in vivo* model to test the efficacy of human hTERT-transduced CTL clones to eradicate autologous melanoma cells in an *in vivo* environment. In the present report, we describe adoptive transfer of hTERT-transduced CTL clones in RAG-$2^{-/-}$ IL-2R$\gamma^{-/-}$ (RAG/γcKO) mice bearing human melanoma lung tumors. The effect of single or multiple doses of two influenza-reactive CTL clones or two MART-1-reactive CTL clones on the growth of a human melanoma *in vivo* was studied in relation to their functional activity *in vitro*. In this model, the criteria for CTL clones to be effective in mediating tumor regression *in vivo* upon adoptive transfer can be defined.

MATERIALS AND METHODS

Mice. Male RAG-$2^{-/-}$ IL-2Rγ (common γ-chain)$^{-/-}$ double knockout (RAG/γcKO) mice on a C57/Bl6 background, as described previously (25), were used at the age of 6–8 weeks. These mice have no functional T, B, and natural killer cells, and are not leaky for these cell types. These mice can be maintained as double knockout mice. The mice were bred under specific pathogen-free conditions, maintained in isolators, and all of the manipulations were performed under laminar airflow.

Tumor Cell Lines. The melanoma cell line melAKR was derived from a melanoma lesion of patient AVL-3 (26). MelAKR-Flu was generated by transduction of melAKR with the retrovirus encoding the influenza matrix peptide GILGFVFTL that binds to HLA-A2 molecules, a series of murine CTL epitopes, and the green fluorescent protein (GFP) gene connected by the internal ribosomal entry site (IRES) sequence, as described (27). MelAKR, melAKR-Flu, the EBV-transformed B cell line JY, which expresses HLA-A2, and the erythroleukemia cell line K562 were cultured in Iscove's modified Dulbecco's medium (Invitrogen Life Technologies, Breda, The Netherlands), supplemented with 8% FCS (Invetrogen Life Technologies), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Roche Diagnostics, Mannheim, Germany).

Isolation of Human T-Cell Clones. $CD8^+$ T-cell clones INFA13 and INFA24 were derived from the peripheral blood mononuclear cell (PBMC) of an HLA-A2-positive healthy donor. $CD8^+$ T cells isolated from the PBMCs by MACS sort using anti-CD8 antibody (Ab)-coated beads, were stimulated with the CD8-negative PBMC fraction pulsed with the influenza virus matrix peptide 58–66 (GILGFVFTL) in the presence of 20 IU/ml recombinant human (rh)IL-2 (Proleukin, Chiron, Amsterdam, the Netherlands). Subsequently, $CD8^+$ T cells that recognize the influenza peptide in HLA-A2 were detected by binding of the HLA-A2/influenza tetramer, and cloned by fluorescence activated single cell sorting (FACSstar Plus; Becton Dickinson, San Jose, CA). Clones INFA13 and INFA24 were identified to specifically recognize influenza virus matrix peptide 58–66 (GILGFVFTL) on HLA-A2-positive target cells. The T-cell receptor (TCR) Vβ chain expressed by the T-cell clones was determined by TCR Vβ chain-specific Ab staining (IOtest β Mark; TCR Vβ Repertoire kit; Immunotech, Marseille, France). T-cell clones INFA24 AND INFA13 were both characterized by the expression of TCR Vβ17, which has described as the dominant Vβ used by human influenza-specific T-cell clones (28).

$CD8^+$ T-cell clone AKR4D8 is a subclone of clone AKR4 that was derived from patient AVL-3 (26) after stimulating PBMCs with the autologous melanoma cell line melAKR that was genetically engineered to produce IL-7 (17). After stimulation, the cells were cloned by single cell sorting, and clone AKR4 was identified to recognize the MART-1 peptide analog 26–35 (ELAGIGILTV) that binds to HLA-A2 molecules (29), and to a lesser extend the unmodified MART-1 epitope 26–35 (EAAGIGILTV). Clone AKR4D8 was isolated from two consecutive rounds of subcloning of clone AKR4. As expected, the AKR4D8 subclone was also reactive with the MART-1 epitope presented in HLA-A2 (17). $CD8^+$ T-cell clone AKR103 was derived from the PBMC of patient AVL-3 that had been stimulated with the autologous melanoma line melAKR transduced with the costimulatory molecule CD80, and subsequently cloned by single cell sorting.[1] Clone AKR103 was identified to recognize both the MART-1 peptide 26–35 EAAGIGILTV, as well as the MART-1 peptide analog ELAGIGILTV, presented by HLA-A2. T-cell clone AKR4D8 expressed TCR Vβ8, whereas clone AKR103 did not bind to any of the Vβ chain-specific antibodies of the TCR Vβ repertoire kit (IOtest β Mark; TCR Vβ Repertoire kit; Immunotech), indicating that the clones AKR4D8 and AKR103 represented different CTL clones isolated from patient AVL-3.

T-Cell Culture and Transduction. T-cell clones were cultured in Yssels medium (30), supplemented with 1% human serum, 20 IU/ml rhIL-2, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded weekly at 0.3×10^6 cells/ml in the presence of a feeder mixture consisting of 0.1×10^6/ml irradiated (80 Gy) JY cells, 1×10^6/ml irradiated (40 Gy) allogeneic PBMC, and 0.1×10^6 cells/ml irradiated (80 Gy) melAKR-Flu cells. Alternatively, the melAKR-Flu cells in the feeder mixture were replaced by 100 ng/ml phytohemagglutinin (HA16; Murex Biotech, Dartford, United Kingdom) in one stimulation every 3 weeks. Cultures were performed in 24-well plates at 1 ml/well, or in 125-cm^2 tissue culture flasks containing 150–300 ml culture volume (1–2 ml culture volume/cm^2). All of the CTL clones were transduced with a retrovirus encoding the hTERT gene and the GFP gene connected by the IRES sequence, as described (17). Briefly, T cells were stimulated with the feeder cell mixture containing phytohemagglutinin, as described above, 2 days before transduction. The cells were transduced with supernatant containing the retrovirus encoding hTERT-IRES-GFP, in fibronectin fragments-coated plates (Retronectin, Takara, Japan) in the presence of 20 IU/ml rhIL-2. During transduction the plate was spun at 2500 rpm for 90 min at 25°C. Subsequently, half of the transduction supernatant was replaced by freshly thawed retroviral supernatant, and the transduction was cultured overnight at 37°C and 5% CO_2. After transduction, the cells were washed and cultured, as described above. hTERT expression by the transduced CTL clones was determined as the GFP expression level by flow cytometry.

Chromium Release Assay. The cytotoxic activity of the CTL clones was tested in a 4-h ^{51}Cr release assay with 500 targets/well and E:T ratios ranging from 1:1 to 30:1, as described (31). Unlabeled K562 cells were added in a 50-fold excess to suppress nonspecific target cell lysis by the T-cell clones.

[1] E. Hooijberg and J. J. Ruizendaal, unpublished observations.

Preincubation of target cells with 100 μM peptide was performed during chromium labeling followed by three wash steps. For functional avidity testing, the peptides were added to the test in 10-fold dilutions ranging from 10^2 to 10^{-8} μM. Assays were performed in a volume of 150 μl/test. ^{51}Cr release was measured in 25 μl of the test supernatant dried on a LUMA scintillation plate (Bio-Rad, Hercules, CA) in a β-radiation counter (Topcount NXT; Packard, Randburg, South Africa).

HLA-Peptide Tetramers. Allophycocyanin-conjugated tetramers composed of HLA-A2 and the MART-1 peptide analog 26–35 (ELAGIGILTV) or the influenza virus matrix peptide 58–66 (GILGFVFTL) were synthesized as described (32). Binding of tetramers to CTL was tested by incubation of 2×10^5 T cells with 0.1 μg tetramer for 10 min at 37°C, followed by incubation with phycoerythrin-conjugated anti-CD4, anti-CD8, or anti-TCR$\alpha\beta$ Abs (Becton Dickinson) on ice for 30 min. Cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson). Propidium iodide was added to exclude nonviable cells.

ELISA. T cells (3300 cells/well) and tumor cells (6600 cells/well) were cocultured overnight in triplicate cultures in a 96-well round-bottomed plate in a total volume of 200 μl/well Yssels medium, supplemented with 1% human serum, 20 IU/ml rhIL-2, 100 IU/ml penicillin, and 100 μg/ml streptomycin. JY cells were preincubated with 100 μM influenza virus matrix peptide 58–66 (GILGFVFTL) for clones INFA13 and INAF24, or with the MART-1 peptide 26–35 (EAAGIGILTV) for clones AKR4D8 and AKR103, washed, and cocultured with T cells. The supernatants were collected after overnight culture and analyzed for the presence of IL-4, IL-10, and IFNγ by ELISA. The concentration of IL-4, IL-10, or IFNγ in the supernatant was determined in triplicate by cytokine-specific ELISA (PeliKine; Sanquin Reagents, Amsterdam, the Netherlands) using a standard curve of diluted recombinant cytokine provided in the kit.

Adoptive Transfer Protocol. Six to 8-week-old male RAG/γcKO mice were injected i.v. in the tail vein with 1×10^6 or 0.5×10^6 melAKR-Flu tumor cells. The mice were treated with a dose of 5×10^6 T cells by i.v. injection in the tail vein on day 3, followed by a s.c. rhIL-2 depot in the flank. This rhIL-2 depot consists of a suspension of 2×10^5 IU rhIL-2 in 40 μl Iscove's modified Dulbecco's medium (Invitrogen Life Technologies), and 80 μl incomplete Freund's adjuvant (Difco Laboratories, Detroit, IL). One group of 8 mice/experiment received another i.v. dose of 5×10^6 T cells in the tail vein at day 5 and at day 10. A control group of 8 mice in each experiment received only tumors cells and the rhIL-2 depot. The mice were sacrificed on day 17, and the lungs were excised. Half of the left lung was isolated and kept in medium for the detection of the injected T cells by fluorescence-activated cell sorter analysis. The rest of the lung was filled with paraformaldehyde via the trachea to open the alveoli. This manipulation increases the morphology of the lung structure and was used for immunohistochemical analysis to determine the tumor size in the lung.

Detection of Human T Cells by Flow Cytometry. The lung tissue was cut into small pieces and mashed into a single cell suspension, followed by total lymphocyte isolation on a Ficoll gradient. After washing, the cells were incubated with phycoerythrin-conjugated antimurine CD45 Ab (Becton Dickinson), PerCP-conjugated antihuman CD8 Ab (Becton Dickinson), and allophycocyanin-conjugated antihuman CD45 Ab (Becton Dickinson) for 30 min on ice. Human T cells were detected as the population-expressing GFP and human CD45, but not murine CD45, which were also tested in parallel for human CD8 expression. The kinetics of the injected human T cells was measured in groups of 8 RAG/γcKO mice injected with 0.5×10^6 melAKR-Flu tumor cells and treated after 3 days with a single dose of 5×10^6 INFA24 T cells. Two mice were sacrificed 3 h, 3 days, 7 days, or 14 days after T-cell transfer, to analyze the presence of injected T cells the lungs and in the peripheral blood by flow cytometry. Long-term experiments of T-cell survival were performed in 14 mice injected with a single dose of 5×10^6 INFA24 T cells, together with a rhIL-2 depot, and sacrificed after 3 weeks, or after 3, 6, 9, or 11 months. Autopsy was performed on the mice to detect any malignancies or other abnormalities. The presence of human T cells in the lungs was analyzed by flow cytometry.

Detection of Melanoma Cells by Immunohistochemistry. The right lung was fixed with formalin and embedded in paraffin to cut longitudinal sections. The sections were stained with polyclonal rabbit Ab S-100 (DAKO, Glostrup, Denmark) to detect melanoma cells. Briefly, the sections were pretreated with Pronase (Sigma Aldrich, Zwijndrecht, the Netherlands) for 10 min, washed, and preincubated with 5% normal goat serum (Sanquin Reagents). Sections were subsequently incubated with the S-100 antibody overnight at 4°C in a humidified chamber. After washing, the biotin-labeled goat-antirabbit IgG Ab (DAKO) was added for 30 min. The sections were washed and incubated with a streptavidin-biotin complex conjugated to horseradish peroxidase (DAKO) for 30 min. Bound antibody was detected by incubation with 3,3′-diaminobenzidine (DAKO) for 5 min, which is visible as a brown staining pattern. The sections were counterstained with hematoxylin.

Quantification of the Number of Microtumors and Total Tumor Size. For each mouse, the number of microtumors was counted in two whole longitudinal lung sections of the right-side lung by two independent observers in two separate sessions. The total tumor size in the lung was quantified as the total area of S-100 staining cells in the two longitudinal lung sections per mouse, using the computer-aided detection program KS-400 (Zeiss, Weesp, the Netherlands), which measures the area of staining per vision field, which is observer independent and gives an objective analysis of the tumor size. The total tumor size was expressed as the sum of the staining area of all of the vision fields in two whole longitudinal sections that were sampled from the center of the lung tissue.

Statistical Analysis. Student's *t* test was used to determine the significance of the differences in the number of microtumors and the total tumor size between the different groups of mice.

***In Vivo* Imaging of Tumor Xenografts.** The luciferase gene isolated from the PGL3 vector (Promega, Madison, WI) was cloned into the retroviral vector pMX in an IRES-YFP configuration. MelAKR-Flu tumor cells were transduced with a retrovirus encoding the luciferase-IRES-YFP construct. Luciferase-transduced cells (melAKR-Flu-Luc) were selected based on YFP expression by fluorescence-activated cell sorting 48 h after transduction. Adoptive transfer was performed as described above. Mice were injected with 0.5×10^6 melAKR-Flu-Luc cells and received an i.v. injection on day 3 with 5×10^6 untransduced cells or hTERT-transduced cells of CTL clone INFA13, as well as a s.c. rhIL-2 depot. Control mice received only tumor cells and the rhIL-2 depot. Tumor growth was monitored *in vivo* by bioluminescence imaging between day 3 and day 20. Mice were anesthetized with isoflurane (Abbott Laboratories, Queensborough, United Kingdom). An aqueous solution of the substrate luciferin (150 mg/kg; Xenogen, Alameda, CA) was injected into the peritoneal cavity 6 min before imaging. Animals were placed into the light-tight chamber of the CCD camera (IVIS; Xenogen). A gray-scale photographic image of the animal was taken in the chamber under dim illumination. After switching off the light source, the photon counts produced by active luciferase within the melAKR-Flu-Luc cells were acquired during a defined period of time ranging up to 2 min. Signal intensity was quantified as the sum of all of the detected photon counts within the region of interest after subtraction of background luminescence, using the software program Living Image (Xenogen). A pseudocolor image representing the spatial distribution of photon counts within the animal (blue, least intense and red, most intense) was generated in Living Image and overlayed on the gray-scale reference image, allowing anatomical localization of the tumors. At day 20, the mice were sacrificed and the lungs were injected via the trachea with a suspension of India ink (15% India ink and 0.01% concentrated ammonium hydroxide in distilled water). Lungs were then removed and bleached with Fekete's solution (58% ethanol 95%, 20% distilled water, 8% formaldehyde solution 37%, and 4% glacial acetic acid). Tumor nodules appeared as discrete white nodules against the black background of normal lung tissue.

RESULTS

Characterization of the Functional Activity of Human CTL Clones with Extended Life Span *in Vitro*. To compare different human CTL clones for their individual efficacy to eradicate autologous human melanoma cells *in vivo*, we generated two influenza virus-specific CTL clones, INFA13 and INFA24, and two melanoma-specific CTL clones, AKR4D8 and AKR103. All four of the CTL clones were transduced with the hTERT-IRES-GFP encoding retrovirus. During subsequent culture, we observed an accumulation of hTERT-IRES-GFP-expressing cells in all of the transduced T-cell cultures, which contained only transduced T cells after 1 or 2 months. The mean fluorescence intensities of GFP expression level of the

hTERT-IRES-GFP transduced clones AKR4D8, AKR103, INFA24, and INFA13 were 120, 310, 405, and 255, respectively. hTERT transduction extended the life span of the CTL cultures far beyond the life span of the untransduced cell cultures, which was between 38 and 42 PD for all of the CTL clones, as we have described previously (17, 18). All of the clones were 100% GFP positive, indicating they were all hTERT positive when the growth curves were determined. The CTL clones AKR4D8, INFA24, and INFA13 were growing at a comparable rate of 1.9, 1.5, and 1.8 PD per stimulation, respectively. Clone AKR103 was growing faster at a rate of 2.8 PD per stimulation. All of the hTERT-transduced clones were able to grow in large culture volumes of 150–300 ml containing 1–2 $\times$ 10^6 T cells/ml upon weekly stimulation with the feeder cell mixture to obtain sufficient T cells for adoptive transfer therapy.

CTL clones INFA24 and INFA13 lysed JY cells only when pulsed with the HLA-A2-binding influenza virus matrix peptide (58–66) GILGFVFTL (Fig. 1*A*). These CTL clones did not recognize HLA-A2-positive melAKR melanoma cells, but lysed melAKR-Flu cells, which express the influenza virus (58–66) epitope (Fig. 1*A*). HLA-A2-positive targets loaded with irrelevant peptide were not recognized by these CTL clones (data not shown). These results show that the clones specifically recognized the influenza epitope. Moreover, both CTL clones expressed CD8 and bound HLA-peptide tetramers composed of HLA-A2 and the influenza virus matrix peptide, but not HLA-A2 tetramers containing the MART-1 peptide analog (Fig. 1*B*). Both CTL clones INFA13 and INFA24 displayed an equally high functional avidity, as determined by the half maximal lysis of peptide-loaded JY cells at 100 pM of influenza virus matrix peptide (58–66) GILGFVFTL (Fig. 1*C*).

CTL clones, AKR4D8 and AKR103, both specifically lysed the autologous melanoma cell line melAKR, as well as melAKR-Flu cells (Fig. 1*A*). Autologous EBV-transformed B cells (data not shown) or JY cells were lysed only when preloaded with MART-1 peptide (26–35) EAAGIGILTV, or with the MART-1 peptide analog ELAGIGILTV (Fig. 1, *A* and *C*). HLA-A2-positive melanoma cells lacking MART-1 expression were not recognized, nor JY cells loaded with irrelevant peptide (data not shown). The slightly enhanced cytolysis by clone AKR103, as compared with clone AKR4D8, may reflect a difference in the intrinsic cytolytic capacity. However, redirected cytotoxicity assays of both clones against the Fc-receptor-expressing P815 cells preloaded anti-CD3 antibody revealed that the

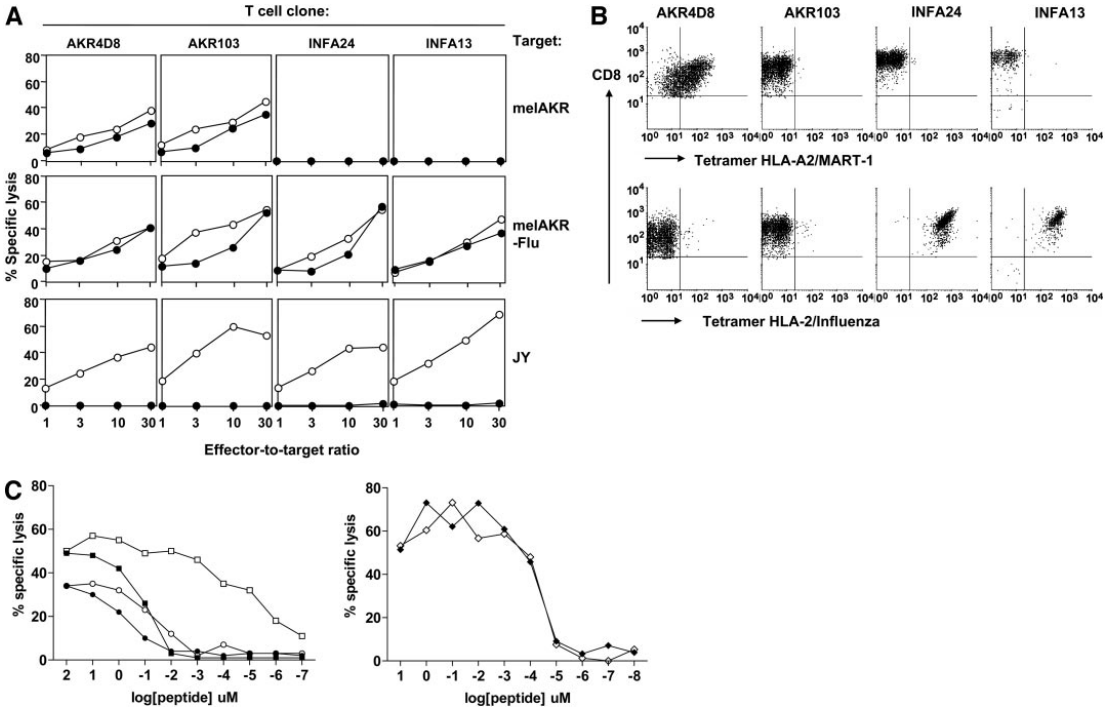

Fig. 1. Specific target cell recognition and tetramer binding by human telomerase reverse transcriptase-transduced MART-1 or influenza virus-specific CTL clones. *A,* recognition of melAKR (*top*), melAKR-Flu (*middle*), or JY (*bottom*) by MART-1-specific CTL clones AKR4D8 and AKR103 or influenza virus-specific CTL clones INFA24 and INFA13, was tested in a 4-h ^{51}Cr release assay. Nonspecific target cell lysis by the CTL was suppressed by a 50-fold excess of unlabeled K562 cells. ● indicate the lysis of target cells in the absence of peptide; ○ indicate the target cells lysis after preloading with MART-1 peptide analog ELAGIGILTV in assays of clones AKR4D8 and AKR103 or influenza virus matrix peptide 58–66 (GILGFVFTL) in assays of clones INFA24 and INFA13. Results are representative of five independent ^{51}Cr release assays performed 6–8 days after stimulation of the T-cell culture. *B,* specific tetramer binding by the human telomerase reverse transcriptase-transduced CTL clones. Graphs show the binding of HLA-A2/MART-1 26–35 (*top*) or HLA-A2/influenza 58–66 (*bottom*) tetramers, and the expression of CD8 by CTL clones AKR4D8, AKR103, INFA24, and INFA13, as tested by flow cytometry. Graphs are representative of eight independent tetramer-binding assays performed 8–10 days after stimulation of the T-cell culture. *C,* functional avidity of the CTL clones. *Right graph*, lysis of JY cells by CTL clone AKR4D8 (*squares*) or clone AKR103 (*circles*) in the presence MART-1 peptide EAAGIGILTV (*closed symbols*) or peptide analog ELAGIGILTV (*open symbols*) at concentrations ranging from 100 μM to 0.1 pM was tested in a chromium release assay. Graph shows the percentage specific lysis upon 4-h incubation at an E:T ratio of 30:1. Functional avidity was determined by the peptide concentration at which half the maximal lysis was observed. Clone AKR103: 10–100 nM ELAGIGILTV, 100 nM -1 μM EAAGIGILTV. Clone AKR4D8: 1–10 pM ELAGIGILTV, 100 nM EAAGIGILTV. *Left graph*, lysis of JY cells by CTL clone INFA13 (◆) or clone INFA24 (◇) in the presence influenza virus matrix peptide 58–66 (GILGFVFTL) at concentrations ranging from 10 μM to 0.1 pM at an E:T ratio of 5:1. Graphs show an equal avidity of clone INFA13 and INFA24 at 100 pM.

cytolytic capacities of these two CTL clones upon anti-CD3 cross-linking were comparable (data not shown). Although both CTL clones recognized the MART-1 peptide analog ELAGIGILTV in cytotoxicity assays, only clone AKR4D8 bound tetramers composed of the MART-1 peptide analog ELAGIGILTV and HLA-A2 molecules (Fig. 1*B*). Both CTL clones expressed comparable levels of TCR and CD8, as judged by anti-TCR$\alpha\beta$ or anti-CD8 antibody binding, indicating that the absence of tetramer binding of clone AKR103 was not due to a lower T-cell receptor or CD8 expression level (Fig. 1*B*). Additional analysis revealed that the absence of tetramer binding to clone AKR103 was most likely due to the very low affinity of its TCR for the MART-1 peptide analog ELAGIGILTV that was used to make the tetramer (Fig. 1*C*). Whereas clone AKR4D8 showed a high functional avidity for the MART-1 peptide analog ELAGIGILTV with a half-maximal lysis at 1–10 pM peptide (Fig. 1*C*), the avidity of clone AKR103 for this peptide was 10,000-fold lower (10–100 nM). A smaller difference in avidity between the CTL clones was found for the naturally processed epitope of the MART-1 protein, EAAGIGILTV, which is presented by the melAKR cells (Fig. 1*C*). Thus, it can be expected that both clones would bind tetramers of the natural peptide equally well. However, this could not be tested because due to the lower affinity of this natural MART-1 peptide for HLA-A2, it could not be used to generate tetramers. The difference in affinities of the AKR4D8 and the AKR103 TCR for the MART-1 peptide analog suggested already that these clones expressed different TCR. This was confirmed by specific antibody staining of the TCR Vβ chain (data not shown). The functional *in vitro* cytolytic activities of all four of the hTERT-transduced CTL clones was identical to that of the untransduced CTL clones, as we have described previously (17), indicating that hTERT transduction had not changed the specific target cell recognition of the CTL clones.

The cytotoxicity assays showed that melAKR-Flu cells were lysed to a similar extent by the influenza virus-specific CTL clones, and the CTL clones AKR103 and AKR4D8 (Fig. 1A). Stimulation with plate-bound anti-CD3 antibody induced comparable levels of IFNγ in all four of the CTL clones (Fig. 2), indicating that the intrinsic capacities of the influenza virus-specific and the MART-1-specific CTL clones to produce IFNγ were the same. However, the influenza virus-specific CTL clones produced 3–4-fold higher levels of IFNγ upon recognition of melAKR-Flu cells than the MART-1-specific CTL clones (Fig. 2). Likewise, recognition of JY cells loaded with specific peptide resulted in higher levels of IFNγ production by the influenza-specific clones, as compared with the MART-1-specific CTL clones. None of the CTL clones produced IL-4 or IL-10 on activation (data not shown). Although we cannot rule out that the influenza epitope expression on the melAKR-Flu cells may have been higher than the MART-1 expression, the enhanced IFNγ production by the influenza virus-specific CTL clones upon recognition of peptide loaded JY cells (Fig. 2) may have resulted from the higher avidity of these CTL clones, as compared with the MART-1 CTL clones (Fig. 1*C*). It is likely that the difference in avidity between the clones is caused by the higher TCR affinity of the influenza virus-specific clones and not by differences in the capacity to form adhesions, because MelAKR-Flu cells expressed CD58, CD54 (ICAM1), CD102 (ICAM2), and CD50 (ICAM3), the ligands of which, CD2, CD11a (LFA-1), activated CD11a, or CD18, were expressed at comparable levels on all four of the CTL clones.

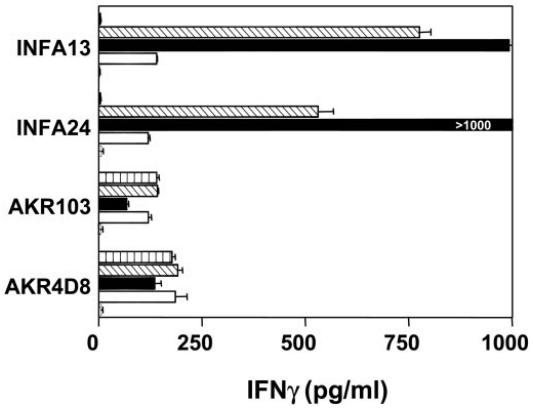

Fig. 2. IFNγ production by the human telomerase reverse transcriptase-transduced CTL clones upon activation. The level of IFNγ production by the CTL clones upon activation was determined after overnight stimulation with melAKR cells (*vertically hatched bars*), melAKR-Flu cells (*hatched bars*), JY cells loaded with influenza virus peptide GILGFVFTL (INFA13 and INFA24, *black bars*) or loaded with the MART-1 peptide EAAGIGILTV (AKR4D8 and AKR103, *black bars*), plate bound anti-CD3 antibody (*white bars*), or in the absence of target cells (*dotted bars*). IFNγ released in the culture supernatant was analyzed by ELISA. The graph shows the average of the cytokine levels of triplicate cultures; *bars,* $\pm$SD.

Effect of Adoptive Transfer of Specific T Cells on the Growth of Human Melanoma in RAG/γcKO Mice. We developed a transplantation protocol of human melanoma cells in the RAG/γcKO mice. When injected i.v. in the tail vein, melAKR melanoma cells were found to grow as microtumors in the lungs after 17 days (Fig. 3*A*). The microtumors were growing in between the alveoli, extravasating from the blood vessels (Fig. 3*B*), as well as in the pleural cavity. The number of lung microtumors varied between 200 and 900 in two entire longitudinal lung sections in different experiments. Injection of 1×10^6 melAKR cells gave rise to tumors in all of the mice, which were sufficient in number and size after 17 days to detect possible effects of treatment. The transduced cell line melAKR-Flu was observed to grow with an approximately two times shorter doubling time in culture. Injection of various tumor cell doses revealed that injection of 0.5×10^6 melAKR-Flu cells per mouse gave rise to lung microtumors at day 17 that were equivalent in number to those obtained with 1×10^6 melAKR cells. Therefore, experiments involving melAKR-Flu cells were performed with 0.5×10^6 cells/mouse.

We investigated the feasibility of this model to test the efficacy of hTERT-transduced influenza virus-specific CTL to affect the growth of tumor cells with (melAKR-Flu) or without (melAKR) specific antigen expression *in vivo*. Adoptive transfer of human CTL was performed 3–10 days after tumor transplantation. When a single dose of 5×10^6 INFA24 T cells was given i.v. at day 3, a reduction of 24% (Fig. 4*A*) in the number of melAKR-Flu lung microtumors and 48% decrease in total tumor size (Fig. 4*A*) was observed, as compared with the untreated control group. Fig. 3*C* illustrates the observed decrease in tumor size of the microtumors in a section of the lung after CTL treatment. The antitumor effect of the influenza-specific T cells was even more pronounced in the adoptive transfer of CTL clone INFA13, which mediated regression of 95% of the tumors (Fig. 4*A*). Interestingly, this difference in *in vivo* efficacy between the CTL clones was not apparent from the *in vitro* cytotoxicity assays, in which melAKR-Flu cells were lysed to the same extent by both CTL clones. As expected, treatment of melAKR tumor-bearing mice with single or repeated doses of influenza virus-specific CTL clone INFA24 neither reduced the number of microtumors nor the total tumor size (Fig. 4*A*), indicating that the antitumor effect of the CTL clones was dependent on specific tumor cell recognition by the CTL. These experiments clearly demonstrated the feasibility of our *in vivo* model to test the efficacy of *in vitro*-generated and expanded CTL clones. Importantly, the experiments also show that transduction of the telomerase gene and the resulting life span extension do not lead to an abrogation of the *in vivo* activities of the CTL clones.

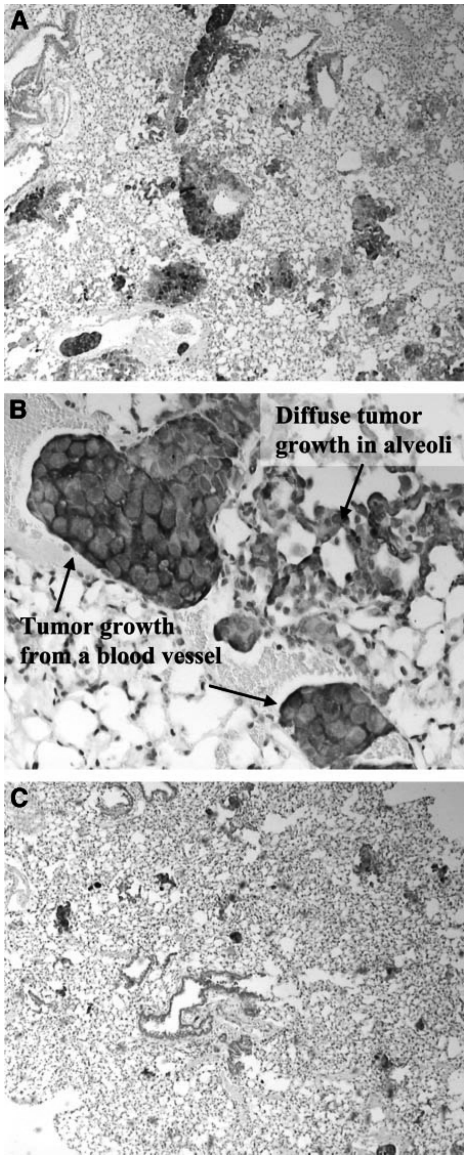

Fig. 3. Growth of human melanoma cells after i.v. injection in RAG/γcKO mice. *A,* growth of melAKR cells in the lungs of untreated mice 17 days after injection of 1×10^6 cells. Original magnification, ×100. Injection of 0.5×10^6 melAKR-Flu cells gave similar results. *B,* detail of microtumors in the lungs showing diffuse tumor growth in the alveoli and the growth of microtumors in blood vessels. Original magnification, ×400. *C,* reduction of the number and size of melAKR-Flu microtumors in the lungs of mice treated with influenza virus-specific CTL clone INFA24, as compared with untreated controls. Original magnification, ×100.

Having established that CTL clones can specifically affect tumor growth *in vivo*, we tested the effects of the MART-1-specific clones on the growth of wild-type melAKR tumors. Treatment of melAKR tumor-bearing mice with a single dose of clone AKR4D8 reduced the growth of melAKR microtumors. A significant reduction of 27% in the number of microtumors and 46% reduction in total tumor size were observed when three doses of CTL AKR4D8 were given (Fig. 4*B*). Repeated adoptive transfer of AKR4D8 T cells may, thus, reduce both tumor cell seeding and tumor growth. Injection of clone AKR103 gave a significant reduction in the number of microtumors of 24% at a single dose and of 41% at three doses (Fig. 4*B*), which was also evident from the significant reduction in the total tumor size, showing a reduction of 29% at a single dose and 42% at multiple doses. Tumor growth inhibition mediated by clone AKR103 was significant at a single dose of CTL, which indicates that clone AKR103 was more effective in mediating tumor regression *in vivo* than clone AKR4D8. Therefore, we performed additional experiments with clone AKR103. Surprisingly, treatment of mice bearing melAKR-Flu lung tumors with one or three doses of CTL clone AKR103 did not result in any tumor regression, neither in the number of lung microtumors, nor in the total tumor size (Fig. 4*B*). Immunohistochemical analysis of the melAKR-Flu tumor-bearing lung sections after treatment with CTL AKR103 showed an intact expression of MART-1 in the tumor cells, indicating that the lack of tumor growth inhibition was not due to the selective loss of MART-1 antigen expression. Thus, although melAKR and melAKR-Flu cells were both lysed by clone AKR103 *in vitro*, clone AKR 103 failed to affect the growth of melAKR-Flu tumors *in vivo*. Moreover, the equal levels of melAKR-Flu and melAKR target cell lysis *in vitro*, render it unlikely that competition of the influenza virus peptide and the endogenous MART-1 peptide for binding to HLA-A2 decreased the recognition of melAKR-Flu cells by the MART-1-specific CTL clones *in vivo*. These results show that cytolytic activity of CTL *in vitro* is not always predictive of a

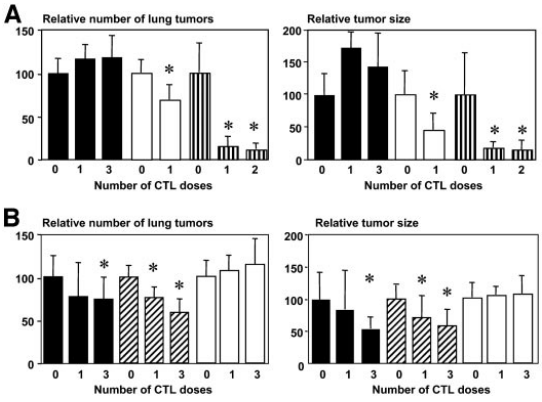

Fig. 4. Reduction of tumor growth by adoptive transfer of specific CTL clones *in vivo*. *A,* tumor growth inhibition of melAKR-Flu tumors in groups of 8 RAG/γcKO mice treated with a single (*1*) dose of CTL clone INFA24 (□), or a single (*1*), or two (*2*) doses of clone INFA13 (▨). ■, absence of tumor growth inhibition in the treatment of melAKR tumor with one or three doses of CTL INFA24. The average number of lung micro-tumors (*left graph*) and the total tumor size (*right graph*) in the control group was set at 100%, and the number and size of the tumors in the treated mice were calculated relative to the untreated control group for each experiment. Graphs show the average relative tumor growth reduction of three independent experiments; *bars,* ±SD. Significant decreases, as tested by the student *t* test, are indicated by ∗: one dose of clone INFA24 *versus* untreated mice, number of tumors: $P = 0.009$, tumor size: $P = 0.001$; one dose or two doses of clone INFA13 *versus* untreated mice, number of tumors: $P = 0.000$ and $P = 0.000$, tumor size: $P = 0.002$ and $P = 0.001$, respectively. *B,* tumor growth inhibition of melAKR tumor cells in RAG/γcKO mice treated with a single (*1*) dose or three (*3*) doses of CTL clone AKR4D8 (■) or clone AKR103 (▨). □, treatment of melAKR-Flu tumor-bearing mice with one or three doses of clone AKR103. Graphs show the relative average number of microtumors in the lungs (*left*) or the relative total tumor size (*right*) at day 17 in three independent experiments with groups of 8 mice, as described in *A*. Significant decreases in tumor growth are indicated by ∗: three doses of clone AKR4D8 *versus* untreated mice, number of tumors: $P = 0.048$, tumor size: $P = 0.029$; one or three doses of clone AKR103 *versus* untreated mice, number of tumors: $P = 0.005$ and $P = 0.000$, tumor size: $P = 0.039$ and $P = 0.008$, respectively.

capacity to effect tumor regression *in vivo*. The efficacy of treatment with influenza-specific T cells indicated that melAKR-Flu melanoma tumors were susceptible to CTL treatment *in vivo*. It should, however, be noted that the effect of CTL on the tumor growth *in vivo* measured at day 17 is the net result of the inhibitory effect of the CTL and the growth rate of the tumor. The inhibitory effect of CTL AKR103 on the melAKR-Flu tumors may have been comparable with the parental melAKR tumors, similar to what was observed in cytotoxicity assays. However, as melAKR-Flu tumors were growing faster *in vivo* than the parental melAKR tumors, it is possible that the relatively modest inhibitory effect of CTL AKR103 treatment during the first few days after CTL injection was lost by the faster growth rate of the melAKR-Flu cells during the next days.

Other aspects that influence the success rate of adoptive transfer of CTL are the localization of the CTL to the tumor and T-cell survival *in vivo*. These variables determine the time frame in which the CTL must exert their cytolytic activity against the tumor cells. To investigate these aspects in our *in vivo* model, we analyzed the lungs of the treated and control mice at day 17 for the presence of injected CTL by flow cytometry. In our adoptive transfer experiments, a small population of injected viable CTLs that bound human CD45-specific and human CD8-specific antibodies, as well as specific tetramers, was still present in the lungs at day 17. The number of CTLs varied among mice within a group, but the average number did not differ significantly between groups of mice treated with the four different CTL clones (data not shown). This suggests that the greater tumor growth inhibition by the influenza virus-specific CTL clones, as compared with the MART-1-specific CTL clones, probably did not result from an increased tumor localization or T-cell survival. To follow the kinetics of CTL in the circulation and lungs after injection in more detail, we measured the presence of CTL clone INFA24 after 3 h, 3 days, 7 days, and 14 days after injection of 5×10^6 T cells in melAKR-Flu tumor-bearing mice (Fig. 5). Viable CTLs were detectable in the peripheral blood at a concentration of 25 cells/10,000 murine PBMC (0.25%), and ~9,500 injected T cells (0.18%) are present in the lungs during the first 3 days after injection, followed by rapid clearance between day 3 and day 7 after injection. The tumor cells in the lungs started to increase in number after day 3 when most of the injected CTLs were cleared. These results show that the injected INFA24 CTLs mediated the antitumor effect at very low E:T cell ratios and were able to effect up to 40% reduction in tumor growth (Fig. 5). Moreover, these results suggest that the CTLs mediated their antitumor effect during the first 3 days after injection.

We have described previously that ectopic hTERT expression immortalizes human T cells, and that the hTERT-transduced T cells remain dependent on TCR-mediated stimulatory signals to enter cell cycle *in vitro* (17, 18). Our *in vivo* results of the hTERT-transduced CTL detection in the lungs of the treated mice showed that the hTERT-transduced cells had not given rise to T-cell malignancies during the adoptive transfer experiments. These results suggest that hTERT-transduced CTLs had not started to grow in an uncontrolled manner upon injection *in vivo*. Because these experiments lasted only 17 days, we injected hTERT-transduced CTLs in a group of 14 nontumor-bearing mice and examined the presence of T cells after 3–11 months. Viable human CTLs were detectable in the lungs of these mice after 3 and after 6 months, albeit at low numbers. No viable T cells were found in the mice 9 or 11 months after CTL injection, which is probably due to the absence of exogenous rhIL-2 during this prolonged time of follow up. The mice did not have any abnormalities that might have been caused by the injection of hTERT-transduced T cells, suggesting that a part of the CTLs survived in these mice, but had not developed into malignancies within 11 months.

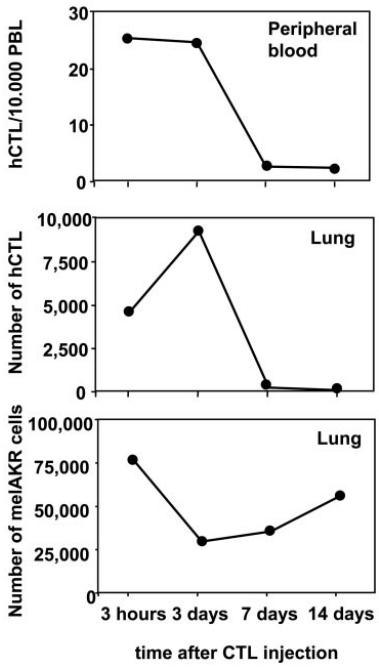

Fig. 5. Kinetics of injected human CTL. RAG/γcKO mice injected with 0.5×10^6 melAKR-Flu tumor cells and treated after 3 days with a single dose of 5×10^6 INFA24 T cells. Injected T cells and tumor cells were detected by flow cytometry. Graphs show the presence of injected CTL INFA24 in the peripheral blood (*top graph*), and in the lungs (*middle graph*), as well as the number of tumor cells in the lungs (*bottom graph*) at 3 h, 3 days, 7 days, or 14 days after T-cell transfer. Values are the average of 2 mice/time point.

Tumor Growth Inhibition Mediated by hTERT-Transduced CTL, as Compared with Untransduced CTL. After having determined that the hTERT-transduced CTL clones are able to reduce the growth of tumors, it was important to determine whether the hTERT transduction reduced or potentiated the *in vivo* activities of the CTL clones. Therefore, we compared the effect of adoptive transfer of hTERT-transduced CTLs with untransduced CTLs. Due to the limited life span of cloned CTLs (15), sufficient cells of the untransduced CTLs for adoptive transfer could only be generated of clone INFA13. We performed this experiment in a novel model of noninvasive *in vivo* tumor growth detection in mice (33). This method would allow us to determine whether there would be differences between hTERT and untransduced CTLs in *in vivo* activities at different time points. To this end, melAKR-Flu cells were transduced with a retrovirus encoding the luciferase gene (melAKR-Flu-Luc), which allows detection of the tumor cells *in vivo* by the bioluminescence signal by the luciferase activity upon enzymatic conversion of the substrate luciferin. This method enables the monitoring of tumor growth during the experiment at multiple time points and represents a quantitative measure of the tumor load (33). Fig. 6 shows that the hTERT-transduced cells of clone INFA13 almost completely inhibited the growth of melAKR-Flu-Luc tumors in RAG/γcKO mice. This tumor growth inhibition was comparable with the effect of the untransduced CTLs of clone INFA13, indicating that hTERT transduction did not affect the *in vivo* efficacy of human CTLs *in vivo*. These results are consistent with our previously published *in vitro* data showing that ectopic hTERT ex-

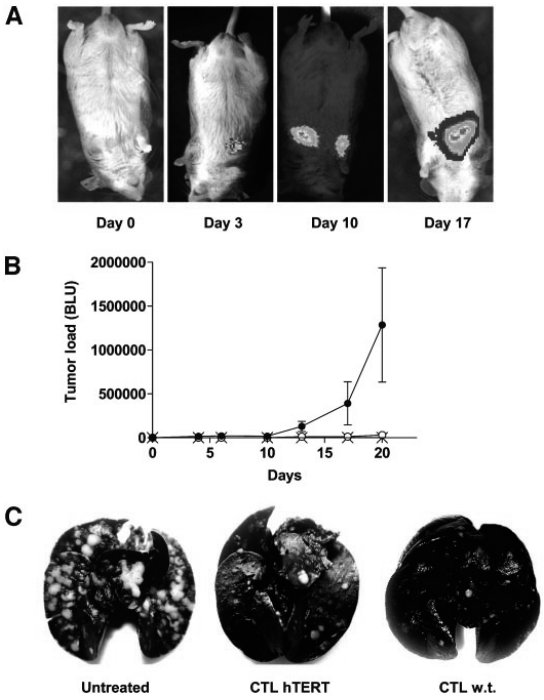

Fig. 6. Bioluminescence imaging of tumor growth inhibition mediated by human telomerase reverse transcriptase-transduced or untransduced CTL. *A,* pseudocolor image representing the spatial distribution of photon counts within the animal (bl*ue,* least intense and *red,* most intense) overlayed on the gray-scale reference image, allowing anatomical localization of the melAKR-Flu-Luc tumors. Pictures show a representative picture of the tumor load at days 0, 3, 10, and 17 in untreated mice. *B,* RAG/γcKO mice injected with 0.5×10^6 melAKR-Flu-Luc tumor cells and treated after 3 days with a single dose of 5×10^6 hTERT-transduced INFA13 T cells (○), or untransduced INFA13 T cells (*), or left untreated (●). Tumor growth was monitored at days 4, 6, 10, 13, 17, and 20 by injection of luciferin and detection of the bioluminescence (*BLU*). Graph shows the average values of 5 mice/group. *C.* India ink staining of the lungs of melAKR-Flu-Luc tumor-bearing mice treated with untransduced INFA13 T cells (*right*), hTERT-transduced INFA13 T cells (*middle*), or untreated mice (*left*); *bars,* ±SD.

pression does not change the functional activity of human T cells (17, 18, 24).

DISCUSSION

In the present report we have shown that hTERT-transduced human CTLs are capable of inhibiting human tumor growth *in vivo*. A single dose of CTL was already sufficient to mediate significant reduction in tumor growth, and tumor growth inhibition was dependent on specific recognition of the tumor cells by the CTL. To our knowledge, this is the first report of the *in vivo* functional activity of hTERT-transduced human T cells. Our results with hTERT-transduced human CTL *in vivo* are consistent with the results of human CTL therapy in other xenograft models in immunodeficient mice, showing tumor growth inhibition by the infused CTL (1, 3–5). However, the mice used in these previous studies were nude mice, or SCID mice, in which minimal levels of T- and B-cell development or the presence of natural killer cells may have hampered interpretation of the antitumor effect mediated by the infused T cells. We used mice that completely lacked T, B, and natural killer cells. Moreover, RAG/γcKO mice do not develop thymomas, which usually occur in NOD/SCID mice, allowing the use of these mice in long-term experiments. Most reports on adoptive transfer of human CTLs in xenograft models describe the antitumor effect of one single CTL population or CTL clone *in vivo* (1, 3–5). In the present study, we have compared different human CTL clones for their growth-inhibiting effect in one tumor model, and observed that infusion of CTL clones, which are functionally active *in vitro,* can have variable outcomes *in vivo*. It is important to note here that these experiments could only be made because the hTERT transduction allowed for generation of unlimited numbers of CTLs.

Clinical studies have shown the success of adoptive transfer of human T cells to mediate tumor regression in melanoma and renal cell carcinoma patients. The potential application and success rate of adoptive transfer depends, however, on the isolation of tumor-reactive T cells of each patient. Moreover, successful treatment may require a more diverse population of both CD8+ and CD4+ T cells with various antigen specificities. Multiepitope targeting by T cells may avoid immune escape of tumor cells that have lost the expression of the targeted antigen. Furthermore, the addition of CD4+ T cells to the infused cells has been shown to enhance human CTL graft survival in immunodeficient mice (34), and may be important to maintain CTL effector function. To apply adoptive transfer of mixtures of well-defined monoclonal T-cell populations to more patients, it is important to expand a large portion of the patient-derived T-cell population to select for T cells recognizing the tumor cells. Ectopic hTERT expression allows the expansion of human T cells to enable long-term adoptive transfer treatment with repeated doses of T cells without the limitation of the life span of human T cells. Therefore, T cells can be selected for the tumor reactivity upon hTERT transduction without additional selection on replicative age. We have described previously that hTERT-transduction does not change the antigen specificity and functional activity of human CD8+ and of CD4+ T cells *in vitro*. In the present report we show that hTERT-transduced CTL clones are capable of inhibiting tumor growth *in vivo* and mediate tumor growth inhibition to the same extent as the untransduced CTL. Therefore, ectopic hTERT expression allows the expansion of human CTL without affecting the *in vivo* functionality. hTERT-transduced T cells may be considered for application in adoptive transfer procedures, provided appropriate assessment of the possible risk of hTERT-transduced T cells to acquire a malignant phenotype *in vivo*. To decrease this risk and in view of the observation that hTERT expression is not required for *in vivo* function, additional research will focus on ways to eliminate the ectopic hTERT expression in the T cells after large-scale expansion and before adoptive transfer.

Our *in vivo* study has shown several aspects of adoptive transfer of human T cells that influence the *in vivo* efficacy. When comparing different CTL clones both *in vitro* and *in vivo*, we have observed that the *in vitro* assays, such as specific tetramer binding and cytotoxicity assays were not fully predictive of the *in vivo* efficacy of CTL clones (Table 1). Both influenza virus-specific CTL clones, INFA24 and INFA3, equally lysed melAKR-Flu cells *in vitro*, and displayed equal functional avidity, IFNγ production, and tetramer binding. However,

Table 1 *Correlation between results of in vitro functional assays and in vivo antitumor efficacy*

	CTL clone			
	AKR4D8	AKR103	INFA13	INFA24
Lysis of melAKR cells *in vitro*	+	+	−	−
Inhibition of melAKR tumor growth *in vivo*	+	+	−	−
Lysis of melAKR-Flu cells *in vitro*	+	+	+	+
Inhibition of melAKR-Flu tumor growth *in vivo*	Not tested	−	++	+
IFNγ production[a]	+	+	++	++
Specific tetramer binding	+	−	++	++

[a] In cocultures with melAKR-Flu cells.

CTL clone INFA13 was significantly more effective *in vivo* than clone INFA24. This difference was not apparent from the above-mentioned *in vitro* assays and may be the result of other yet-to-be-defined functional differences of the CTL that may affect the *in vivo* activity. These findings illustrate the limitations of *in vitro* assays to predict *in vivo* efficacy of CTL and the importance of testing CTLs for their functionality *in vivo*. Furthermore, although melAKR-Flu tumor cells were equally well killed *in vitro* by both the influenza virus-specific CTL clones and the MART-1-specific CTL clones, the influenza virus-specific CTL clones produced more IFNγ upon recognition of melAKR-Flu cells. These results may indicate a rate-limiting effect in the cytotoxicity assays caused by the level of sensitivity of target cells to lysis by CTL, which, therefore, do not reveal quantitative differences between highly lytic CTL clones. The importance of the CTL avidity for the *in vivo* efficacy upon adoptive transfer was demonstrated for tyrosinase-related protein 2-specific murine CTL in syngeneic mouse model (7) and for gp100-specific human CTL clones in a nude mouse model (6). These findings are consistent with our data. The influenza virus-specific CTL displayed a higher tetramer binding intensity and functional avidity, and produced more IFNγ upon activation with specific antigen, as compared with the MART-1-specific CTL clones. This correlated well with the higher efficacy of the influenza virus-specific CTL in mediating tumor growth inhibition *in vivo*. The elevated level of IFNγ production by the influenza virus-specific CTL upon recognition of the melAKR-Flu tumor cells may have additionally enhanced the *in vivo* efficacy of these clones. Local IFNγ production in the tumor leads to an increased HLA class I expression on tumor cells, allowing better tumor cell recognition by the CTL. Adoptive transfer of murine CTL transduced with the IFNγ gene was found to be more effective in mediating tumor regression in a syngeneic setting than the parental CTL (35), suggesting a positive effect of IFNγ production by the CTL on the therapeutic efficiency.

In conclusion, the discrepancies found between *in vitro* assays and *in vivo* antitumor effect of CTL (Table 1) suggest that the efficacy of CTL clones to eradicate tumors *in vivo* depends on the balance between CTL effector function and tumor growth *in vivo*. The adoptive transfer therapy model described in the present report reveals the efficacy of human CTL clones *in vivo*, and allows selection of CTLs capable of mediating superior therapeutic effects. Furthermore, our model allows evaluating adoptive transfer strategies of $CD8^+$ T cells combined with tumor-specific $CD4^+$ T cells or dendritic cells to enhance the antitumor effect.

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Chapter 5

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

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

Introduction

Adoptive cell therapy (ACT) with TCR modified T cells is no longer a mere preclinical strategy but is now analyzed in phase I clinical trials. The rationale behind the development of TCR modified T cell therapy is persuasive. For tumor-associated antigens for which the endogenous T cell repertoire is limited in size or activity due to self-tolerance, it seems reasonable to supply this repertoire by infusion of genetically engineered tumor specific T 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 antigens[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 utilized to enhance the expression of the introduced T cell receptor. These latter two approaches can also suppress the formation of mixed TCR dimers that are composed of endogenous and exogeneous 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 antigen encounter *in vivo*[14-16], even when the endogenous T cell repertoire is non-responsive[17,43]. More recent work has provided first evidence for the clinical potential of TCR gene therapy[18]. In this phase I clinical trial, patients with metastatic melanoma were treated with autologous T lymphocytes engineered to express a TCR specific for the melanocyte differentiation antigen MART-I. Notably, following T cell infusion, tumor regression was observed in 2 patients and these clinical responses appeared to correlate with the magnitude of the TCR modified T cell response upon infusion.

While these preclinical and clinical data suggest that the underlying rationale behind this therapy is valid, it is important to emphasize that substantial improvements are required to transform TCR gene transfer into a clinically meaningful strategy. Specifically, the clinical data obtained to date have shown that persistence of TCR gene modified T cells in individual patients is variable, and that the expression of the introduced MART-I-specific TCR was markedly lower than TCR expression from the endogenous loci[18]. Perhaps because of this, with a response rate of 2/17, clinical effectiveness of TCR gene transfer was clearly less than that of prior trials by the Rosenberg group that involved infusion of *ex vivo* expanded tumor-infiltrating lymphocytes[19,20]. The results from murine studies support the notion that the current protocols for adoptive therapy with TCR modified T cells are still suboptimal. Specifically, while infusion of TCR modified T cells can be used to halt the outgrowth of transplantable[17] and spontaneously developing tumors[43] 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 anti-tumor activity of TCR modified T cells *in vivo*. We reasoned that improvements in TCR gene therapy could involve one of either three factors: First, alterations within the format of the introduced TCR genes; Second, modification of the cell graft; Third, adjustment of the host environment that the gene modified T cells encounter upon infusion. Within this study we chose to analyze one parameter representing each of these three different aspects.

Materials & Methods

Mice. RIP-OVA^{hi} mice[21] 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-Iα-IRES-OT-Iβ retrovirus encoding the non-modified OT-I TCR genes (pMX-OT-I_{wt}) has been described[17]. Optimized OT-I TCR genes were produced by GeneArt (GeneArt GmbH, Regensburg, Germany) and cloned into the retroviral vector pMX to create pMX-OT-Iαopt-IRES-OT-Iβopt (pMX-OT-I_{opt}). Mouse splenocytes were modified by retroviral transduction

as described previously[14]. Mice received an adoptive transfer of $1x10^6$ OT-I TCR transduced or mock transduced $CD8^+$ T cells. Where indicated, TCR modified T cells were mixed with a 9-fold excess of mock-transduced cells, either unmanipulated or depleted for $CD8^+$, $CD4^+$ or $CD25^+$ cells. For depletion, passenger cells were incubated with PE-labeled anti-CD25, anti-CD8 or anti-CD4 mAb (all from BD Pharmingen) respectively. Subsequently, cells were incubated with anti-PE beads (Miltenyi Biotec, Bergisch-Gladbach, Germany), and negative selection was performed by autoMACS (Miltenyi Biotec) according to manufacturer's guidelines. Depletion of $CD8^+$ and $CD4^+$ cells was performed after T cell activation. Depeletion of $CD25^+$ 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 ovalbumin (OVA) (aa 161-385) and the murine CD4 molecule as a marker gene product[17] was cultured in RPMI supplemented with 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin. Prior to inoculation, cells were washed three times with HBSS (Gibco, Auckland, New Zealand) to remove serum components and $1x10^5$ cells were injected subcutaneously in the right flank. Tumors were measured with calipers and mice were killed once tumors reached an average diameter of 10mm. For *ex vivo* analysis of antigen expression, sliced tumors were incubated in medium supplemented with collagenase IV (0.2 mg/ml; Worthington, Lakewood, NJ) and DNaseI (25 μg/ml; Roche, Mannheim, Germany) for 20-30 minutes at 37°C. Single cell suspensions were generated with the aid of a cell strainer (BD Biosciences, Erembodegem, Belgium). Erythrocytes were removed by NH_4Cl treatment, and cells were subsequently cultured in RPMI supplemented with 10% FCS and antibiotics. After 1-3 days of culture, expression levels of the CD4 marker gene product on cells recovered from tumor material were measured as a surrogate marker of OVA expression, and were compared to CD4 expression levels on cultured B16-OVA and B16 cell lines after corresponding times of *in vitro* culture.

Flow cytometry. Surface TCR expression was measured 24 hours after retroviral transduction by flow cytometry. Cells were stained with FITC- or PE-conjugated anti-TCR Vα2 and anti-TCR Vβ5 mAbs (the Vα and Vβ segments used by the OT-I TCR), and APC-conjugated anti-CD8α mAb (Pharmingen). Propidium iodide (Sigma) 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 (Microvette CD 300 Li-Heparine, Omnilabo, Breda, The Netherlands) at the indicated days post-transfer. Following removal of erythrocytes by NH_4Cl treatment, the cells were stained with the indicated antibodies and analyzed by flow cytometry. Data acquisition and analysis was done on a FacsCalibur (Becton Dickinson, MountainView, CA) with CellQuest and FCS express (De Novo Software, Thornhill, Ontario, Canada) software.

Irradiation-induced host conditioning and viral vaccination. Irradiation-induced host conditioning was achieved by 5 Gy total body irradiation (TBI) with a radiobiology constant potential X-ray unit (Pantak HF-320; Pantak Limited, Reading, United Kingdom), one day before adoptive cell transfer. For viral vaccination, mice were infected intraperitoneally at the indicated timepoints with $1x10^6$ PFU of a recombinant vaccinia strain that expresses ovalbumin (rVV-OVA)[22].

Measurement of blood glucose levels and treatment of diabetes. To monitor the onset and severity of diabetes, mice were weighed regularly throughout experiments and in case of weight loss, blood glucose levels were monitored by Accu-Check Compact (Roche Diagnostics, Germany) measurement. Mice were considered diabetic when blood glucose levels reached > 20 mmol/L. To allow long-term follow-up, diabetic mice were treated by subcutaneous introduction of insulin implants according to the manufacturer's protocol (LinShin Canada, Inc.).

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

Results

ACT with TCR transduced T cells upon irradiation-induced host conditioning.

Two fundamentally distinct strategies can be used to drive the expansion of adoptively transferred T cells *in vivo*. When the cognate antigen of the introduced T cells is provided by vaccination, TCR triggering is induced and the resulting T cell proliferation and T cell differentiation parallels that seen during physiological T cell responses. As an alternative to antigen-specific vaccination, host conditioning regimens such as non-myeloablative chemotherapy or irradiation can be utilized to promote the outgrowth of infused T cell populations. The mechanisms that drive T cell proliferation and differentiation in the latter case are thought to be substantially more diverse. First, the reduction in T cell and NK cell numbers that is achieved by host conditioning leads to an enhanced availability of IL-7 and IL-15, cytokines that can induce T cell proliferation independent of the presence of cognate antigen. In addition, depletion of regulatory T cells and release of adjuvants from intestinal bacteria may further drive T cell activation. Finally, in case tumor-specific T cells are infused into tumor-bearing hosts, release of cognate antigen as a consequence of tumor cell death may be an added contributing factor. Importantly, due to the fact that T cell expansion upon vaccination and host conditioning is driven by distinct mechanisms, both the persistence and functional properties of the induced T cell population can differ[23,24]. Specifically, while vaccination results in the rapid emergence of a highly differentiated pool of effector T cells[25], T cell populations induced by host conditioning display properties of memory T cells[23], possibly translating in an enhanced capacity for long-term persistence.

Irradiation- and chemotherapy-induced host conditioning prior to adoptive T cell transfer has been used to enhance the *in vivo* expansion and anti-tumor effect of TCR-transgenic T cells in mouse models[26] and of tumor-infiltrating lymphocytes (TIL) in melanoma patients[19]. Likewise, in the phase I TCR gene therapy trial by Morgan and colleagues, chemotherapy-induced host conditioning was used with the aim to facilitate engraftment of the infused TCR modified T cells. However, in preceding preclinical studies of TCR gene transfer in mouse models[14,17], vaccination rather than host conditioning has been used to drive activation and expansion of the transferred TCR modified T cells, and a comparison of the two strategies has not been made.

To first develop a mouse model that allows a comparison of the relative value of host conditioning regimens, vaccination regimens and other variables in TCR gene transfer, RIP-OVA^{hi} were injected subcutaneously with B16 tumors expressing OVA. As documented previously, RIP-OVA^{hi} mice are tolerant towards the self antigen ovalbumin. As a consequence, the endogenous T cell repertoire is unable to influence the outgrowth of B16-OVA tumors, even upon 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-OVA^{hi} mice were challenged with B16-OVA tumor cells. Subsequently, mice were either left untreated, or were treated on day 6 by 5 Gy total body irradiation (TBI, leading to sublethal lymphodepletion), followed by transfer of $1x10^6$ 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. 1A-B; average survival of 24 versus 60 days; $p< 0.005$). Furthermore, in recipients of OT-I transduced cell populations a highly dominant CD8$^+$ cell population expressing the 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 month) (Fig. 1C). Infusion of OT-1 TCR transduced cells into non-conditioned recipients had no substantial effect on tumor growth or survival as compared to 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 antigen can lead to a prolonged anti-tumor effect in an otherwise self tolerant setting. Furthermore, this combination yields a T cell repertoire that is markedly skewed towards tumor reactivity.

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

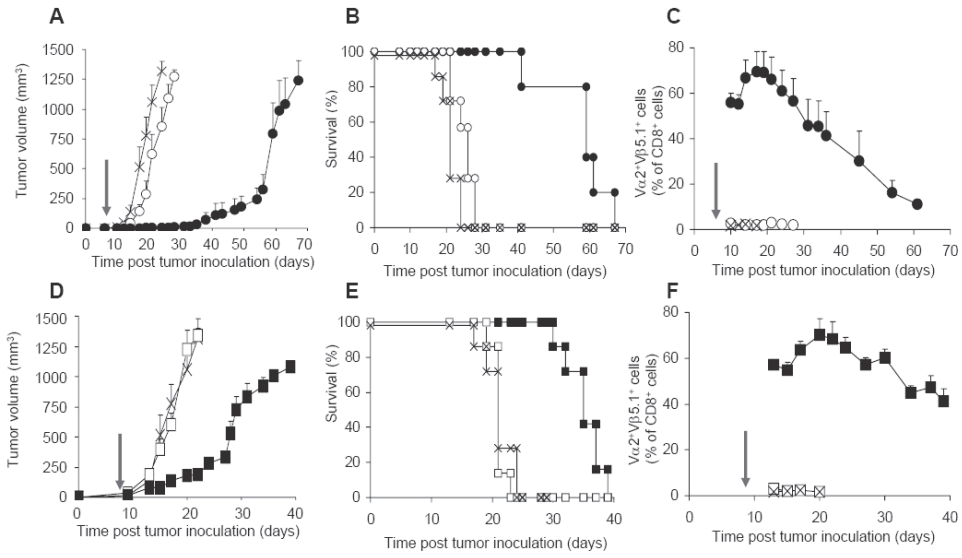

Figure 1. Transfer of TCR transduced T-cells in combination with irradiation-induced host conditioning. RIP-OVA^{hi} mice (N=5-7 per group) were inoculated with 1x10^5 B16-OVA tumor cells subcutaneously, followed by sublethal TBI at day 6 (A-C) or day 9 (D-F) and transfer of 1x10^6 OT-Iwt TCR transduced CD8+ T cells (filled symbols) or an equal amount of mock transduced T cells (open symbols) at day 7 (A-C) or day 10 (D-F). Tumor growth was compared to that in control mice (depicted by crosses). (A, D) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. Arrow indicates time point of adoptive transfer. (B, E) Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 10mm or when tumors started bleeding. P-values of B: irradiation vs irradiation + ACT: 0.0014; no treatment vs irradiation + ACT: 0.0014; no treatment vs irradiation 0.016 (Mantel-Cox test); P-values of E: irradiation vs irradiation + ACT: 0.0002; no treatment vs irradiation + ACT: 0.0002; no treatment vs irradiation 0.35 (Mantel-Cox test) (C, F) Analysis of blood cells of irradiated RIP-OVA^{hi} mice at indicated time points post adoptive cell transfer. Bars indicate SD.

Irradiation-induced host conditioning outperforms vaccination as an engraftment regimen for TCR modified T cells.

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

In vivo activation of OT-I transduced T cells by vaccination with rVV-OVA resulted in a very rapid burst in both the number and frequency of TCR modified T cells, with a peak frequency of TCR modified T cells of 10.3% of $CD8^+$ cells on day 5 after transfer. Comparison of TCR modified T cell numbers in these mice that received rVV-OVA with those in mice that had been pretreated by TBI showed that the absolute number of TCR modified T cells early after transfer was indeed significantly higher in mice that received viral vaccination ($p<0.005$ at day 7). However, within the second week post transfer, numbers of TCR modified T cells significantly declined in rVV-OVA vaccinated mice. Because of this contraction, and because of the continuing homeostatic T cell proliferation in recipients treated by TBI, $V\alpha2^+V\beta5.1^+$ $CD8^+$ T cell numbers in TBI-treated mice exceeded those in rVV-OVA vaccinated mice on day 10 post adoptive transfer and onwards ($p<0.05$ at days 12 and 17).

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

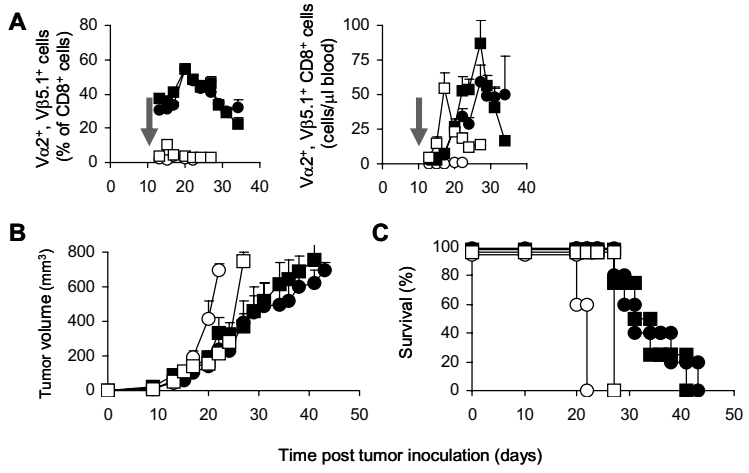

Figure 2: Enhanced persistence and anti-tumor effect of TCR transduced T cells after irradiation-induced host conditioning as compared to active vaccination. RIP-OVA^{hi} mice (N=5-7 per group) were inoculated with $1x10^5$ B16-OVA tumor cells subcutaneously, and received an adoptive transfer $1x10^6$ OT-I TCR transduced $CD8^+$ T cells (filled circles, filled squares, open squares) or an equal amount of mock transduced T cells (open circles) at day 10. Transferred T cells were boosted either by sublethal TBI at day 9 (filled circles, open circles), vaccination with rVV-OVA at day 10 (open squares) or sublethal TBI at day 9, followed by vaccination with rVV-OVA at day 13 (filled squares). (A) Analysis of $V\alpha2^+V\beta5.1^+$ $CD8^+$ cells in peripheral blood (percentage in left panel, absolute numbers right) at indicated time points post adoptive transfer. Bars depict SEM. (B) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. (C) Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 10mm or when tumors started bleeding. P-values: irradiation versus ACT + vaccination or ACT + irradiation or ACT + irradiation + vaccination: <0.005; ACT + irradiation vs ACT + vaccination: 0.0055; ACT + irradiation vs ACT + irradiation + vaccination: 0.5; ACT + irradiation + vaccination vs ACT + vaccination: 0.01 (Mantel-Cox test).

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

Gene optimization results in a moderate increase in TCR expression but marked increase in anti-tumor efficacy.

As described previously, modification of T cell receptor formats such as inclusion of a second interchain disulfide bond, incorporation of the murine constant domains and optimization of gene sequences can all lead to enhanced expression of the introduced T cell receptor. Furthermore, for the latter type of gene optimization this was accompanied by a clear increase in the number of TCR-modified T cells detected upon infusion into recipient mice[8]. To assess whether alterations that enhance the expression of introduced TCR genes also enhance the *in vivo* anti-tumor activity of TCR modified T cells, a gene-optimized variant of the OT-I TCR (termed OT-I_{opt}) was created, and RIP-OVA^{hi} derived splenocytes were retrovirally transduced with either the wild type OT-I TCR or the gene optimized variant (Figure 3A). Gene optimization resulted in a 1.4 fold increase in transduction efficiency as revealed by anti-Vα2 and anti-Vβ5 staining (50% versus 69% of $CD8^+$ T cells after correction for endogenous $V\alpha2^+V\beta5^+$ cells), and this was accompanied by a 1.3 fold increase in average TCR expression (MFI of 455 versus 603 for the TCRα chain; 37 versus 50 for the TCRβ chain).

To determine the effect of OT-I TCR gene optimization on the anti-tumor activity of OT-I TCR transduced T cells *in vivo*, $1x10^6$ OT-I, OT-I_{opt} or mock transduced $CD8^+$ T cells were transferred into tumor bearing, sublethally irradiated RIP-OVA^{hi} mice. Within the first weeks post infusion, the percentage of $V\alpha2^+V\beta5.1^+$ $CD8^+$ T cells was slightly increased in mice that received OT-I_{opt} TCR transduced T cells, as compared to recipients of T cells expressing the parental OT-I TCR (Figure 3B, left panel), likely reflecting the somewhat higher transduction efficiency. However, the increase in absolute numbers of $V\alpha2^+V\beta5.1^+$ $CD8^+$ T cells did not reach significance (day 10-21; $p=0.2$-0.4) (Figure 3B, right panel).

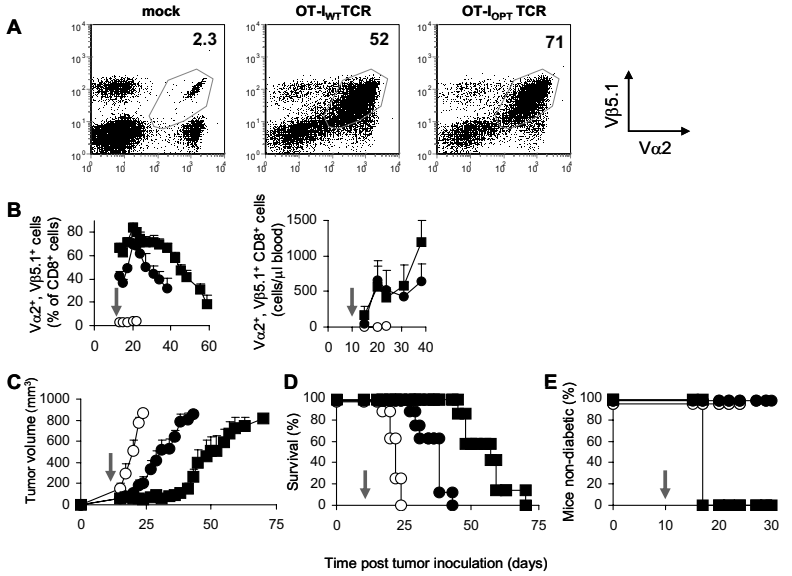

Figure 3: TCR gene optimization increases *in vivo* anti-tumor activity. (A) Flow cytometric analysis of mock (left), OT-I_{wt} (middle) or OT-I_{opt} (right) transduced T cells prior to adoptive transfer. The number in the upper right corner of each dot-plot reflects the percentage of $V\alpha2^+V\beta5^+$ cells within the $CD8^+$ population. (B-E) RIP-OVA^{hi} mice (N=7-8 per group) were inoculated with $1x10^5$ B16-OVA tumor cells subcutaneously, sublethally irradiated at day 9 and received an adoptive transfer of $1x10^6$ OT-I_{wt} TCR transduced $CD8^+$ T cells (filled circles), $1x10^6$ OT-I_{opt} TCR transduced $CD8^+$ T cells (filled squares), or an equal amount of mock transduced T cells (open circles) at day 10. (B) Analysis of $V\alpha2^+V\beta5^+$ $CD8^+$ cells in peripheral blood (percentages in left panel, absolute numbers in right panel) at indicated time points post adoptive transfer. Bars depict SEM. (C) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. (D) Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 10mm or when tumors started bleeding. P-values: irradiation versus irradiation + ACT OT-I_{wt} or irradiation + ACT OT-I_{opt}: <0.005; irradiation + ACT OT-I_{wt} vs irradiation + ACT OT-I_{opt}: <0.005 (Mantel-Cox test) (E) Induction of diabetes. Mice were considered diabetic once blood glucose levels exceeded 20 mmol/l.

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

Precursor frequency of TCR modified T cells determines anti-tumor effect.

While the fraction of T cells that becomes antigen-responsive upon transduction of murine T cells with mouse TCRs such as the OT-I TCR is markedly high, the percentage of antigen-responsive or MHC tetramer-positive T cells that is obtained upon transduction of

human T cells with human tumor-specific TCRs generally appears to be substantially lower. Although infusion of large numbers of TCR modified T cells is still feasible with the transduction efficiencies that can be achieved in a clinical setting[18], the resulting cell grafts do contain a higher number of non modified 'passenger' cells.

To examine whether the presence of a large number of passenger cells in such grafts could affect the *in vivo* potential of the TCR modified T cells, we prepared T cell grafts containing an equal amount ($1x10^6$) of OT-I_{opt} TCR transduced T cells but with different amounts of 'passenger cells'. Rather than generating such grafts by transduction with different amounts of retrovirus (in which case the reduced expression of the TCR transgene seen at lower virus doses would be a confounding factor), a single batch of TCR modified T cells was prepared, which was then either used directly, or was mixed with a 9-fold excess of mock-transduced cells (referred to as the 'low passenger group' and 'high passenger group', respectively; 56% $V\alpha2^+V\beta5.1^+$ cells of $CD8^+$ T cells and 5.6 % $V\alpha2^+V\beta5.1^+$ cells of $CD8^+$ T cells. Subsequently, the cells were transferred to B16-OVA bearing RIP-OVA^{hi} mice that had been conditioned by TBI, and T cell responses and tumor outgrowth were monitored.

Comparison of peripheral blood samples of recipients of high passenger or low passenger cell grafts revealed that the 10-fold difference in TCR modified T cell frequency prior to ACT was compressed to a difference of less than ~3-fold (79% $V\alpha2^+V\beta5.1^+$ of $CD8^+$ T cells in the 'low passenger group' versus 33% $V\alpha2^+V\beta5.1^+$ of $CD8^+$ T cells in the 'high passenger group') (Fig. 4A, left panel). The preferential outgrowth of T cells that express the OT-I TCR that is observed in particular upon infusion of cell grafts with low TCR modified T cell frequencies suggests that part of the *in vivo* proliferation is driven by TCR-specific interactions. This is consistent with the possibility that recognition of the cognate OVA antigen by TCR modified T cells provides an additional stimulus beyond that given by the lymphopenic environment. Alternatively, this preferential outgrowth of TCR modified T cells could reflect recognition of MHC molecules presenting endogenous epitopes, which have previously been shown to contribute to homeostatic proliferation in lymphopenic hosts[27].

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

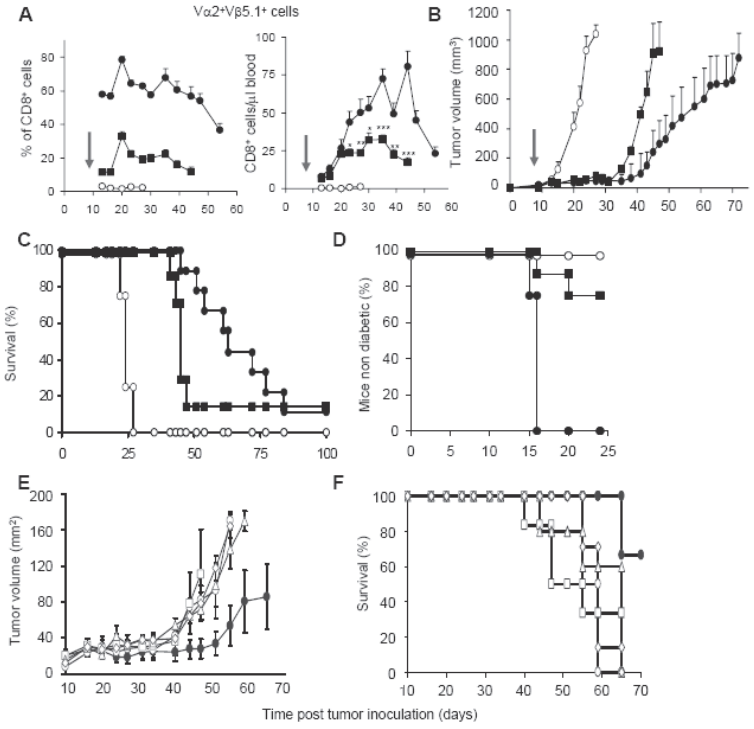

Figure 4: Precursor frequency of TCR transduced T cells affects tumor control. RIP-OVA^{hi} mice (N=7-8 per group) were inoculated with 1x10^5 B16-OVA tumor cells subcutaneously and sublethally irradiated at day 9. To determine the effect of the precursor frequency of TCR transduced T cells, 1x10^6 OT-I$_{opt}$ TCR transduced CD8$^+$ T cells were either transferred directly ('low passenger group') (filled circles, transfer of 3.6x10^6 cells in total) or 1x10^6 OT-I$_{opt}$ TCR transduced CD8$^+$ T cells were diluted 10 times with mock transduced splenocytes ('high passenger group') (filled squares, transfer of 3.6x10^7 cells in total). Control mice received 3.6x10^6 mock transduced T cells (open circles). (A) Analysis of V 2$^+$V 5.1$^+$ CD8$^+$ cells in peripheral blood (percentage in left panel, absolute numbers right) at indicated time points post adoptive transfer. Bars depict SEM. T-tests were performed to determine differences between low and high passenger groups; * represents P-value <0.05; ** <0.005; *** <0.0005. (B) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. (C) Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 10mm or when tumors started bleeding. P-values: irradiation versus 'low passenger group': <0.0001; irradiation versus 'high passenger group' 0.002; 'low passenger group' group versus 'high passenger group': 0.15 (Mantel-Cox test). (D) Induction of diabetes. Mice were considered diabetic once blood glucose levels exceeded 20 mmol/l. (E,F) Tumor bearing and sublethally irradiated mice received 1x10^6 OT-I$_{opt}$ TCR transduced CD8$^+$ T cells (filled circles, transfer of 4.2x10^6 cells in total), or 1x10^6 OT-I$_{opt}$ TCR transduced CD8$^+$ T cells diluted with total mock transduced splenocytes (open circles, transfer of 1.4x10^7 cells in total). To determine the effect of passenger cell subpopulations, mice received 1x10^6 OT-I$_{opt}$ TCR transduced CD8$^+$ T cells diluted with mock transduced splenocytes depleted of CD8$^+$ (open squares, transfer of 8.7x10^6 cells in total), CD4$^+$ (open triangles, transfer of 7.8x10^6 cells in total) or CD25$^+$ cells (open diamonds, transfer of 1.4x10^7 cells in total). (E) Analysis of tumor development. Tumor growth was measured 3 times a week, bars indicate SEM. (F) Kaplan-Meyer survival plot. Mice were sacrificed once tumors reached an average diameter of 15mm or when tumors started bleeding. The experiment was terminated at day 70.

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 co-transfer 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 focussed 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. 4E-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 co-transferred cells will be the selective purification of the desired TCR-modified T cells.

Immuno-editing by TCR modified T cells.

In mice treated with a combination of TBI and infusion of OT-I_{opt} transduced T cells tumor progression was eventually observed in the large majority of mice (Fig. 3 and Fig. 4). Notably, analysis of individual tumor growth curves at this late phase revealed that after varying periods of one to two months in which tumor progression was essentially absent, tumors in individual mice suddenly progressed with kinetics that were comparable to those observed in untreated mice (Fig. 5A). This rapid late outgrowth of tumors in treated mice suggested an acute loss of tumor control, possibly consistent with the selection of escape variants. To address whether tumor outgrowth after prolonged control by TCR modified T cells could be explained by antigen loss, we collected tumors in a series of experiments and analyzed the expression of the CD4 marker that is translated from the same mRNA as the OVA antigen. This analysis revealed that antigen expression was substantially reduced in tumors obtained from mice that had received TCR modified T cells as compared to tumors obtained from control mice (Figure 5B; $p<1x10\text{-}6$). Furthermore, in tumors that escaped immune control after more prolonged periods, evidence for antigen loss became increasingly apparent, consistent with an ongoing process of immune selection.

Thus, even though the B16-OVA cell line used was derived from a single cell clone selected for high CD4 expression, the prolonged selection pressure in mice treated by TBI plus TCR modified T cell infusion resulted in the appearance of escape variants with low antigen expression. These data suggest that -with regard to the possibility of tumor escape- the targeting of tumor associated antigens such as WT-1[28] or PRAME[29] that contribute to cellular transformation may be preferred. Alternatively, and in analogy with developments in antibody therapeutics, the simultaneous use of two or three TCRs directed against different TAAs will likely suffice to minimize the chance of tumor escape through antigen loss. Clinical implementation of such 'oligoclonal TCR gene transfer' will be an interesting future challenge from both a logistic and regulatory point of view.

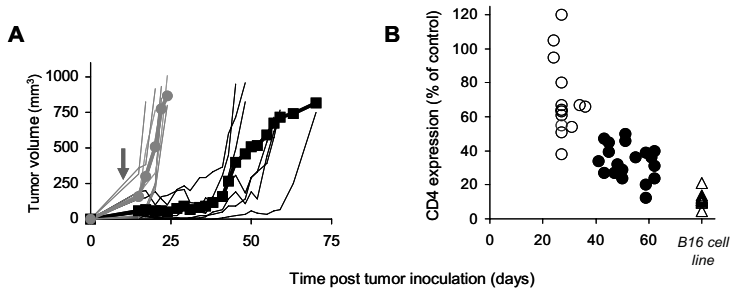

Figure 5: Immuno-editing by TCR modified T cells. (A) Individual tumor growth curves of RIP-OVA^{hi} mice depicted in Fig 3. Thin lines represent individual growth curves, thick lines with symbols represent group averages. Mice challenged with B16-OVA cells either received an adoptive transfer of $1x10^6$ OT-I_{opt} TCR transduced $CD8^+$ T cells (black squares/lines) or an equal amount of mock transduced T cells (grey circles/lines) at day 10. Arrow indicates timepoint of adoptive transfer. Note that after a variable period of stasis, tumors in mice treated with OT-I transduced T cells ultimately grow out with kinetics that are comparable to those seen in control mice. (B) Antigen expression on a collection of tumors obtained in a series of experiments. Mice were sacrificed when the average tumor diameter exceeded 10 mm. Expression of the CD4 marker gene was used as a surrogate marker for OVA expression and is expressed as a fraction of CD4 surface expression on cultured B16-OVA cells (redetermined at each time point of analysis). Open circles represent tumors derived from mice that received mock transduced T cells, filled circles represent tumors derived from mice that received OT-I_{opt} TCR transduced T cells, open triangles represent *in vitro* cultures of the B16 cell line.

Discussion

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

The host environment & engraftment of TCR-modified T cells - We here demonstrate that host conditioning by TBI leads to superior engraftment and anti-tumor efficacy of TCR modified T cells. Whereas viral vaccination resulted in a more pronounced early boost in the number of TCR modified T cells, irradiation-induced host conditioning led to a substantial improvement in the persistence of TCR modified cells. It is noted that because of the rapid growth kinetics of the transplantable tumor model used here, a rapid development of T cell responses is likely to be of greater importance in this model than it will be in the clinical setting, where tumor progression is markedly slower. Thus, the improved tumor control in mice conditioned by TBI as compared to mice receiving viral vaccination seen here may still underestimate the clinical value of chemotherapy- or irradiation-induced host conditioning in TCR gene transfer-based protocols. It seems likely that further improvements can be made in conditioning regimens for ACT. For instance, murine data suggest that myeloablative conditioning plus stem cell support results in an enhanced expansion and function of adoptively transferred TCR transgenic T cells[31]. Alternatively, the selective depletion of the

cellular subsets that compete for homeostatic cytokines may yield a more targeted approach to facilitate cell engraftment. Finally, blockade of inhibitory pathways by combination with monoclonal antibody therapy against CTLA-4 or PD-1/ PD-L1 may be considered.

TCR transgene design - Alterations in TCR transgene design fall into two classes, those that aim to change the specificity or affinity of the TCR for its cognate antigen and those that aim to increase the expression of the desired TCR αβ heterodimer upon T cell modification. Efforts to achieve the latter have stemmed from the observation that non-modified TCR heterodimers are generally expressed at low levels upon introduction in human peripheral blood T cells. Recent elegant work by Heemskerk and others has shown that this low expression is due to competition of exogenous TCR chains with endogenous TCR chains for assembly with CD3 components, and due to the formation of mixed dimers of endogenous and exogenous TCR chains. Interestingly, the ability of the exogenous and endogenous TCR to compete for surface expression can vary widely between different TCRs, most likely reflecting the efficiency with which the different TCR heterodimers fold[32]. The OT-I TCR used here can be considered a 'dominant' TCR in that retroviral transduction of mouse T cells with the unmodified TCR leads to TCR transgene expression in a high proportion of cells. Nevertheless, TCR gene optimization still resulted in a modest increase in transduction efficiency and a quite marked effect on the in vivo activity of T cells modified with this TCR. Based on these data it seems plausible that other strategies that have yielded similar increases in TCR expression in vitro (10-12) will also be of significant value to enhance the in vivo function of TCR modified T cells, and a combination of the different strategies may in fact be preferred.

Composition of the cell graft - In a final set of experiments we demonstrated that the frequency of TCR modified T cells within the cell graft determines the efficacy of ACT, even when infused numbers of TCR modified T cells are kept constant. We have considered two non-mutually exclusive explanations for this observation. First, the co-infusion of a large number of unmodified cells may lead to a reduced proliferation and differentiation of the TCR modified T cells by decreasing the availability of cues for homeostatic expansion. Specifically, an increased availability of the IL-7 and IL-15 cytokines has been shown to play an essential role in the enhancement of T cell mediated tumor immunotherapy after lymphodepleting host conditioning and the co-transfer of irrelevant T cells and NK cells may simply limit this effect[33,34]. Alternatively, regulatory T cells (T-regs) have been shown to suppress immune responses towards B16 melanoma[35,36], and the infusion of large numbers of passenger cells may result in a more rapid restoration in regulatory T cell number following host conditioning. In the experiments shown here, 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 prior to 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[37] or reversible MHC tetramer-based isolation[38] 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[39].

Finally, while we have here focussed on the frequency of TCR modified T cells within the graft, it seems plausible that alterations in the type of T cells that is used for viral modification may also be beneficial. For instance, the selective modification of T cells with a high capacity for immune reconstitution may potentially be attractive[40]. As a somewhat more futuristic approach, more defined populations of TCR modified T cells for adoptive therapy may conceivably also be generated in systems in which TCR modified T cells can be obtained *in vitro* from hematopoietic progenitor cells[41,42], with the added benefit that endogenous TCR rearrangement is at least partially suppressed[41].

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

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Chapter 6

Selecting highly affine and well-expressed TCRs for gene therapy of melanoma.

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Selecting highly affine and well-expressed TCRs for gene therapy of melanoma

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A recent phase 1 trial has demonstrated that the generation of tumor-reactive T lymphocytes by transfer of specific T-cell receptor (TCR) genes into autologous lymphocytes is feasible. However, compared with results obtained by infusion of tumor-infiltrating lymphocytes, the response rate observed in this first TCR gene therapy trial is low. One strategy that is likely to enhance the success rate of TCR gene therapy is the use of tumor-reactive TCRs with a higher capacity for tumor cell recognition. We therefore sought to develop standardized procedures for the selection of well-expressed, high-affinity, and safe human TCRs. Here we show that TCR surface expression can be improved by modification of TCR alpha and beta sequences and that such improvement has a marked effect on the in vivo function of TCR gene-modified T cells. From a panel of human, melanoma-reactive TCRs we subsequently selected the TCR with the highest affinity. Furthermore, a generally applicable assay was used to assess the lack of alloreactivity of this TCR against a large series of common human leukocyte antigen alleles. The procedures described in this study should be of general value for the selection of well- and stably expressed, high-affinity, and safe human TCRs for subsequent clinical testing. (Blood. 2007;110:3564-3572)

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Introduction

In the majority of human cancers, the shared tumor-associated antigens that are available for immunotherapy are nonmutated self-antigens. While T-cell reactivity against certain self-antigens can be observed and is in fact rather common for melanoma-associated antigens,[1] the boosting of such reactivity by vaccination has largely been unsuccessful to date.[2] Work over the past years by Dudley et al has demonstrated that objective tumor regressions can be obtained in approximately 50% of patients with metastatic melanoma by infusion of ex vivo–expanded tumor-infiltrating lymphocytes (TILs) after pretreatment with lymphodepleting chemotherapy.[3,4] While the demonstration of the clinical effectiveness of TIL infusion can be considered one of the major breakthroughs of cellular immunotherapy, the isolation of antigen-specific cells from resected tumor material and the in vitro expansion of these cells is feasible only for a fraction of melanoma patients. In addition, the scarcity of tumor-reactive T cells in tumor material of other human cancers precludes clinical application for most other human malignancies.

As an alternative to the use of naturally occurring tumor-reactive T cells, tumor-reactive cells may be generated by the transfer of tumor-specific T-cell receptor (TCR) genes. Such TCR gene transfer circumvents the requirements for isolation and in vitro expansion of T cells, and would allow the use of high-affinity tumor-reactive TCRs in larger patient groups.[5,6] Retrovirus-mediated delivery of TCR genes has been used to convey virus and tumor reactivity to human T cells,[7-11] and TCR-modified cytotoxic T cells and helper T cells have displayed in vivo functionality in several mouse models.[12-15]

Recently, the feasibility of TCR gene transfer was assessed in a clinical phase 1 study.[16] In this trial, peripheral blood lymphocytes of patients with metastatic melanoma were transduced with a Mart-1–specific T-cell receptor, and reinfused following lymphodepleting chemotherapy. While the infusion of TCR-transduced cells was effective in that these cells persisted for more than 2 months in most individuals, objective tumor regression was observed in only 2 of 15 patients. These data indicate that TCR gene transfer is feasible in a clinical setting, but that a substantial improvement of this approach is required to become a clinically meaningful treatment strategy.

One possible factor in the low response rate observed in this trial is a suboptimal capacity of the TCR-modified T cells for tumor cell recognition. First, tumor-specific T cells can differ widely in their capacity for tumor cell recognition, even when recognizing the same antigen.[17,18] Second, the expression of retrovirally introduced human TCRs in human T cells is suboptimal,[19] often requiring cotransduction of vectors encoding TCR alpha or beta genes together with a selectable marker, rather than with the bicistronic vectors that have been used in the clinic. Here, we set out to develop and test generally applicable strategies for the isolation of TCRs for clinical use. The resulting 3-step approach has been used to select a melanoma-reactive TCR with improved expression, a higher capacity for antigen recognition, and lack of detectable alloreactivity.

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Materials and methods

Mice

Six- to 10-week-old female C57BL/6 mice (H-2^b) and pmel-1 TCR transgenic mice[20] 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 (DEC).

Cell lines and peripheral blood mononuclear cells

FLYRD18 is a human fibrosarcoma retroviral packaging cell line (ECACC no. 95091902). The Jurkat/MA cell line is a Jurkat cell line lacking endogenous TCR expression.[21] T2 is an HLA-A2$^+$ cell line that is deficient for TAP (transporter associated with antigen presentation). The panel of single major histocompatibility complex (MHC) class I allele–expressing K562 cell lines (the SAL panel) has been described previously.[22] All cell lines were cultured in Iscove modified Dulbecco medium (IMDM) (GIBCO, Invitrogen, Carlsbad, CA) with 5% fetal calf serum (PAA, Pasching, Austria), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Melanoma cell lines Mel 526 (HLA-A2$^+$, Gp100$^+$, Mart$^+$) and Mel 938 (HLA-A2$^-$, Gp100$^+$, Mart$^+$) were described previously.[23] Mel GDO and Mel AKR (HLA-A2$^+$, Gp100$^+$, Mart$^+$) were established in the NKI from resected tumor lesions. Melanoma cell lines were maintained in RPMI (GIBCO, Invitrogen) in the presence of 5% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL).

Peripheral blood mononuclear cells PBMCs from anonymous healthy donors derived via the local blood bank were isolated by leukapheresis and subsequent Ficoll-Isopaque density centrifugation. Following transduction, PBMCs were cultured in Yssels medium[24] supplemented with 20 U/mL IL-2 (Proleukin; Chiron, Emeryville, CA). Every 14 days, transduced PBMCs were stimulated with irradiated JY cells and allogeneic PBMCs, plus 100 ng/mL phytohemagglutinin and 20 U/mL IL-2.

TCR gene optimalization and cloning

Sequences of the Gp100-specific pmel-1 TCR were kindly provided by N. Restifo (NIH, Bethesda, MD). Sequences of the Mart-1–specific 1D3 and 2C2 TCRs have been described previously.[25] The Mart-1–specific DMF4 TCR[26] and the Gp100-specific R6C12 TCR[27] were isolated at the NIH. Modified TCR genes were designed and produced by GeneArt (Regensburg, Germany). DNA sequences are provided in Figure S2, available on the *Blood* website; see the Supplemental Figures link at the top of the online article. Wild-type wt and gene-optimized TCR alpha and beta chains were cloned into the retroviral vector pMX[28] containing an internal ribosomal entry site, or in the indicated vector.

Production of retroviral supernatants and retroviral transduction

FLYRD18 packaging cells were plated in 6-well plates at 1.5×10^5 cells/well. After one day, cells were transfected with 2.5 μg retroviral vector DNA using FuGENE TM6 (Roche Diagnostics, Indianapolis, IN). After 48 hours, retroviral supernatant was pooled, centrifuged, and frozen at − 80°C. PBMCs were activated with 20 U/mL IL-2 and 2 mg/mL phytohemagglutinin, at 1×10^6 cells/mL. Forty-eight hours after stimulation, PBMCs were resuspended in retroviral supernatant, transferred to RetroNectin-coated plates at 0.5×10^6 cells/mL, and centrifuged for 90 minutes at 430*g*. Jurkat/MA cells were transduced without the centrifugation step. Mouse splenocytes were transduced as described previously.[12]

Flow cytometric analysis

Surface expression of pmel-1 TCR-transduced murine splenocytes was measured using D^b-tetramers, or by double staining with fluorescein isothiocyanate (FITC)-labeled anti-Vβ13 monoclonal antibody (mAb) and PE-labeled anti-Vβ2, 3, 4, 5, 8, 9, 10, and 11 mAb (anti-Vβ-pool), in combination with anti-CD8α mAb (all mAbs from BD Pharmingen, San Jose, CA). Surface expression of TCR-transduced PBMCs was measured by staining with MHC-tetramers, using MHC-tetramers generated through ultraviolet-peptide exchange,[29,30] or by staining with anti-Vβ12 (for DMF4), anti-Vβ14 (for 1D3 and 2C2), or anti-Vβ8 (for R6C12) antibody (Immunotech, Westbrook, ME), in combination with anti-CD8 or anti-CD4 antibody (Becton Dickinson, San Jose, CA). Cells were analyzed and sorted using a FACSCalibur and FACSAria (Becton Dickinson).

Adoptive transfer and viral infection

Mice received an intravenous adoptive transfer of transduced splenocytes, nontransduced splenocytes, or in vitro–activated splenocytes from pmel-1 transgenic mice. To induce lymphodepletion, mice received total body irradiation (TBI) of 5 Gy, one day before adoptive transfer. Mice were vaccinated at the day of adoptive transfer by intraperitoneal injection of 1×10^7 plaque-forming units of recombinant vaccinia virus encoding hGp100$_{(25\text{-}33)}$, kindly provided by N. Restifo.[31] For the measurement of T-cell responses, peripheral blood samples were taken at the indicated days after treatment.

IFN-γ assay

T2 cells were pulsed with peptides for 1 to 2 hours at 37°C. Next, 0.5×10^6 TCR-transduced PBMCs were incubated with 0.5×10^6 peptide-pulsed T2 cells or 0.5×10^6 SALs, in the presence of 20 U/mL IL-2 and 1 μL/mL Golgiplug (BD Biosciences, Basel, Switzerland). After 4- to 5-hour incubation at 37°C, cells were washed and stained with FITC-labeled anti-CD8 antibody and phycoerythrin (PE)-labeled anti-CD4 antibody, and analyzed for IFN-γ production by intracellular cytokine staining.

Chromium release assay

Target cells were labeled for 1 hour at 37°C with 100 μCi (3.7 MBq) ^{51}Cr (Amersham, Gent, Belgium). Labeled target cells were incubated with effector cells at indicated ratios for 4 hours at 37°C in 200 μL medium, in the presence of a 50-fold excess of unlabeled K562 cells.

Results

Increased expression and in vivo function of gene-optimized TCRs

A specific issue in TCR gene transfer has been the relatively low level of transgene expression that is obtained after retroviral modification of human T lymphocytes. This is at least partially due to competition of introduced and endogenous TCR gene products for the limited pool of CD3 components, and inefficient heterodimer formation of the introduced TCR chains.[32,33]

It has previously been demonstrated that in vitro TCR expression can be improved by the use of synthetic genes with an optimized codon usage.[34] However, the in vivo consequences of TCR gene optimization—which will determine its utility in a clinical setting—have not been assessed. To address this issue, the nucleic acid sequence of the murine Gp100–specific pmel-1 TCR was modified to conform to the codon bias observed in highly expressed mammalian genes,[35] thereby avoiding *cis*-acting sequence motifs.[36]

To distinguish pmel-1 expression from endogenous TCR expression, mouse splenocytes, transduced with retroviruses containing the wt or optimized (opt) pmel-1 TCR genes, were stained with a combination of anti-Vβ13 antibodies and a pool of anti-Vβ antibodies, thereby allowing detection of T cells with dual TCR expression.[15] Analysis of TCR-transduced cells via this strategy

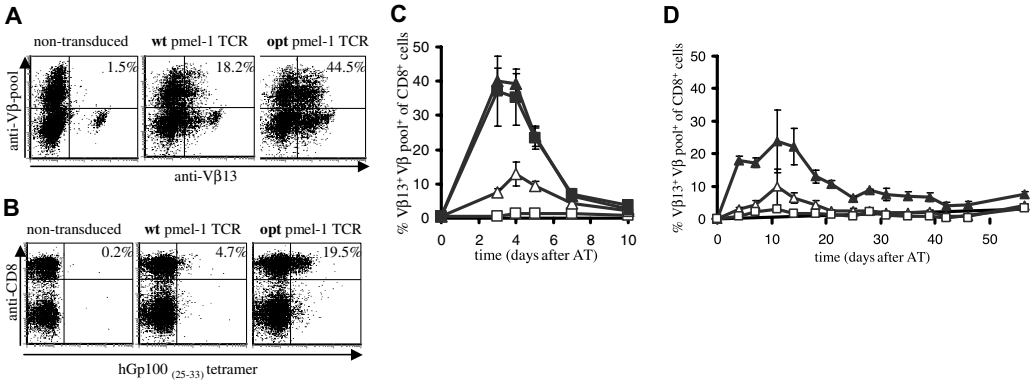

Figure 1. In vivo antigen expansion of wild-type and gene-optimized TCR-transduced murine splenocytes. Activated B6 splenocytes were transduced with vectors encoding wild-type or gene-optimized pmel-1 TCR. (A) TCR expression was determined by ow cytometry using anti-CD8α, anti-Vβ13, and a pool of anti-Vβ2, anti-Vβ3, anti-Vβ4, anti-Vβ5, anti-Vβ6, anti-Vβ8, anti-Vβ10, and anti-Vβ11. All uorescence-activated cell sorting (FACS) plots show events that are gated for CD8 expression. The numbers indicate the percentage of CD8$^+$ T cells with detectable pmel-1 TCR expression, calculated as follows: %Vβ13$^+$Vβpool$^+$ cells/%Vβpool$^+$ cells × 100%. (B) Detection of TCR expression using anti-CD8 antibodies and D^b-hGp100$_{25\text{-}33}$ tetramer. The numbers indicate the percentage of tetramer$^+$ CD8$^+$ cells. (C,D) B6 recipients received splenocytes containing either 1×10^6 pmel-1 TCR transgenic CD8$^+$ cells (■, only in panel C), 1.5×10^6 wild-type TCR-transduced CD8$^+$ cells (△), or 1.5×10^6 optimized TCR-transduced CD8$^+$ cells (▲). Control mice received 20×10^6 activated, nontransduced cells (□). Mice received an intraperitoneal injection of 1×10^7 pfu rVV-hGp100$_{(25\text{-}33)}$ virus at the day of adoptive transfer (C), or mice received a sublethal dose of total body irradiation one day before adoptive transfer (D). At the indicated time points, peripheral blood was collected and analyzed by ow cytometry using APC anti-CD8 α, FITC anti-Vβ13, and PE anti-Vβpool. The percentage of Vβ13$^+$Vβpool$^+$ CD8$^+$ cells of total Vβpool$^+$ CD8$^+$ cells is plotted. For mice that received pmel-1 TCR transgenic cells, the percentage of Vβ13$^+$ CD8$^+$ cells is plotted. Error bars represent standard deviations (n = 4).

(Figure 1A) or with MHC-tetramers (Figure 1B) revealed a substantial increase in the percentage of cells expressing the pmel-1 TCR upon gene optimization (Vβ staining 44.5% versus 18.2%; MHC-tetramer staining 19.5% versus 4.7%). The level of expression was affected to a lesser extent (mean fluorescence intensity [MFI]: 63 versus 52). The markedly lower percentage of MHC-tetramer$^+$ cells compared with the percentage of Vβ$^+$ cells can be explained by the fact that T cells with relatively low TCR expression can escape detection by MHC-tetramer staining. Furthermore, T cells in which the introduced TCR beta chain is expressed as a mixed heterodimer with endogenous alpha chains are detected by Vβ staining, but not MHC-tetramer staining.[32,33] In addition, when single anti-Vβ staining is used (as for human cells; Figure 2A), T cells with endogenous TCRs expressing this beta chain will also be detected.

To test whether gene optimization affected the in vivo function of TCR-modified T cells, B6 mice received an adoptive transfer of wt or opt TCR-transduced cells. As a control, mice received nontransduced cells or splenocytes of pmel-1 transgenic mice. Remarkably, although mice received an equal number of TCR-transduced cells, T-cell responses upon infection with a recombinant vaccinia strain encoding the hGp100$_{(25\text{-}33)}$ epitope were markedly higher in recipients of opt pmel-1 TCRs than in recipients of wt pmel-1 TCRs, and comparable with responses in recipients of pmel-1 TCR-transgenic cells (Figure 1C, peak T-cell responses of 13% and 39% for wt and opt, respectively).

Adoptive transfer of transduced cells to irradiated recipients, a setting resembling TCR gene transfer in lymphodepleted patients, confirmed the marked difference between wt and opt pmel-1–transduced cells and revealed long-term persistence of cells transduced with gene-optimized TCRs (Figure 1D). Furthermore, an increase in in vivo antigen-specific T-cell responses upon TCR gene optimization is likewise observed for a second TCR (OT-I, de Witte et al, unpublished observations, December 2006).

To assess the effect of TCR gene optimization of human melanoma–specific TCRs, we generated wild-type and gene-optimized versions for 2 human TCRs (Mart-1–specific DMF4 and Gp100-specific R6C12). As for the murine TCR, gene optimization led to a substantial increase in the percentage of cells with detectable transgene expression in transduced PBMCs (Figure 2A: Vβ staining, Figure 2B: MHC-tetramer staining). Gene optimization of the DMF4 TCR resulted in a 2-fold increase in the percentage of Vβ12-expressing cells (36.8% compared with 17.0%) and an almost 3-fold increase in tetramer-binding cells (24.7% compared with 9.0%). In R6C12 TCR–transduced cells, gene modification resulted in a 3-fold increase in Vβ8-expressing cells (32.9% versus 11.3%) and a more than 7-fold increase in tetramer-binding cells (22.4% versus 3.0%). Gene optimization predominantly affected the percentage of T cells with detectable transgene expression, whereas the level of TCR expression was enhanced to a lesser extent (MFI MHC-tetramer$^+$ cells opt versus wt TCR: DMF4, 127 versus 100; R6C12, 98 versus 63). This effect of gene optimization was observed using a series of independent transductions (n = 4) and independent DNA batches (n = 2).

As the effect of gene optimization is thought to be in part due to enhanced RNA stability, it was possible that the improved expression of gene-modified TCRs was partially caused by more efficient production of retroviral particles. To be able to distinguish between an effect of gene optimization on TCR protein production and on viral titers, transductions were performed in a cell line that is devoid of endogenous TCR cell surface expression. In these Jurkat/MA cells, exogenous TCR alpha and beta chains are expressed at the cell surface in the absence of competition with endogenous TCR chains.

Whereas transduction with wild-type and gene-optimized TCRs resulted in a marked difference in expression in PBMCs, transduction of Jurkat/MA cells with serial dilutions of retrovirus revealed comparable expression for gene-optimized and wild-type TCRs, both for the DMF4 (Figure 2C) and the R6C12 TCR (Figure 2D). This indicates that gene optimization has an effect primarily on TCR protein production, whereas retroviral titers are not measurably influenced.

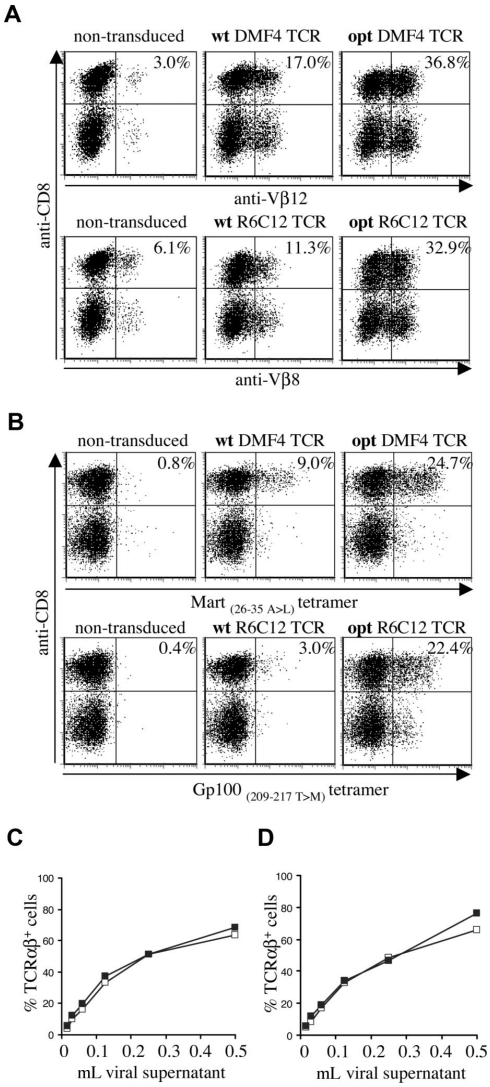

Figure 2. Expression of wild-type and gene-optimized human melanoma–specific TCRs. Activated PBMCs were transduced with wild-type or codon-optimized Gp100-speci c R6C12 and Mart-1–speci c DMF4 TCRs. TCR expression was determined 4 days after transduction by ow cytometric analysis using (A) anti-CD8 and anti-Vβ12 (top panels) or anti-Vβ8 (bottom panels), or (B) anti-CD8 and A2.1-Mart-1$_{(26\text{-}35,\ 27\ A>L)}$ tetramer (top panels) or A2.1-Gp100$_{(209\text{-}217,\ 210\ T>M)}$ tetramer (bottom panels). The numbers indicate the percentage of Vβ$^+$ or tetramer$^+$ CD8$^+$ cells. (C,D) Retrovirus encoding wt (□) or opt (■) DMF4 (C) or R6C12 TCR (D) was titrated on Jurkat/MA cells by adding the indicated amount of viral supernatant to a total volume of 1 mL. Four days after transduction, cells were analyzed by ow cytometry for TCR expression by anti-TCRαβ antibody staining.

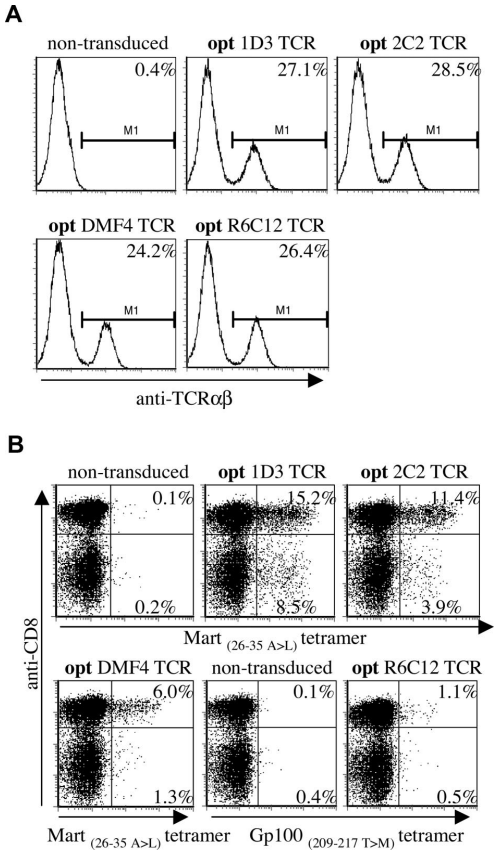

Figure 3. Differential expression of a panel of optimized melanoma-specific TCRs in peripheral blood T cells. (A) Jurkat/MA cells were transduced with titrated aliquots (Figure S1) of viral supernatants of vectors encoding the gene-optimized Mart-1–speci c TCRs 1D3, 2C2, and DMF4 or the Gp100-speci c TCR R6C12. Transduced cells were stained with anti-TCRαβ 4 days after transduction. Histograms show levels of TCR expression for the different TCRs. (B) Retroviral aliquots as used in panel A were used to transduce human peripheral blood T cells. TCR expression was determined by staining with anti-CD8 and A2.1-Mart-1$_{(26\text{-}35,\ 27\ A>L)}$ or A2.1-Gp100$_{(209\text{-}217,\ 210\ T>M)}$ tetramers. The numbers in the upper-right and lower-right corners indicate the percentage of tetramer$^+$ CD8$^+$ and tetramer$^+$ CD8$^-$ cells, respectively.

Expression patterns and activity of a panel of melanoma-specific TCRs

Having established the in vitro and in vivo benefit of TCR gene optimization, we sought to select the TCR with the highest expression and functional activity from a panel of 4 gene-optimized TCRs. This panel consisted of 3 Mart-1–specific TCRs (1D3, 2C2, and DMF4) and a Gp100-specific TCR (R6C12). The 1D3 and 2C2 TCRs were derived from highly tumor-reactive cytotoxic T lymphocyte (CTL) clones isolated from a melanoma patient vaccinated with Mart-1$_{26\text{-}35}$ peptide.[25] The DMF4 TCR was isolated from a dominant T-cell clone in a patient who experienced an objective tumor regression upon adoptive transfer of autologous TILs.[3,26] This receptor was used in the recent TCR gene transfer trial. The R6C12 TCR was derived from a CTL clone of a melanoma patient vaccinated with Gp100$_{209\text{-}217}$ peptide.[27]

First, we assessed the relative efficacy with which the 4 different TCRs were expressed at the cell surface. To rule out differences in retroviral titers of the TCR panel, retroviral supernatants were titrated in the Jurkat/MA cell line.

Virus dilutions resulting in comparable expression of the 4 different TCRs in Jurkat/Ma (Figure 3A, titrations shown in Figure

S1A,B) were used to transduce PBMCs, and TCR expression was determined by MHC-tetramer staining (Figure 3B; note, transduction efficiencies using diluted virus are lower than when using untitrated virus). A marked hierarchy in expression of the introduced TCRs in $CD8^+$ cells was reproducibly detected in 4 independent experiments, with 1D3 giving the highest percentage of MHC-tetramer$^+$ cells (15.2%, MFI: 233), followed by 2C2 (11.4%, MFI: 225) and DMF4 (6.0%, MFI: 152). Transduction with the Gp100-specific R6C12 TCR resulted in only a small fraction of MHC-tetramer$^+$ cells (1.1%, MFI: 75), even though expression of this TCR in Jurkat/Ma was efficient.

Interestingly, transduction with 1D3 and 2C2 resulted in a substantial population of MHC-tetramer$^+$ $CD4^+$ cells (as confirmed by staining with anti-CD4 antibody, data not shown). Expression of the introduced TCR in $CD4^+$ cells was most efficient for the 1D3 TCR (8.5%, MFI: 205), followed by the 2C2 TCR (3.9%, MFI: 120). MHC-tetramer staining of $CD4^+$ cells following transduction with DMF4 and R6C12 was close to background levels (1.3% and 0.5%, respectively).

Together, these results show that viral titers that yield equal TCR expression in the absence of competition with endogenous TCR chains yield substantially different expression levels in human PBMCs. Of the 4 melanoma-specific TCRs tested here, 1D3 shows the highest expression in both $CD8^+$ and $CD4^+$ cells.

To determine the capacity for antigen recognition of T cells expressing the melanoma-specific TCRs, intracellular IFN-γ production of PBMCs, transduced with titrated virus, was determined. Incubation with Mart-$1_{(26\text{-}35)}$ peptide–loaded targets resulted in cytokine production in $CD8^+$ cells transduced with either one of the Mart-1–specific receptors (Figure 4A). However, the 1D3 receptor was approximately 10-fold more sensitive than 2C2 or DMF4 (EC_{50} 1D3: 3-10 nM, EC_{50} 2C2 and DMF4: 30-100 nM). Furthermore, modification of PBMCs with the 1D3 receptor also provided $CD4^+$ cells with the capacity for IFN-γ production upon antigen recognition (EC_{50}: 30-100 nM), whereas this was not observed for the 2C2 or DMF4 receptor (Figure 4B). Transduction of PBMCs with the R6C12 receptor resulted in only low levels of IFN-γ production in $CD8^+$ cells upon incubation with Gp$100_{(209\text{-}217)}$ peptide–loaded targets (Figure 4C, EC_{50}: 1 nM), and no detectable production in $CD4^+$ cells (Figure 4D). Of the Mart-1–specific TCRs, the 1D3 TCR was selected for further study, as it was well expressed, had a higher sensitivity than the 2 other Mart-1–specific receptors, and induced IFN-γ production in both $CD8^+$ and $CD4^+$ cells.

Peptide titrations are not useful to compare the relative effectiveness of T-cell receptors recognizing distinct epitopes (ie, the 1D3 and R6C12 TCRs recognizing the Mart-1 and Gp100 epitope, respectively), as differences in epitope density on target cells are not taken into account. To address this issue, the cytotoxic activity of peripheral blood T cells modified with either the Mart-1–specific 1D3 or the Gp100-specific R6C12 receptor was tested against several HLA-$A2^+$ human melanoma cell lines that express both target antigens. Transduction of human PBMCs with the 1D3 TCR endowed these cells with the capacity to lyse HLA-A2–positive melanoma cell lines, whereas HLA-A2–negative target cells were not killed (Figure 4E). In contrast, lysis by cells transduced with the R6C12 TCR was substantially lower, and only slightly above background (Figure 4F).

The use of gene optimization and subsequent in vitro comparison of different TCRs yielded a synthetic 1D3 alpha-beta gene pair that is well expressed and highly active. To formally test the value of these optimizations, PBMCs transduced with titrated virus encoding the opt 1D3 receptor were compared with PBMCs transduced with the wt DMF4 TCR that was recently used in a clinical trial.[17] In addition, the opt DMF4 TCR was included to determine whether gene optimization affects primarily the yield of cells showing detectable transgene expression, or also T-cell sensitivity.

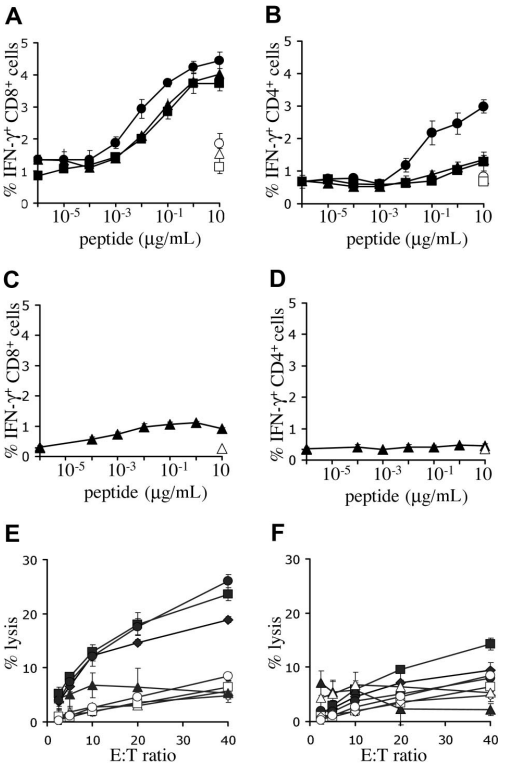

Figure 4. Functional analysis of melanoma-specific TCRs. (A,B) Human PBMCs were transduced with titrated aliquots of virus as detailed in Figure 3. Five days after transduction, cell cultures transduced with the Mart-1–speci c TCRs DMF4 (▲), 1D3 (●), or 2C2 (■) were incubated with T2 cells loaded with the indicated concentrations of Mart-$1_{(26\text{-}35)}$ peptide. As a control, transduced cells were incubated with T2 cells loaded with the highest concentration of Gp$100_{(209\text{-}217)}$ peptide (open symbols). (C,D) Five days after transduction, cell cultures transduced with Gp100 R6C12 TCR (◆) were incubated with T2 cells loaded with the indicated concentrations of Gp$100_{(209\text{-}217)}$ peptide. As a control, transduced cells were also incubated with T2 cells loaded with the highest concentration of Mart-$1_{(26\text{-}35)}$ peptide (open symbols). After 5 hours of incubation, cells were stained with FITC anti-CD8 and PE anti-CD4, and intracellular cytokine production was determined using APC anti-IFN-γ. The percentage of IFN-γ–positive CD8 cells (A,C) and CD4 cells (B,D) is shown. Error bars in panels A-D represent standard deviations (n = 3). (E,F) Lysis of melanoma cell lines in a ^{51}Cr-release assay. Twelve days after transduction, 1D3 TCR–transduced (E) or R6C12 TCR–transduced (F) cells were cocultured with different HLA-$A2.1^+$, $Mart^+$, and $Gp100^+$ cell lines: AKR (●), GDO (■), and 526 (◆). The HLA-$A2.1^-$, $Mart^+$, and $Gp100^+$ cell line 938 (▲) was used as a control. Coculture of nontransduced cells with melanoma cell lines is indicated by open symbols: AKR (○), GDO (□), 526 (◇), and 938 (△). Cells were incubated at the indicated effector-target ratios for 4 hours, after which the percentage of lysis was determined. Error bars represent standard deviations (n = 3).

MHC-tetramer staining confirmed expression patterns observed in previous experiments (Figure 5A), with gene-optimized 1D3 showing the highest percentage of MHC-tetramer$^+$ $CD8^+$ cells (11.7%), and gene-optimized DMF4 showing a markedly higher percentage than its wild-type counterpart (9.1% vs 2.9%). Furthermore, only 1D3 showed a substantial population of MHC-tetramer$^+$ $CD4^+$ cells (8.8%).

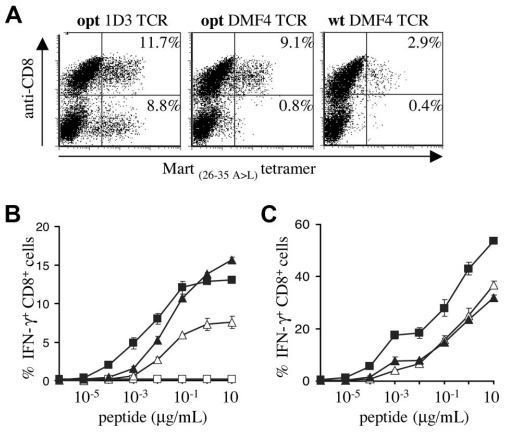

Figure 5. Comparison of the gene-optimized 1D3 TCR and wild-type DMF4 TCR. Activated PBMCs were transduced with titrated viral supernatants of pMX vectors encoding the DMF4 wild-type or gene-optimized receptor or the 1D3 gene-optimized receptor. (A) Four days after transduction, expression in PBMCs was determined by staining with anti-CD8 and A2.1-Mart-$1_{(26\text{-}35,\ 27\ A>L)}$ tetramer. The numbers in the top-right and bottom-right corners indicate the percentage of tetramer$^+$ CD8$^+$ and tetramer$^+$ CD8$^-$ cells, respectively. (B) Transduced cells were incubated with T2 cells loaded with the indicated Mart-$1_{(26\text{-}35)}$ peptide concentrations. After 5 hours of incubation, cells were stained with anti-CD8, and intracellular cytokine production was determined by anti-IFN-γ staining. The percentage of IFN-γ$^+$ CD8$^+$ cells is shown for wild-type DMF4 (△), gene-optimized DMF4 (▲), gene-optimized 1D3 (■), or nontransduced lymphocytes (□). Error bars represent standard deviations (n = 2). (C) Tetramer$^+$ CD8$^+$ cells were sorted and 1 week later incubated with T2 cells loaded with the indicated Mart-$1_{(26\text{-}35)}$ peptide concentrations. After 5 hours of incubation, cells were stained with anti-CD8, and intracellular cytokine production was determined by anti-IFN-γ staining. The percentage of IFN-γ$^+$ CD8$^+$ cells is shown for sorted wild-type DMF4 (△), gene-optimized DMF4 (▲), and gene-optimized 1D3 (■) transduced PBMCs.

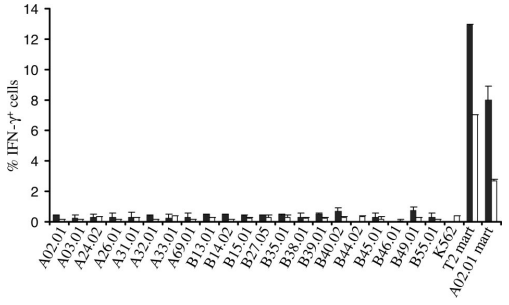

Figure 6. Assessment of alloreactivity of TCR-transduced PBMCs against single MHC allele–expressing cells. 1D3 TCR–transduced cells were incubated with 21 single MHC allele-expressing cell lines for a period of 5 hours. T2 cells and A02.01 K562 cells loaded with the Mart-$1_{(26\text{-}35)}$ peptide were used as a positive control. After incubation, cells were stained with anti-CD8 and PE CD4, and intracellular cytokine production was determined using anti-IFN-γ. The percentage of IFN-γ$^+$ CD8$^+$ cells (■) and CD4$^+$ cells (□) is depicted. Bars represent range (n = 2).

In line with the data shown in Figure 4A, optimized 1D3 TCR–transduced cells were approximately 10-fold more sensitive than cells transduced with the optimized DMF4 TCR. Comparison of wild-type and optimized DMF4 revealed equal sensitivity, indicating that peptide sensitivity is not measurably affected by gene optimization (Figure 5B).

The percentage of T cells transduced with the unmodified DMF4 TCR that was detected in functional assays was high compared with the percentage of transgene expression as detected by MHC-tetramer staining. This could either reflect the fact that MHC-tetramer staining underestimates the frequency of TCR-modified T cells or that T cells expressing the unmodified DMF4 are more likely to be functionally active compared with T cells expressing other Mart-1–specific TCRs. To test this, 1D3 opt, DMF4 opt, and DMF4 wt TCR-transduced cells were sorted on the basis of MHC-tetramer and CD8 staining (resulting in 84%, 80%, and 82% tetramer$^+$ CD8$^+$ cells, respectively; data not shown), and IFN-γ production was determined upon peptide stimulation (Figure 5C). Sorted PBMCs transduced with either wild-type or gene-optimized DMF4 receptor showed similar percentages of IFN-γ–producing cells. Furthermore, PBMCs transduced with gene-optimized 1D3 maintained their higher sensitivity after sorting.

Recognition of allogeneic MHC molecules

As discussed previously,[5] TCR gene transfer in the clinical setting entails a partial MHC mismatch between the TCR recipient and the original "TCR donor." Consequently, during thymic selection, the introduced TCR has not been selected against reactivity toward all of the MHC alleles expressed by the recipient, and recognition of allogeneic MHC molecules complexed with self-antigens could result in autoimmune pathology. Although alloreactivity has not been observed for the TCR used in a first phase 1 trial,[16] the high frequency of T-cell alloreactivity in other settings[37] warrants the development of standardized methods to screen for such reactivity.

To mimic a setting in which there is an MHC mismatch between TCR recipient and donor, PBMCs transduced with the 1D3 receptor were tested against a large panel of K562 cell lines each expressing single HLA-A and -B alleles (the SAL panel)[22] by determining intracellular IFN-γ production upon coculture. Of the 21 SALs tested, none induced detectable IFN-γ production in 1D3-expressing CD8$^+$ or CD4$^+$ cells, whereas incubation with Mart-$1_{(26\text{-}35)}$ peptide–loaded T2 cells or HLA-A2$^+$ SALs did result in a substantial population of CD8$^+$ and CD4$^+$ cells producing IFN-γ (Figure 6). These results show that for the receptor and MHC alleles tested, alloreactivity does not seem to play an important role.

Long-term in vitro culture and vector comparison

As long-term persistence of adoptively transferred cells is thought to be important for clinical antitumor efficacy,[38] we set out to determine whether PBMCs transduced with the gene-optimized 1D3 receptor would maintain TCR expression and cytotoxic capacity after prolonged in vitro culture.

Every 3 or 4 days, total cell numbers (Figure 7A) and percentage of MHC-tetramer$^+$ cells (Figure 7B) were assessed. A high percentage of MHC-tetramer$^+$ cells was maintained over 4 weeks of culture, even after a 3-log expansion. Interestingly, in line with previous data on the effect of T-cell activation state on activity of the retroviral LTR,[39] the fraction of MHC-tetramer$^+$ cells was highest in the period following T-cell restimulation. After prolonged in vitro culture, 1D3 TCR–transduced cells remained capable of lysing HLA-A2–positive melanoma cell lines (Figure 7C).

Finally, having selected a well- and stably expressed, highly affine, melanoma-specific TCR with no detectable alloreactivity against a large series of HLA alleles, we aimed to assess which retroviral vector is most suitable for expression of TCR transgenes in human lymphocytes. To this purpose, 3 different

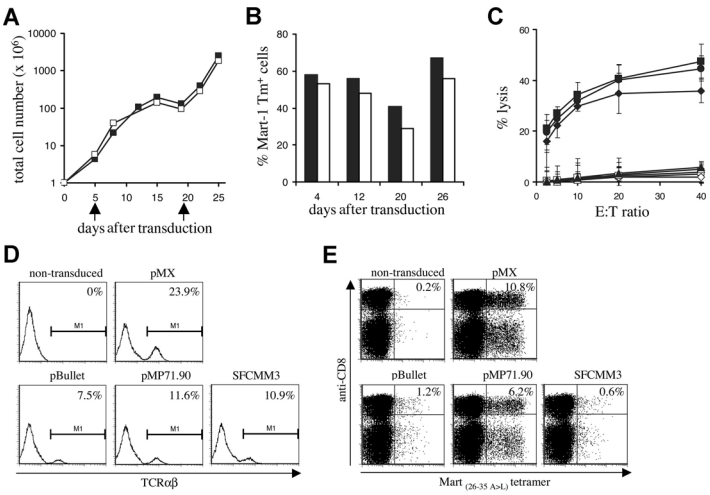

Figure 7. Long-term in vitro culture and vector comparison. (A) PBMCs transduced with the 1D3 TCR (■) and nontransduced (□) PBMCs were restimulated every 14 days (indicated by arrows) by addition of irradiated feeder cells and phytohemagglutinin. At the time points indicated, total cell numbers were determined. (B) TCR expression in 1D3-transduced cells was determined by staining with anti-CD8, anti-CD4, and A2.1-Mart-1$_{(26\text{-}35,\ 27\ A>L)}$ tetramer. The numbers indicate the percentage of tetramer$^+$ CD8$^+$ (■) and tetramer$^+$ CD4$^+$ (□) cells. (C) Four weeks after transduction, cytolytic activity of 1D3 TCR–transduced cells against different HLA-A2.1$^+$, Mart$^+$ cell lines—AKR (●), GDO (■), and 526 (◆)—was determined. The HLA-A2.1$^-$, Mart$^+$ cell line 938 (▲) was used as a control. Cytolytic activity of nontransduced cells is indicated by open symbols: AKR (○), GDO (□), 526 (◇), and 938 (△). Error bars represent standard deviations (n = 3) (D,E) Transient viral supernatant of the indicated vectors all encoding the 1D3 TCR was used to transduce Jurkat/MA cells (D) or PBMCs (E). Four days after transduction, TCR expression in Jurkat/MA cells was determined by staining with anti-TCRαβ. Expression in PBMCs was determined by staining with anti-CD8 and A2.1-Mart-1$_{(26\text{-}35,27\ A>L)}$ tetramer. The numbers indicate the percentage of tetramer$^+$ CD8$^+$ cells.

retroviral vectors that have previously been used in clinical trials (pBullet,[40,41] pMP71.90,[42] and SFCMM3[43,44]) were compared with the pMX vector used in in vivo mouse studies for their ability to yield TCR transgene expression in human PBMCs, and the same set of retroviral vectors was also evaluated in the Jurkat/MA system.

The Mart 1D3 receptor was cloned into each vector in either an α-IRES-β or an α-SV40EP-β configuration. All vectors were capable of inducing expression of the 1D3 receptor in the Jurkat/MA system (Figure 7D). In contrast, only transduction with pMX- and pMP71.90-based vectors yielded efficient expression of the 1D3 TCR in PBMCs, resulting in 10.8% (MFI: 261) and 6.2% (MFI: 323) tetramer-binding cells, respectively (Figure 7E). Transduction with both these vectors resulted in stable expression of the 1D3 TCR during a period of at least 3 weeks (data not shown).

These data support 2 points, first evaluation of TCR expression in cell lines such as Jurkat/MA cells has little predictive value for the capacity of these vectors to yield detectable TCR expression in peripheral blood T cells. Second, for the expression of TCR alpha and beta genes from a single vector, pMP71.90 is the most effective of those vectors previously used in a clinical setting, and comparable with the pMX vector that has successfully been used in a series of in vivo mouse studies.

Discussion

Here, we describe a 3-step approach for the selection of well-expressed, high affine, and safe TCRs. An important step in this approach is the improvement of TCR expression via gene modification, since expression of introduced TCR alpha and beta genes is generally low due to formation of heterodimers of endogenous and introduced chains, resulting in lower expression of the correctly paired heterodimer. Furthermore, correctly assembled chains compete with other heterodimers for binding to the CD3 complex[32,33] that forms the limiting component in the TCR assembly process.

We modified TCR genes to improve stability and translation of the messenger RNA while leaving the amino acid sequence unaltered, thus enabling efficient protein expression. We were able to show that cell surface expression of 2 of 3 murine TCRs and 2 of 2 human TCRs benefits substantially from gene optimalization (this paper and A.J., unpublished observations, September 2006).

Furthermore, gene modification of the murine pmel-1 TCR distinctly improves in vivo antigen recognition, as determined by expansion of the transduced T-cell population upon vaccination. Interestingly, this marked effect on the in vivo expansion of TCR-modified cells was observed even though the increase in the level of expression upon gene optimization was only modest. As a comparable increase in expression is observed upon gene optimization of 2 human TCRs, these data suggest that the in vivo behavior of human TCR-transduced PBMCs may also benefit substantially from gene optimization. Likewise, the current data suggest that the modest increase in expression of exogenous TCRs upon remodeling of the TCRαβ interface[45] may also result in a substantially improved engraftment of TCR-modified cells in vivo.

Notably, the effect of gene optimization was more pronounced for the human Gp100–specific R6C12 receptor than for the Mart-1–specific DMF4 receptor, both with respect to the percentage of cells showing detectable transgene expression and the level of expression. To assess whether it may be possible to predict the value of gene optimization for different TCRs, we determined the percentage of codons that was modified in the variable regions of these receptors. In the gene-optimized R6C12 receptor, 52% of codons were modified in the alpha chain and 47% in the beta chain. In contrast, modifications of DMF4 alpha and beta chains were 32% and 21%, respectively. This may suggest that for TCRs for which a lower level of gene optimization is required, this process results in a smaller increase in TCR expression. However, a substantially larger dataset will be required to rigorously test this notion.

In addition to demonstrating the in vivo effect of TCR gene optimization, the data described in this study show that selection of both viral platforms and individual TCRs should occur by analysis of expression in peripheral blood T cells rather than cell systems that lack endogenous TCR expression, in which the requirements for expression of exogenous TCRs are substantially lower. As an example, while expression of R6C12 and 1D3

receptors is comparable in Jurkat/MA cells, only the latter is efficiently expressed in human PBMCs. Likewise, while all 4 retroviral vector systems tested yielded substantial TCR expression in Jurkat/MA cells, pMX and pMP71 were superior in yielding TCR expression in PBMCs.

Clinical application of TCR gene transfer should be preceded by an evaluation of the possible side effects, caused by either on-target reactivity (mediated via recognition of target antigens on normal tissues) or off-target reactivity (mediated by mechanisms described in the next paragraph). The risk of on-target autoimmunity will primarily depend on the expression pattern of the antigen involved. Melanoma-differentiation antigens, such as Mart-1 and Gp100, are expressed on melanoma cells as well as normal melanocytes, and targeting of these antigens is known to induce autoimmune melanocyte destruction resulting in vitiligo and uveitis.[3,20] TCR gene transfer targeting these antigens has not resulted in severe side effects.[16] However, it remains possible that increased autoimmunity will occur when more potent TCRs or conditioning regimens are used.

Off-target autoimmunity by TCR-transduced cells theoretically can be induced via 3 different mechanisms.[5] First, introduction of exogenous TCR chains can lead to the formation of heterodimers with endogenous alpha and beta chains, which might be reactive toward self-peptides. Second, if ignorant self-reactive T cells are transduced, triggering of these cells via the introduced TCR can result in an expanded population of autoreactive cells. Off-target autoreactivity via these 2 mechanisms would occur irrespective of MHC disparities between TCR donor and recipient. Both mouse models of TCR gene transfer[12,15] and the recent phase 1 clinical trial[16] do not provide evidence that these mechanisms form a substantial reason for concern.

As a third possibility for off-target autoimmunity, MHC mismatches between TCR donor and recipients may result in recognition of allogeneic MHC molecules complexed to self-antigens by the TCR-modified cells. With the aim to develop a generally applicable strategy to screen for such alloreactivity, we developed a simple assay to test TCR-modified T cells against a set of cell lines expressing defined single HLA-A and -B alleles with a high prevalence in the human population. Lack of reactivity against any of the MHC alleles expressed by this panel provides evidence that for this T-cell receptor the risk of type III off-target autoimmunity may be little. However, it may be worthwhile to further expand the set of class I alleles that is tested for a more complete evaluation of MHC alloreactivity, and possibly such an evaluation should also include reactivity against MHC class II alleles. Furthermore, although allorecognition by TCRs is often less peptide dependent,[46] it remains possible that a TCR that is unreactive toward an MHC allele on the cell line used for in vitro testing does recognize this MHC allele when complexed with a tissue-specific antigen. Based on this latter consideration, it may remain useful to set up a database for allowed MHC mismatches for every TCR used in coming clinical trials.

We have used this evaluation and optimization strategy to select a melanoma-specific receptor with improved expression, a higher affinity compared with other receptors, and a lack of detectable alloreactivity. $CD4^+$ T cells transduced with this MHC class I–restricted 1D3 receptor are capable of MHC-tetramer binding and production of IFN-γ. Prior data have shown that the provision of $CD4^+$ T-cell help contributes to both primary $CD8^+$ responses and $CD8^+$ T cell memory formation.[47] Furthermore, recent studies in mouse models have shown that transfer of a $CD8^+$-dependent MHC class I–restricted TCR into $CD4^+$ T cells can be used to generate MHC class I–restricted $CD4^+$ T-cell help.[13,14] However, as optimal function of the modified $CD4^+$ T cells requires the presence of the CD8αβ coreceptor,[13] the selection of TCRs that can function independent of CD8 is considered attractive.[27,48,49] Perhaps more importantly, compared with a TCR evaluated in a previous clinical trial, $CD8^+$ T cells expressing the 1D3 TCR recognize antigen at a 10-fold lower concentration. Furthermore, the high level of expression of the 1D3 TCR may predict a more effective in vivo persistence as based on analogy with the murine melanoma–specific pMel TCR.

The 3-step approach we describe in this study may be of use for the selection of well-expressed, high affine, and safe T-cell receptors for future clinical trials, thereby enhancing the clinical development of TCR gene therapy. Such selection may involve the evaluation either of naturally occurring TCRs or—by analogy with antibody development—of TCRs obtained by technologies that circumvent the limitations of the naturally occurring immune repertoire.[11,49,50]

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Authorship

Contribution: A.J. designed and performed research, analyzed data, and wrote the paper; R.G.-E. designed and performed research and analyzed data; M.D. performed research and analyzed data; W.K., Y.M.Z., I.I.N.D., N.R., P.R., and R.A.M. provided important reagents; T.N.M.S. and J.B.A.G.H. designed research and wrote the paper. A.J. and R.G.-.E. contributed equally to this work.

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Chapter 7

Discussion

Discussion: what defines the success of immunotherapy?

This thesis describes different immunotherapeutic strategies, ranging from activation of the endogenous T cell repertoire to infusion of genetically modified T cells. Until now, cell-based immunotherapy of cancer unfortunately has met with little success. Here, I will describe the factors that contribute to the efficacy of immunotherapy and that might enhance the success rate of immunotherapeutic strategies.

Target selection

One of the main issues in cancer immunotherapy is selection of the right target antigen. As discussed in the introduction, melanoma antigens can be divided into four different categories. Unique antigens, that arise from somatic point mutations, enable strictly selective targeting of tumor cells. They are however less suitable for immunotherapy since they are only expressed on individual tumors. Antigens belonging to the other categories, i.e. cancer/testis, melanocyte-differentiation and overexpressed antigens, are expressed in tumors from different patients. These shared antigens are therefore usually favored targets, but T-cells directed against these antigens often lack tumor specificity and can also target normal cells. In the case of melanoma differentiation antigens, destruction of healthy melanocytes can result in vitiligo and uveitis. However, since uveitis can be treated via the administration of corticosteroids, and vitiligo is considered a relatively mild side effect, the toxicity associated with targeting these antigens seems acceptable. In contrast, when target antigens are expressed on vital tissues, like the ubiquitously expressed p53 antigen, autoimmune destruction of normal cells could cause severe toxicities. Although the higher expression level of p53 on tumor cells is thought to provide tumor selectivity[1], recent experiments have indicated that targeting of this antigen can result in destruction of host peripheral blood and stem cells, leading to lethal cytopenia (M. Lauwen et al, manuscript submitted). Tissue distribution of target antigens is therefore a very important issue in the selection of targets for immunotherapy.

Another factor in target selection, is the tendency of tumors to escape immune attack by loss of antigen expression. The induction of T cell responses against specific antigens can lead to the selection of tumor cell variants that have lost expression of these target antigens, an event that has been observed for melanoma in mice [2] (chapter 5), as well as in humans[3-5]. To circumvent this, one could select target antigens that are essential for cellular transformation, such as PRAME, which is overexpressed in melanoma and is associated with an advantage in cell growth and survival[6]. Alternatively, multiple antigens could be targeted simultaneously to reduce the chance of outgrowth of antigen loss variants. Obviously, these strategies to circumvent antigen loss can only be successful if MHC-expression by tumor cells is maintained, whereas loss of MHC class I expression, caused for example by mutations in the β2-microglobulin gene or defects in antigen processing and transport pathways, will prevent tumor elimination by cytotoxic T cells.

T cell avidity

Since most tumor antigens are self antigens, naive T cells circulating in the periphery will generally only recognize these antigens with low affinity TCR-peptide/MHC interactions, due to the process of negative selection in the thymus. Activation of this endogenous repertoire

via vaccination will therefore be much more difficult than vaccination against pathogens. Also in the case of adoptive transfer of T cell clones or cell lines that have been obtained from patients, the clinical use of T cells specific for self antigens is restricted to the endogenous repertoire, although in vitro selection and expansion enable the infusion of high numbers of activated T cells. TCR gene transfer on the other hand, enables the introduction of high affinity receptors into T cells, thereby circumventing the limitations of the endogenous repertoire.

One strategy to acquire high affinity TCRs is to isolate them from T cells that managed to escape negative selection. This strategy is based on the assumption that TCRs present in some melanoma patients, e.g. patients that showed strong T cell responses or even tumor regression upon immunotherapy, are of an above average quality and would therefore be of value for broader groups of patients. This strategy is only feasible for antigens for which tolerance is not absolute, such as the melanoma differentiation antigens[7-9] (chapter 6).

To circumvent the restrictions of the endogenous repertoire, different strategies can be used that enable isolation of TCRs from a non-tolerant environment. First, high affinity TCRs can be isolated from a setting where the relevant MHC molecule is absent. In such an allogeneic setting, tolerance against self peptides bound by this molecule will not occur, and T cells (TCRs) with a high avidity for the relevant MHC/self peptide complexes will still be present within the endogenous repertoire[10]. Some of these TCRs might display cross-reactivity towards other self antigens, and receptors obtained in this manner should therefore carefully be evaluated before using them for clinical application.

A second strategy is to make use of a setting where negative selection is absent. In an in vitro display of libraries of TCRs, T cells do not undergo selection in the thymus and this will enable the screening of a large number of TCRs for increased affinity towards a specific antigen. Also here, cross-reactivity is an important issue of concern since negative selection is completely absent. Indeed, loss of specificity is known to occur after in vitro receptor selection[11].

Finally, high affinity TCRs can be isolated from a setting where the thymus does not present the relevant peptide. One example of such a setting is the isolation of TCRs specific for minor histocompatibility antigens (mHags). These antigens are derived from polymorphic genes, and are thought to be responsible for the graft-versus-leukemia effect upon MHC-matched stem cell transplantation. TCRs with a high affinity for a certain mHag can be isolated from an individual that does not express this antigen and subsequently used to target $mHag^+$ tumors[12]. Also in mice transgenic for human HLA genes, human epitopes that are not conserved between mice and men will be absent during thymic selection, and tolerance towards these antigens will therefore not occur[13,14]. TCRs obtained by this approach will be of murine origin, which could result in anti-receptor immune reactions. In current TCR gene transfer protocols, lymphodepleting pre-conditioning of the host will presumably prevent reactivity towards TCR modified cells and preliminary data from the Rosenberg lab indeed confirm this (R. Morgan, personal communication). However, in settings without lymphodepletion, the immunogenicity of infused TCR modified cells might be partially circumvented by replacing the murine constant domains with their human counterparts[15]. The generation of mice transgenic for human TCR loci, analogous to mice carrying human Ig loci[16], would completely solve this problem and markedly simplify the isolation of high affinity TCRs.

Although TCR gene transfer offers the possibility to employ receptors with an increased affinity compared to the endogenous repertoire, the level of expression of introduced TCRs is generally low due to the formation of mixed dimers (i.e. heterodimers of exogenous and endogenous TCR chains) and competition for components of the CD3 complex. Since the number of TCRs on the surface of a T cell is thought to (partially) correlate with T cell functionality, the low level of expression of introduced TCRs could potentially prevent optimal effector function of transduced cells[17,18]. One strategy to enhance the level of cell surface expression is the optimization of TCR genes. Although the sensitivity of gene-optimized TCR transduced cells as determined in in vitro assays seems unaffected, the in vivo functionality of these cells is markedly improved (chapter 5&6). Other approaches to enhance TCR expression can also reduce, or perhaps even prevent, the formation of mixed dimers via the introduction of an additional disulphide bond or via the use of murine constant domains[19-21]. A combination of these different strategies would presumably enhance TCR expression even further. We are currently evaluating these approaches for both human and murine TCRs (see section on safety issues).

Instead of introducing full length alpha and beta TCR chains, gene transfer can also be performed using chimeric receptors, which generally consist of an antibody-based external receptor structure linked to the TCR signal transduction domain[22]. These receptors offer the advantage of MHC-unrestricted antigen recognition, and TCR expression of these receptors is not hampered by the formation of mixed dimers. However, the in vivo functionality of cells transduced with chimeric receptors has never been directly compared to full length TCRs, and issues such as the potential immunogenicity of these receptors have not been addressed in in vivo studies.

Recently, it has been shown that microRNA 181a (miRNA181a) is involved in the posttranscriptional regulation of T cell sensitivity during T cell development. High levels of this microRNA are present in immature thymocytes, associated with increased T cell sensitivity that allows interaction with self antigens during thymic selection. On the other hand, miRNA181a is downregulated in mature T cells and targeted expression of miRNA181a in tumor-specific T cells might therefore enhance tumor antigen sensitivity and improve the efficacy of immunotherapy[23]. Careful evaluation of this strategy is needed however, since lowering the activation threshold of T cells could also induce crossreactivity towards other self antigens.

Conditioning regimen

Tumor-specific cells, either transferred or present in the endogenous repertoire, can be activated via active immunization, which will result in a proliferative burst (expansion phase), promptly followed by contraction of the effector T cell pool with only a low number of memory cells remaining in the circulation. Although T cell responses against tumor antigens are detectable upon vaccination, they are often of limited magnitude and persistence of these cells is only short-term[24,25,26].

In contrast, host conditioning via irradiation or chemotherapy can create an environment in which tumor-reactive T cells are activated and induced to undergo homeostatic proliferation[27,28]. T cells that are transferred into an “empty” host have unlimited access to proliferative cytokines and antigen presenting cells, and are less susceptible to suppression by regulatory elements. In melanoma patients, adoptive transfer of high numbers

of tumor infiltrating lymphocytes following lymphodepleting chemotherapy, resulted in long term persistence of adoptively transferred cells and marked tumor regression[29,30].

Chapter 3 describes a setting in which vaccination and irradiation are combined and where only a low number of antigen-experienced cells is transferred to the recipients. In this setting, transferred cells undergo vaccine-induced activation, followed by contraction of the antigen-specific T cell pool. This results in a marked but transient skewing of the T cell repertoire towards tumor recognition, suggesting that the transferred cells are not able to use the empty environment. Different T cell kinetics are described in chapters 5 and 6, where a relatively high number of TCR-transduced cells is transferred into irradiated hosts. In this setting, transferred cells repopulate the host's immune system and persist over a significant period of time. Additional vaccination of recipients has no effect on the persistence or functionality of transferred cells. Although these models differ in several aspects, the data suggest that the composition of the graft, i.e. the percentage and/or number of antigen-specific cells, influences T cell kinetics upon adoptive transfer into irradiated hosts.

Increased intensity conditioning or even complete myeloablation followed by hematopoietic stem cell transfer might further enhance treatment efficacy. Full ablation could lead to more complete removal of regulatory T cells and depletion of cytokine sinks. In addition, high-dose total body irradiation can induce diffuse tissue injury and a generalized inflammatory reaction, which could further drive the T cell response. Recent data showed that lymphodepletion to a level that required hematopoietic stem cell transplantation, combined with adoptive transfer of tumor reactive T cells, resulted in an enhanced anti-tumor effect compared to pretreatment with non-myeloablative conditioning[31]. However, increasing the intensity of conditioning will also increase lymphodepletion-associated toxicities (i.e. prolonged neutropenia, risk of infections, pulmonary complications), and therefore selection of an optimal conditioning regimen and careful monitoring of patients will be crucial[32].

Alternatively, selective depletion of competing cellular subsets using monoclonal antibodies against antigens expressed exclusively on T lymphocytes could provide a milder and therefore safer means of induction of lymphopenia. However, T cell-specific antibodies will not deplete NK cells, so that these cells remain present in the recipients and compete with transferred T cells for cytokines such as IL-7 and IL-15. Furthermore, depleting antibodies will not induce tumor tissue damage and will therefore lack the potential beneficial effect of improved antigen presentation or tumor accessibility.

T cell differentiation state

Prolonged antigenic stimulation during in vitro culture, such as often is required for the adoptive transfer of high numbers of tumor reactive lymphocytes, can cause the generation of exhausted T cells[33-35]. These cells might have an optimal antitumor activity in vitro, their proliferative capacity is however often decreased and these cells show poor survival in vivo. In melanoma patients, adoptive transfer upon extensive proliferation of T cell clones or vaccine-induced tumor specific cells did not result in long term persistence of transferred cells and clinical responses could not be observed[36,37].

Several studies suggest that in vivo persistence can be increased when adoptive transfer is performed with T cells that still express costimulatory and lymphoid homing receptors. These cells are thought to have an improved capacity to home to secondary lymphoid tissue, mediated via the expression of CD62L and CCR7. Interaction with peptide-MHC complexes

on APCs and simultaneous costimulation, e.g. via CD27/CD70, within these lymphoid tissues subsequently induces T cell activation and proliferation, and enables long-term persistence[38-40]. Studies in mice have shown that adoptive transfer of cells in a less differentiated state yields superior antitumor immunity as compared to adoptive transfer of fully differentiated cells[41-44]. Furthermore, although the tumor infiltrating lymphocytes responsible for tumor regression in the successful TIL trial generally had a late stage effector phenotype, the T cells that persisted in these patients for a long period expressed the costimulatory molecules CD27 and CD28, characteristic of a less differentiated state[45]. These data suggest that selection of less differentiated subpopulations before adoptive transfer can result in more effective antitumor immunity, and is a more preferred strategy than the selection of tumor reactive T cells based on in vitro IFNγ production and tumor cell lysis. Besides the expression of costimulatory and homing molecules, telomere length is also correlated with the long-term persistence of transferred cells[46]. To counteract the defective proliferative capacity of in vitro expanded cells, the hTERT gene can be introduced in order to prevent telomere erosion[47] (chapter 4). Although this approach can greatly enhance the number of population doublings, it has also been associated with genomic instability, which may limit its clinical application[48-50].

TCR gene transfer circumvents the need for extensive in vitro culture to generate large numbers of tumor specific T cells, although retroviral transduction does require strong activation of cells in order to induce cell-cycling. Selection of less differentiated subpopulations has not been tested for TCR transduced cells, but this approach seems worthwhile to pursue. As an alternative, infection with lentiviral vectors could be used, since this does not require cell division for integration of the transgene. However, activation of T cells with cytokines is still required for lentiviral transduction because infection of totally quiescent cells is blocked[51,52].

A different approach to prolong the persistence of transferred cells, is to optimize the in vitro stimulation procedure so that fit, rather than exhausted, T cells are generated. Murine TCR transgenic cells cultured in IL-15 maintained expression of CD62L and CCR7, whereas cells cultured in IL-2 had reduced expression of these molecules. Furthermore, subsequent adoptive transfer of IL-15 cultured cells into sublethally irradiated hosts resulted in a more pronounced anti-tumor effect as compared to cells cultured in IL-2[53]. Human T cells modified with a chimeric TCR and cultured in the presence of IL-15 were capable of long-term persistence in SCID-Beige mice and could eradicate established tumors[54]. Also, cell culture in the presence of artificial APCs, which can be engineered to express different co-stimulatory molecules, can generate high numbers of T cells that retain a substantial replicative capacity[55,56].

The presence of exogenous cytokines is not only required during in vitro culture, also in vivo CTLs often depend on the administration of cytokines. Administration of IL-2 can improve CTL persistence and expansion, but high doses of IL-2 are associated with serious toxicities, such as the vascular leak syndrome. These toxicities can be avoided by using lower doses of IL-2, which still support the growth of transferred cells[57]. IL-2 not only activates T cells, but is also known to cause activation induced cell death in vivo, and is involved in the induction of CD4+ CD25+ regulatory T cells[58]. Other γ-chain cytokines, such as IL-15 and IL-7, which only induce the activation of T cells, might be more suitable for exogenous

administration[53,59]. Alternatively, transfer of the IL-2 gene can be used to specifically deliver the cytokine to tumor-specific T cells[60]. This strategy has shown to allow maintenance of human melanoma-reactive T cells in vitro without the need of exogenous IL-2 administration. However, data from a first clinical trial with IL-2 modified TILs have been disappointing (B. Heemskerk, personal communication). Furthermore, constitutive expression of IL-2 might enable autonomous T cell growth and therefore require additional safety measures[61].

Finally, the in vitro production of tumor-specific T cells by TCR gene transfer into hematopoietic stem cells (HSCs) offers the possibility to generate T cells with long telomeres and presumably a much greater proliferative capacity than the memory cells currently used[62,63]. Furthermore, allelic exclusion of endogenous alpha and beta chains in these progenitor cells would also reduce the problem of mixed dimer formation, although allelic exclusion is far from complete for TCR alpha chains[63]. However, retroviral transduction of HSCs may increase the chance of transformation due to insertional mutagenesis, since genes involved in growth and development are active in progenitors, and insertions into these "high risk" genes are therefore more likely to occur. In addition, progenitor cells have a high proliferative potential and transformed cells will thus be more prone to uncontrolled growth. In a gene therapy trial of SCID-X1, leukemia was observed in several patients upon infusion of modified HSCs[64] (see also paragraphs on safety issues). Although there are indications that additional factors were involved in the development of leukemia in these patients[65,66], the potential risks of TCR gene transfer into hematopoietic precursors should be carefully evaluated in mouse models that mimic gene therapy of SCID-X1 and other diseases.

Modification of tumor environment

Progressing tumors often develop strategies to evade tumor recognition, and these mechanisms can lead to T cell dysfunction or anergy, and may thereby prevent effective anti-tumor immunity. The combination of immunotherapy with strategies that counteract tumor immune evasion and other inhibitory mechanisms could therefore improve the anti-tumor activity.

One strategy that tumors use to hamper immune responses is the production of inhibitory molecules such as IL-10, TGFβ, and IDO. The best characterized immunosuppressive cytokine is TGFβ, which is frequently found to be present in high concentrations in cancer patients, and is associated with disease progression and poor responses to immunotherapy. TGFβ supports tumor growth through the promotion of angiogenesis, the inhibition of T cell proliferation and activation, and the induction of regulatory T cells[67]. The immunosuppressive actions of TGFβ can be inhibited via large and small molecule inhibitors, but global blockade of TGF may result in side effects[68]. Alternatively, T cells can be provided with a dominant-negative TGFβ receptor, a strategy that has shown to induce preferential tumor infiltration and elimination with minimal side effects[69-71]. The infusion of TCR-transduced cells that were also modified with the TGFβDN receptor, led to an increase in T cell responses, but also to an increase in mortality that requires more attention before the possible merits of TGFβ blockade can be studied further (M. de Witte, unpublished observations, see also discussion of safety issues).

Besides secretion of immunosuppressive molecules, tumors can also express inhibitory ligands, such as FasL, PD-L1 (B7-H1) and B7-H4, on their cell surface and in this way hamper immune responses via the inhibition of T cell activation and proliferation and the

induction of T cell apoptosis. To circumvent Fas-mediated tumor immune evasion, T cells can be rendered resistant to FasL by gene transfer of small interfering RNA (siRNA). Human EBV-specific CTLs modified via this strategy were no longer sensitive to Fas-induced apoptosis, maintained their polyclonality upon prolonged cultured and remained dependent on antigen-specific stimulation for their proliferation and survival[72]. The inhibitory effects of programmed death receptor ligand -1, PD-L1, can be counteracted via the administration of blocking antibodies, and has shown to enhance tumor eradication in in vivo mouse studies[73,74]. However, PD-L1 is also expressed on normal tissue and the PD-L1/PD-1 pathway is involved in the regulation of peripheral tolerance, so extensive testing is warranted before clinical application.

T-cell mediated kill of tumors can also be prevented by the lack of expression of costimulatory molecules. The presence of costimulatory signals is thought to be required for both the activation of tumor-specific cells, as well as for efficient tumor cells lysis by transferred cells[75,76]. In the absence of costimulatory molecules such as CD28, costimulation to infused cells can be provided by modification of these cells with CD28-derived chimeric receptors. These single chain receptors contain an antigen-specific domain fused to the signal transduction domain of CD28, which can supply T cells with a costimulatory signal in the absence of B7-positive tumor cells[77]. The design of these chimeric receptors can be further optimized so that they provide both activation and costimulatory signals (CD28/CD3ζ)[78]. As for the regular chimeric receptors (see above), the functionality of T cells transduced with these receptors needs more extensive in vivo evaluation.

The transfer of TCR genes into T cells specific for viruses that have a latent persistence in vivo, might enhance the survival of modified cells by providing both antigen-dependent activation and costimulation via targets expressing viral antigens. Heemskerk et al. showed that modification of CMV-specific cells with a TCR specific for the minor antigen HA-2 generated cells that could recognize both HA-2^+ and CMV^+ targets[79]. An additive advantage of this strategy is that the formation of mixed dimers will be limited due to gene transfer into a T cell population with a restricted TCR repertoire. Furthermore, using virus-specific cells as recipients also limits the chance of activating ignorant, self-reactive cells via the introduction of an exogenous TCR (see safety issues). This approach has not been tested in vivo but clinical application should be feasible based on the presence of CMV or EBV specific cells in the majority of individuals, and the possibility to isolate these cells using MHC-tetramers[80-82]. A potential problem in this approach could be the in vivo selection of T cells that have a high expression of the viral-specific TCR and therefore a low expression of the introduced TCR.

Lastly, tumors can suppress immune responses via the induction of regulatory T cells. These cells are responsible for the induction and maintenance of peripheral tolerance towards self antigens and in that way they can also prevent effective anti-tumor immunity. For many different cancer types, including melanoma, an increased frequency of regulatory T cells has been observed in the peripheral blood of patients[83,84]. Furthermore, accumulation of regulatory T cells within ovarian tumors has been associated with a decreased survival[85]. As discussed previously, regulatory T cells can be eliminated by lymphodepleting chemotherapy or total body irradiation. However, this effect will only be transient when regulatory cells are reinfused with the graft or induced via the administration of IL-2[86]. Several mouse models have demonstrated that systemic depletion of regulatory T cells by targeting CD25, CTLA-4, or GITR results in an enhanced anti-tumor response[87-89]. Although administration of

antibodies against CTLA4 resulted in tumor regression in melanoma patients, it was also occasionally associated with severe autoimmunity[90,91].

Safety issues

An increasing number of immunotherapy trials is being performed in recent years, and some of these have resulted in clinical success. Before embarking on such studies, several factors concerning safety should be considered. A first safety issue in cancer immunotherapy is the choice of target antigens. The development and severity of on-target toxicity (i.e. reactivity towards the target antigen expressed on healthy tissue), will depend on the expression pattern of these antigens. As discussed previously, expression on tissues such as the skin is not likely to result in serious toxicity, whereas expression on vital tissues could potentially lead to more severe problems.

In TCR gene therapy, there are several additional safety issues, associated with either the process of T cell transduction via retroviral integration, or with the introduction of an exogenous TCR into mature T cells. Retroviral transductions could potentially lead to malignant transformation when the expression profile of oncogenes is altered by integration of the therapeutic gene. This was considered to be mainly a hypothetical risk, but became reality in a gene therapy trial of X-SCID where 3 out of 11 children developed leukemia upon retroviral integration in the LMO2 oncogene[64]. However, the risk of transforming events upon infusion of TCR-modified cells is likely to be much smaller than in the X-SCID trial, since there are several factors specific for this trial that might have contributed to the occurrence of leukemia. First, transduction of hematopoietic stem cells is more likely to result in integration in genes involved in self-renewal and growth as compared to transduction of mature T cells. In fact, there are no reports of side effects due to insertional mutagenesis in preclinical or clinical studies with mature T cells[92,93]. Second, γc-transduced cells have a strong growth advantage due to the severe immunodeficiency in X-SCID patients. Finally, the γc transgene itself may be tumorigenic, as suggested by a recent study in mice where overexpression of this gene caused the induction of T cell lymphomas[65].

Nevertheless, several approaches could be followed to minimize the risk of transforming events upon TCR gene transfer. For example, lentiviral or self-inactivating vectors might be considered instead of the retroviral vectors currently used in most clinical trials. Lentiviruses preferentially integrate downstream of transcriptional start sites, and in self-inactivating vectors transgene expression is driven from an internal promoter instead of the strong viral LTR[94,95]. In our hands, however, T cell transduction using different self-inactivating vectors did not result in substantial TCR expression (R. Gomez, unpublished observations). Possibly, with the development of engineering approaches that yield more robust TCR expression (see below), the use of lentiviruses may have more potential.

Another approach that is likely to enhance safety is to induce efficient TCR transgene expression while minimizing the number of viral integrations. The use of stronger promoter/enhancer elements may enhance transgene expression, but could also result in an enhanced effect on neighboring genes. Alternatively, transgene expression can be increased at the post-transcriptional level. As shown in chapter 5, gene-optimization enhances TCR expression without influencing viral titers, suggesting that the number of retroviral integrations is also unaffected. In addition, usage of the Woodchuck Hepatitis post-transcriptional regulatory element (WPRE) has been reported to strongly enhance transgene

expression, although this effect presumably also depends on an increase in viral titers[96]. However, in our own experience, introduction of WPRE into different retroviral vectors abolished expression of the DMF4 Mart-1-specific TCR (A. Jorritsma, unpublished observations).

Finally, inclusion of a suicide switch in retroviral vectors would allow the selective elimination of transformed cells. Introduction of the herpes simplex virus thymidine kinase (HSV-TK) gene in combination with administration of ganciclovir has shown to control graft versus host disease upon donor lymphocyte infusions[97,98]. However, immunogenicity of the HSV-TK gene product limits application of this safety switch[99,100]. A non-immunogenic alternative consists of the pro-apoptotic caspase 9 molecule fused to a FK506 binding domain, in combination with a chemical dimerizer[101,102]. In the RIP-OVA mouse model, autoimmune diabetes caused by OT-1 transgenic cells expressing the suicide switch could be blocked upon infusion of the dimerizer, although this approach has not yet been tested for TCR-transduced cells[103].

Besides the risk associated with retroviral integrations, the introduction of a new TCR into mature T cells could in itself lead to side-effects. Theoretically, three different mechanisms could result in the induction of autoimmunity[104]. First, introduction of exogenous TCR chains can lead to the formation of heterodimers with endogenous alpha and beta chains. This can generate TCRs with new specificities that might be reactive towards self-peptides. Second, if ignorant self-reactive T cells are transduced, triggering of these cells via the introduced TCR can result in an expanded population of autoreactive cells. Finally, MHC-mismatches between the TCR donor and recipients may result in recognition of allogeneic MHC molecules complexed to self antigens. For both human (chapter 6) and murine[105] transduced T cells, settings with an MHC-mismatch between TCR donor and recipient did not demonstrate alloreactivity, although these observations cannot guarantee safety of TCR-peptide/MHC combinations other than the ones tested.

Until now, both preclinical and clinical studies with TCR modified T cells have not shown any signs of off-target reactivity. However, recent experiments within our lab demonstrated that in a setting that strongly promotes the proliferation and activation of transferred T cells, i.e. in a lymphodepleted environment in combination with additional adjuvants such as IL-2 or TGFβ-blockade, severe pathology was induced upon infusion of TCR-modified cells, characterized by bone marrow failure and other signs of graft versus host disease. These side effects occurred in the absence of target antigen and ongoing experiments suggest that this pathology may also arise when a different TCR is used, indicating that the effect may be TCR-independent and not caused by cross-reactivity of the introduced TCR (G. Bendle, unpublished observations). Since introduction of exogenous TCRs is known to result in the formation of mixed dimers[106], these new TCRs could be responsible for the observed off-target autoimmunity. In this scenario, mixed dimers with self-reactive specificities would induce bone marrow failure analogous to settings of MHC-mismatched lymphocyte infusions[107-108].

Currently, experiments are being performed to assess whether mixed dimers are indeed the cause of the autoimmune pathology observed in our models. If so, several strategies could be employed to avoid damage by these heterodimers. First, pairing of exogenous and endogenous chains may be reduced or prevented by remodeling of the TCRαβ interface via

introduction of an extra disulphide bond or usage of murine constant domains[19-21]. Preliminary experiments with the Mart-1 –specific 1D3 TCR showed that these modifications could enhance the level of expression of the intended 1D3 heterodimer, but the effect on mixed dimer formation has not been assessed yet (R. Gomez, unpublished observations). Alternatively, cells expressing the correct αβ heterodimer could be selected using specific MHC-tetramers[82], thereby presumably minimizing the presence of autoreactive cells that express mixed dimers within the graft. Finally, the formation of mixed dimers can be avoided by the usage of single chain chimeric receptors[109], the transduction of γδ T cells[110], or by –at least to some extent- the transduction of hematopoietic stem cells (see section on T cell differentiation state). However, these approaches have not yet been evaluated in in vivo models.

Concluding remarks

Immunotherapy provides new possibilities for the treatment of tumors that do not, or no longer, respond to conventional therapies. Active immunization, which acts by stimulating the naive T cell repertoire, can be a simple, "off the shelf" method for the induction of T cell responses against tumors expressing viral antigens. The development of strategies such as DNA tattoo vaccination resulted in a more rapid and potent induction of immune responses, and combination of this strategy with host conditioning is likely to improve the clinical efficacy of vaccination.

However, when the endogeous T cell repertoire is affected by tolerance, such as is the case for melanoma and many other tumors, passive immunization via the transfer of TCR genes seems to be a more preferable approach. The selection of high affinity TCRs and the development of TCR formats that are well expressed without competition with endogenous receptors, are likely to improve the anti-tumor efficacy of TCR modified cells, while minimizing chances of autoimmunity. In addition, optimization of the host conditioning regimen, the selection of "fit" T cell subpopulations, and modification of the tumor environment can lead to further improvements. These factors should be taken into consideration when designing new clinical TCR gene transfer protocols.

If current limitations can be solved and future clinical trials are successful, TCR gene therapy has the potential of becoming a potent addition to conventional cancer therapies such as surgery, chemotherapy and radiation. The modification of patient's T cells with TCR genes has to be performed in specialized laboratories, but since this procedure is relatively simple, "off the shelf" application will become possible.

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Summary

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Summary

This thesis describes different immunotherapeutic strategies that can be used for the treatment of cancer in general, and of melanoma in particular. Tumor-specific T cell responses can be induced via either active or passive immunization, and both approaches are discussed within this thesis.

Chapter 2 describes a new method for active immunization using a tattoo device to deliver DNA into the skin. Tattoo vaccination results in faster and more robust antigen specific T cell responses compared to conventional intramuscular DNA vaccination. Moreover, this vaccination strategy could efficiently target established tumors in mice and provide protection against infection with influenza A.

In chapter 3, DNA tattoo vaccination is combined with sublethal irradiation of recipients and adoptive transfer, a strategy that results in a marked, although transient, skewing of the T cell repertoire towards tumor recognition. In vivo, this combined treatment results in a more pronounced anti-tumor effect compared to DNA vaccination alone.

Active immunization is dependent on the endogenous T cell repertoire and may therefore not be the preferred strategy when targeting tumor self antigens. Chapter 4 describes a passive immunization strategy that uses the adoptive transfer of a tumor-specific T cell clone to generate effective anti-tumor responses. The life span of these transferred human T cells is extended by the introducion the telomerase reverse transcriptase (hTERT) gene. hTERT-transduced cells can be cultured far beyond the number of population doublings observed for wild type cells, while maintaining their anti-tumor functionality in vivo.

An attractive alternative to the use of naturally occurring tumor-reactive T cells is passive immunization via the transfer of T cell receptor (TCR) genes, since this circumvents isolation and extensive in vitro culture of T cells and allows broader clinical application. In chapter 5 we use a mouse model to elucidate the requirements for efficient targeting of self antigens by TCR gene transfer. Factors such as host conditioning regimen, the format of the introduced TCR genes, and the properties of the infused T cell graft have a marked effect on the anti-tumor efficacy of TCR modified cells, indicating that these factors should be taken into consideration when designing clinical TCR gene therapy trials.

Chapter 6 shows that gene-modification of melanoma-reactive TCRs can markedly enhance TCR expression and in vivo functionality. This chapter also describes a procedure for the selection of a human TCR that is well expressed and highly affine, and that is therefore likely to enable more efficient targeting of human melanoma.

In summary, this thesis describes different strategies for the induction of anti-tumor immune responses. Passive immunization via TCR gene transfer is the preferred strategy when targeting tumor self antigens, because this can circumvent limitations of the endogenous T cell repertoire and does not require extensive in vitro culture. In addition, we have identified several factors that can improve the anti-tumor activity of TCR modified cells, and implementation of these factors in the design of clinical trials is likely to positively affect the clinical efficacy of TCR gene therapy.

Bijsluiter Immuuntherapie

Inleiding

Hoewel de behandeling van kanker de laatste jaren sterk is verbeterd, is deze ziekte nog steeds één van de belangrijkste doodsoorzaken in de westerse wereld. Chirurgie, bestraling en chemotherapie kunnen het ziekteproces in een vroeg stadium vaak wel stoppen, maar in het geval van uitzaaiingen zijn deze standaardbehandelingen niet in staat de tumor tot en met de laatste cel uit te roeien. Daarom wordt er op dit moment veel onderzoek gedaan naar nieuwe behandelingsmethoden die, eventueel in combinatie met standaardtherapieën, wel tot volledige genezing kunnen leiden.

Een mogelijk veelbelovend alternatief is immuuntherapie: hierbij wordt het afweersysteem van het lichaam gebruikt in de strijd tegen kanker. Het afweersysteem speelt een belangrijke rol bij de bescherming tegen ziekteverwekkers zoals bacteriën en virussen, maar het kan ook tumorcellen opsporen en vernietigen. T-cellen hebben hierin een groot aandeel. Dit zijn witte bloedcellen die circuleren door het lichaam en die alles wat potentieel gevaarlijk is kunnen herkennen en opruimen. Deze herkenning vindt plaats door middel van een structuur aan het oppervlak van de T-cel, de T-cel receptor (TCR). De TCR kan zich binden aan een klein stukje eiwit van een bacterie, virus of tumor dat aanwezig is op het oppervlak van een geïnfecteerde of kwaadaardige cel. Als de binding tussen de TCR en dit stukje eiwit (antigen) sterk genoeg is, wordt de T-cel aangezet tot het uitscheiden van stoffen die een geïnfecteerde cel of tumorcel kunnen doden.

Het probleem bij tumoren is echter dat ze meestal ontstaan uit gezonde menselijke cellen, waardoor ze voor het afweersysteem lastig te herkennen zijn als mogelijk gevaarlijk. Dit heeft als gevolg dat het verdedigingsmechanisme vaak te weinig of te laat geactiveerd wordt, zodat de in gang gezette afweerreactie niet in staat is de tumorgroei te remmen. Immuuntherapie is er daarom op gericht het afweersysteem te helpen bij het opsporen en kapot maken van tumorcellen. Dit kan op twee verschillende manieren. Ten eerste door middel van vaccinatie, ook wel actieve immuuntherapie genoemd. Door patiënten te vaccineren wordt het afweersysteem zelf aangezet tot een reactie tegen de tumor. Dit proefschrift beschrijft een nieuwe manier van actieve immuuntherapie waarbij een vaccin bestaande uit DNA met behulp van een tattoo-apparaat in de huid wordt gebracht (DNA tattoo vaccinatie). Een andere methode is passieve immuuntherapie. Hierbij worden grote aantallen reeds geactiveerde T-cellen ingespoten bij de patiënt, zodat het lichaam niet zelf de afweerreactie in gang hoeft te zetten. In dit proefschrift wordt een vorm van passieve immuuntherapie beschreven waarbij gebruik wordt gemaakt van genetisch gemodificeerde T-cellen (verderop aangeduid als TCR gentherapie). De komende jaren zullen beide behandelingsmethoden getest gaan worden bij kankerpatiënten. In de bijsluiter hieronder worden deze vormen van immuuntherapie daarom verder toegelicht.

Lees deze bijsluiter zorgvuldig door voordat u start met het gebruik van dit geneesmiddel.

- *Bewaar deze bijsluiter, het kan nodig zijn om deze nogmaals door te lezen.*
- *Heeft u nog vragen, raadpleeg dan uw arts of apotheker.*
- *Dit geneesmiddel is aan u persoonlijk voorgeschreven, geef dit geneesmiddel niet door aan anderen. Dit geneesmiddel kan schadelijk voor hen zijn, zelfs als de verschijnselen dezelfde zijn als waarvoor u het geneesmiddel heeft gekregen.*

● *Wanneer één van de bijwerkingen ernstig wordt of in geval er bij u een bijwerking optreedt die niet in de bijsluiter is vermeld, raadpleeg dan uw arts of apotheker.*

Inhoud van deze bijsluiter

1. Hoe werkt dit geneesmiddel?

DNA tattoo vaccinatie

Vaccinatie tegen een tumor gebeurt eigenlijk op dezelfde manier als bij vaccinaties tegen bijvoorbeeld de griep of Hepatitis B. Er wordt een stukje eiwit (antigen), afkomstig van een virus of tumor, in het lichaam van de patiënt gebracht. Deze antigenen zijn op zichzelf niet ziekteverwekkend, maar ze kunnen wel T-cellen activeren. De geactiveerde T-cellen kunnen dan aan de slag gaan om schadelijke tumorcellen op te ruimen.

TCR gentherapie

Voor een goede aanval tegen de tumor zijn grote hoeveelheden T-cellen nodig die de tumor kunnen herkennen. Een manier om aan voldoende T-cellen te komen is door T-cellen te isoleren uit de tumor van de patiënt, en deze vervolgens in het laboratorium te kweken tot zeer grote aantallen. Het is echter lang niet altijd mogelijk de juiste cellen te isoleren omdat er maar heel weinig T-cellen in het lichaam aanwezig zijn die de tumor kunnen herkennen. Bovendien kost het kweken van de cellen veel tijd en moet deze procedure voor iedere patiënt apart herhaald worden. Deze problemen kunnen omzeild worden door middel van gentherapie. In plaats van het isoleren en kweken van geschikte T-cellen wordt een TCR gen dat kan zorgen voor herkenning van de tumor overgezet naar T-cellen afkomstig uit het bloed van de patiënt. Dit een hele simpele manier om willekeurige T-cellen te veranderen in cellen die de tumor kunnen herkennen en aanvallen. Het is hierdoor niet langer nodig T-cellen te isoleren en langdurig te kweken, en bovendien kunnen veel patiënten met hetzelfde TCR gen behandeld worden.

2. Wat is de samenstelling van dit geneesmiddel?

DNA tattoo vaccinatie

Bij deze vorm van vaccinatie bevat het vaccin DNA dat codeert voor tumor antigenen. Dit DNA wordt in het lichaam omgezet in stukjes eiwit (antigenen) die een afweerreactie kunnen oproepen.

TCR gentherapie

Bij deze vorm van immuuntherapie bevat het geneesmiddel T-cellen waarin een TCR gen is gezet. De T-cellen worden hiervoor eerst geisoleerd uit het bloed. Vervolgens worden ze in het lab behandeld met een aangepast virus dat er voor zorgt dat het TCR gen wordt ingebouwd in het DNA van de T-cellen.

3. Waarvoor wordt het gebruikt?

Bij actieve immuuntherapie kan de vaccinatie alleen goed werken wanneer er in het lichaam al T-cellen aanwezig zijn die de tumor kunnen herkennen. Sommige tumoren zijn echter gemakkelijker te herkennen dan andere. Tumoren die worden veroorzaakt door een virus, zoals baarmoederhalskanker (veroorzaakt door het humaan papiloma virus), zijn over het algemeen goed te herkennen door het afweersysteem. Dit komt doordat ze kleine stukjes van het virus op hun oppervlak hebben (virus antigenen). Bij dit soort tumoren is het relatief gemakkelijk om een afweerreactie op te wekken door middel van vaccinatie. De meeste tumoren, zoals bijvoorbeeld melanoom, worden echter niet veroorzaakt door een virus en hebben daarom geen virus antigenen op hun oppervlak. Omdat deze tumorcellen heel

erg lijken op normale cellen, ontsnappen dit soort tumoren over het algemeen aan de aandacht van het immuunsysteem. Het is dan niet mogelijk de T-cellen in het lichaam van de patiënt te activeren door middel van vaccinatie. In deze gevallen is passieve immuuntherapie, door het toedienen van genetisch gemodificeerde T-cellen, daarom meer geschikt.

4. Hoe moet dit geneesmiddel worden gebruikt?

DNA tattoo vaccinatie

Bij deze vorm van vaccinatie wordt een oplossing met het DNA met behulp van een tattoo-apparaat in de bovenste laag van de huid gebracht**.** Onze huid is de eerste verdedigingslinie tussen het lichaam en de buitenwereld. Er bevinden zich hier veel afweercellen en daarom is het een uitermate geschikte plek voor het opwekken van een afweerreactie. Voor de vaccinatie wordt een kleine hoeveelheid DNA-oplossing op de huid gebracht, waarna de huid gedurende korte tijd getatoeëerd wordt. Deze behandeling wordt na 3 en na 6 dagen herhaald.

TCR gentherapie

Bij TCR gentherapie wordt eerst bloed afgenomen van de patient. Hieruit worden de T-cellen geïsoleerd en deze worden vervolgens in het laboratorium behandeld met het TCR gen. In tussentijd wordt de patiënt voorbehandeld met chemotherapie. Dit maakt de T-cellen die nog in het lichaam zitten kapot, waardoor er ruimte onstaat voor de gemodificeerde cellen, zodat deze zich beter vermenigvuldigen. Vervolgens worden de aangepaste T-cellen geïnjecteerd, en krijgt de patiënt tevens een aantal keer een lage dosis van een middel toegediend dat de groei van de cellen nog verder stimuleert (Proleukine).

5. Wat voor bijwerkingen zijn er bekend?

Zowel bij vaccinatie als bij het toedienen van genetisch gemodificeerde T-cellen kunnen er bijwerkingen optreden die ontstaan doordat er ook een afweerreactie tegen gezonde cellen wordt opgewekt. Dit is afhankelijk van de aanwezigheid van antigenen op deze cellen. Melanoom antigenen zitten bijvoorbeeld ook op gezonde pigmentcellen in de huid. Immuuntherapie tegen melanoom kan daarom leiden tot het kapot maken van deze cellen waardoor er op de huid witte plekken ontstaan (vitiligo). Ook in de ogen komen deze pigmentcellen voor, waardoor immuuntherapie in sommige gevallen kan leiden tot oogontsteking. Dit is over het algemeen echter goed te behandelen met corticosteroiden.

Het tatoeëren van de huid kan pijnlijk zijn en na afloop kan de huid enigszins rood zien en geïrriteerd aanvoelen. Dit is echter van korte duur. Eventueel kan pijnbestrijding worden toegepast.

Het gebruik van genetisch gemodificeerde T-cellen kan in theorie een auto-immuunreactie veroorzaken doordat de aangepaste T-cellen ook andere gezonde cellen aanvallen. Ook is er een kans dat het inbouwen van het TCR gen in de T-cellen van de patiënt leidt tot ongecontroleerde deling van deze cellen doordat bepaalde delen van het DNA verstoord zijn geraakt. In een vergelijkbare Amerikaanse studie bij melanoompatiënten zijn tot nu toe echter geen problemen waargenomen, op basis waarvan het risico voor de patiënt op dit moment gering wordt geacht.

6. Gegevens uit preklinisch onderzoek

In dit proefschrift worden verschillende vormen van immuuntherapie beschreven. Hoofdstuk 2 laat zien dat vaccineren van muizen door middel van tatoeëren een snellere en sterkere afweerreactie kan opwekken dan met de standaardmethode van vaccineren, namelijk injectie in de spier. Bovendien kan deze manier van vaccineren de groei van een tumor in muizen veel beter voorkomen. In hoofdstuk 3 wordt deze methode verder verbeterd door de vaccinatie te combineren

met bestraling. De bestraling zorgt ervoor dat er meer T-cellen terecht komen in de tumor, en dat deze cellen beter in staat zijn de tumor aan te vallen. Hoofdstuk 4 beschrijft een methode die het makkelijker maakt T-cellen die geïsoleerd zijn uit een tumor tot grote aantallen op te kweken. Hiertoe wordt een gen, dat er voor zorgt dat de cellen oneindig vaak kunnen delen, in de T-cellen gezet. In hoofdstuk 5 gebruiken we een muismodel om uit te zoeken hoe T-cellen, die veranderd zijn met een TCR gen, het beste hun werk kunnen doen. Dit hoofdstuk laat zien dat de grootste remming van tumorgroei optreedt als er T-cellen worden ingespoten die veel receptoren op hun oppervlak hebben, en wanneer er zo min mogelijk irrelevante cellen (die niet het TCR gen hebben gekregen) worden meegespoten. Bovendien kan bestraling er voor zorgen dat de T-cellen zich goed kunnen vermenigvuldigen en dat ze lang aanwezig blijven in het lichaam. In hoofstuk 6 wordt er overgeschakeld van een diermodel naar menselijke cellen: hier onderzoeken we welke TCR het beste gebruikt kan worden voor de behandeling van melanoompatiënten. Hiertoe hebben we eerst gekeken op welke manier we de meeste receptoren aan het oppervlak van een T-cel kunnen krijgen. Vervolgens hebben we verschillende receptoren met elkaar vergeleken en hieruit de beste gekozen.

DNA tattoo vaccinatie zoals dat beschreven wordt in dit proefschrift is vooral geschikt voor de behandeling van tumoren die veroorzaakt zijn door een virus. Bij tumoren die geen virus antigenen op hun oppervlak hebben is passieve immuuntherapie een betere keuze. De behandeling van patiënten met genetisch gemodificeerde T-cellen heeft bovendien de meeste kans van slagen wanneer een TCR gebruikt wordt met een sterke herkenning van tumor antigenen, en wanneer de gemodificeerde cellen worden ingespoten onder omstandigheden die optimaal zijn voor de vermenigvuldiging en functie van deze cellen.

Curriculum Vitae

Annelies Jorritsma werd geboren op 21 april 1977 te Groningen. Zij behaalde haar gymnasium diploma op het Praedinius Gymnasium in Groningen, en begon in 1995 met de studie farmacie aan de Universiteit van Utrecht. Na een wetenschappelijke stage bij het Imperial College in Londen (Leukocyte Biology Section, prof. T. Williams), en een stage bij het Slotervaart Ziekenhuis (Apotheek, prof. J. Beijnen), ontstond bij haar enthousiasme voor het wetenschappelijk onderzoek. Na het behalen van haar doctoraal examen in 2000 en het apothekers examen in 2002, begon zij daarom in juli 2002 aan een promotieonderzoek bij de afdeling Immunologie van het Nederlands Kanker Instituut, in de groep van Dr. John Haanen. De resultaten van dat onderzoek zijn te lezen in dit proefschrift. Inmiddels is zij als postdoc/apotheker werkzaam binnen deze afdeling, en in die hoedanigheid betrokken bij het opzetten van een fase I klinische studie.

List of publications

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