Chapter 10

Can Redirected T Cells Outsmart Aggressive Melanoma? The Promise and Challenge of Adoptive Cell Therapy

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Additional information is available at the end of the chapter

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1. Introduction

1.1. The challenge to induce lasting remission in late stage melanoma

In early stages of the disease surgical resection of melanoma lesions is a curative option; a 10-year-survival rate of 75-85% can be achieved in stage I or II of the disease. However, stage III or IV melanoma is associated with low survival rates of less than 1 year upon diagnosis [1]. The poor prognosis in advanced stages of the disease is thought to be particularly due to the properties of melanoma cells to systemically spread into various organs, to form micro-metastases beyond the detection limit of current imaging procedures [2, 3] and to give rise to relapse of the disease. This is even the case after initially complete response to therapy and after more than a decade from initial treatment. Durable remission is so far only achieved in pre-defined patient subsets despite the development of novel drugs and major improvements in therapeutic regimens [4-6]. This unsatisfactory situation is thought to be due to the extra-ordinary property of melanoma cells to persist in "dormancy" for long periods of time which is associated with their resistance to chemo-and radiotherapy [7-10]. Taken together, durable cure from melanoma requires eliminating single melanoma cells in a highly specific and efficient fashion even in dormant micro-metastatic lesions.

In this situation recruiting the cellular immune defense machinery to detect and destroy individual melanoma cells is a powerful alternative to conventional therapeutic regimens. The hope is sustained by the supportive effect of high dose interleukin-2 (IL-2) [11] and anti-cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) antibody [12] as well as interferon (IFN) α-2b to prolong the disease-free survival even in late stages of the disease. However, the response rate is quite low and frequently not curative over time [13, 14].

A number of strategies for sharpening the immune cell response against melanoma are currently explored, some of these with remarkable success. In particular, the adoptive transfer of tumor infiltrating lymphocytes (TILs), isolated from melanoma biopsies and amplified to therapeutic relevant numbers ex vivo, produced encouraging phase II results [15, 16]. In a further development, patients' blood T cells are genetically engineered with pre-defined specificity for melanoma-associated antigens making adoptive cell therapy with melanoma specific T cells possible. In this contribution we will discuss the rationale for adoptive cell therapy of melanoma, evidence for efficacy and current challenges to achieve long-term remission. Upcoming strategies in melanoma stem cell targeting are also discussed.

2. Adoptive therapy with ex vivo amplified TILs can induce regression of melanoma

An effective immune response can control melanoma. This notion is supported by the observation that spontaneous and complete melanoma regressions can occur and that immune compromised patients suffer from a higher frequency of melanoma [17, 18]. The conclusion is moreover sustained by the clinical observation that treatment with high dose IL-2 produces an objective response even in late stage melanoma, some patients with long-term complete response for years [11, 19]. Although about 16%, the response rate is remarkable compared to the low and short-lived response rates of classical therapeutic regimens.

First described in 1969 [20], melanoma is infiltrated by T cells of both effector and helper cell origin which can be expanded to high numbers ex vivo in the presence of IL-2. Pioneered by the NCI-Surgery Branch, such tumor infiltrating T cells (TILs) were selected for melanoma reactivity by incubation on feeder cells expressing melanoma-associated antigens [21] and re-administered in substantial numbers together with high dose IL-2 to the patient (Figure 1). Initial trials produced an objective response rate of 11/20 patients [22] which is remarkable since TILs are obviously capable to fight melanoma even in late stage patients who experienced multiple lines of therapy. Responses, however, were of short duration and TILs did not persist for longer period in the peripheral blood after administration. Subsequent trials identified that the key to successful TIL therapy was the number of TILs administered to the patient, the activity of those cells against melanoma and the rapidity of T cell amplification ex vivo [23, 24]. During the subsequent years the initial protocols were optimized with respect to these and other issues and adopted according to GMP standards [25]; a number of trials are currently open in various centers (Table 1).

Persistence of administered TILs in circulation was substantially improved by depletion of the lymphoid compartment of the patient prior to adoptive cell therapy [26-28]. Such pre-conditioning by non-myeloablative chemotherapy had the effect that cytokines sustaining lymphocyte amplification including IL-7 and IL-15 were present in augmented levels ("cytokine sink"). Moreover, space for transferred lymphocytes was created and suppressor cells including regulatory T cells were depleted which additionally helped to improve engraftment of adoptively transferred T cells.

Target antigen	Adoptively transferred T cells and additional treatment	NCT ID	Center
	TILs, IL-2 in variable doses	NCT00001832	NIH
	TILs vs. lymphokine-activated killer (LAK) cells	NCT00002535	StLMC
	TILs, high dose IL-2	NCT00096382	NIH
	TILs, low dose IL-2 s.c.	NCT00200577	NUH
	TILs with vs. without IL-2	NCT00314106	NIH
	TILs, high dose IL-2 with or without dendritic cell immunization	NCT00338377	MDACC
	TILs, high dose IL-2	NCT00604136	HMC
	TILs, high dose IL-2	NCT00863330	AHC
	TILs, high dose IL-2	NCT00937625	HUH
	TILs, high dose IL-2	NCT01005745	MOFFITT
	TILs, low dose IL-2 and intra-tumoral injection of IFN-γ producing adenovirus	NCT01082887	NUH
	TILs, high dose IL-2	NCT01659151	MOFFITT
	TILs, high dose IL-2	NCT01701674	MOFFITT
	TILs, high dose IL-2	NCT01807182	FHCRC
	"re-stimulated"(autologous DCs &anti-CD3 antibody) TILs, low dose IL-2	NCT01883297	UHN
	TILs, low dose IL-2	NCT01883323	UHN
	TILs, dendritic cell vaccination with NY-ESO-1	NCT01946373	KUH
	TILs, high vs. low dose IL-2	NCT01995344	CHNHSFT
	4-1BB selected TILs	NCT02111863	NIH
	“young“ TILs, high dose IL-2	NCT00287131	SMC
	“young“ TILs with or without CD4+ T cell depletion, high dose IL-2	NCT00513604	NIH
	“young“ CD8+ TILs, high dose IL-2	NCT01118091	NIH
	“young“ TILs, high dose IL-2	NCT01319565	NIH
	“young“ TILs, high dose IL-2	NCT01369875	NIH
	“young“ TILs, IL-15	NCT01369888	NIH
	“young“ TILs	NCT01468818	NIH
	“young“ TILs, high dose IL-2, BRAF kinase inhibitor	NCT01585415	NIH
	“young“ TILs, with or without high dose IL-2	NCT01814046	NIH
	“young“ TILs, anti-CTLA-4 antibody	NCT01988077	SMC
	“young“ TILs, high dose IL-2 with standard vs. low dose chemotherapy	NCT01993719	NIH

Aurora Health Care**; CHNHSFT**, Christie Hospital NHS Foundation Trust; **FHCRC**, Fred Hutchinson Cancer Research Center; **HMC**, Hadassah Medical Center; **HUH**, Herlev University Hospital (Copenhagen); **KUH,** Karolinska University Hospital; **MDACC**, M.D. Anderson Cancer Center; **MOFFITT**, H. Lee Moffitt Cancer Center and Research Institute; **MUH**, Mie University Hospital; **NIH**, National Institutes of Health; **NUH**, Nantes University Hospital; **SMC**, Sheba Medical Center; **StLMC**, St. Luke's Medical Center; **UC**, University of California; **UHN,** University Health Network (Toronto)

Table 1. Adoptive cell therapy with tumor infiltrating lymphocytes (TILs) in patients with melanoma

There are still some issues to be addressed, for instance whether clinically most potent TILs can be defined by phenotype and whether these cells can be selectively expanded. There is a common sense that for therapeutic efficacy in the long-term the functional activity of T cells needs to be preserved without signs of exhaustion which is particularly crucial when T cells experienced extensive amplification ex vivo. In the further development of the procedure, TILs were only selected with respect to their proliferative capacities which is independent of their antigen specificity and represents a furthermore simplification of the standard protocol (Figure 1) [29-31]. Those so-called "young TILs" after short-term ex vivo expansions passed through fewer cell division cycles prior to infusion and are thereby in a maturation stage less prone to terminal differentiation and senescence [32]. Those protocols do not further select TILs for their melanoma reactivity based on the observation that infusion of ex vivo activated, IFN-γ^+TILs produced no superior therapeutic efficacy compared to non-responding TILs [16]. These modifications in the protocol resulted in improved persistence of young TILs [33] and about 50% response rates [27, 29, 34], so far in non-randomized trials (Table 1). A series of recent clinical trials with TILs following different lympho-conditioning regimes resulted in objective responses in 56% and complete responses in 22% of patients at the Surgery Branch [35]. Current TIL trials at various centers reproduced objective response rates of 40-50% in melanoma patients, a significant portion of patients free of disease 3-5 years after treatment [36, 37]. Of note, TILs can have anti-tumor activity also towards brain metastases as shown in a NCI trial with 7/17 complete and 6/17 partial remissions [38] sustaining the hope that adoptive cell therapy may be effective towards metastases which are otherwise not accessible.

While most trials apply non-separated TILs, administration of isolated CD8$^+$T cell clones with specificity for Melan-A and gp100 mediated only moderate benefit, required IL-2 and did not persist for longer times [39]. Those CD8$^+$T cells which persisted long-term acquired a phenotype of central memory-type T cells in vivo [40]. It is therefore assumed that CD8$^+$TILs require help of CD4$^+$cells for prolonged persistence making application of non-separated T cell populations more suitable.

Not only the stage of maturation but also the recruitment of T cells through chemokine gradients is crucial for therapeutic success. A recent prospective-retrospective hypothesis-driven analysis revealed that coordinate over-expression of CXCL9, CXCL10, CXCL11, CCL5 in melanoma is associated with responsiveness to treatment after TIL therapy [41].

Melanoma-reactive T cells need to persist in circulation to ensure therapeutic success of TIL therapy [42, 43]. This is reflected by the median survival of patients treated with Melan-A specific TILs of 53.5 months compared to 3.5 months for patients who received TILs of unknown specificity [44]. Some trials are initiated using melanoma specific patient's T cells from the peripheral blood for adoptive cell therapy of melanoma (Table 2). MART-1 or gp100 specific T cell clones isolated and amplified ex vivo produced a 50% response rate [45], however, technical difficulties limit a broad application of such specific T cells since melanoma reactive T cells in the peripheral blood of melanoma patients are extremely rare.

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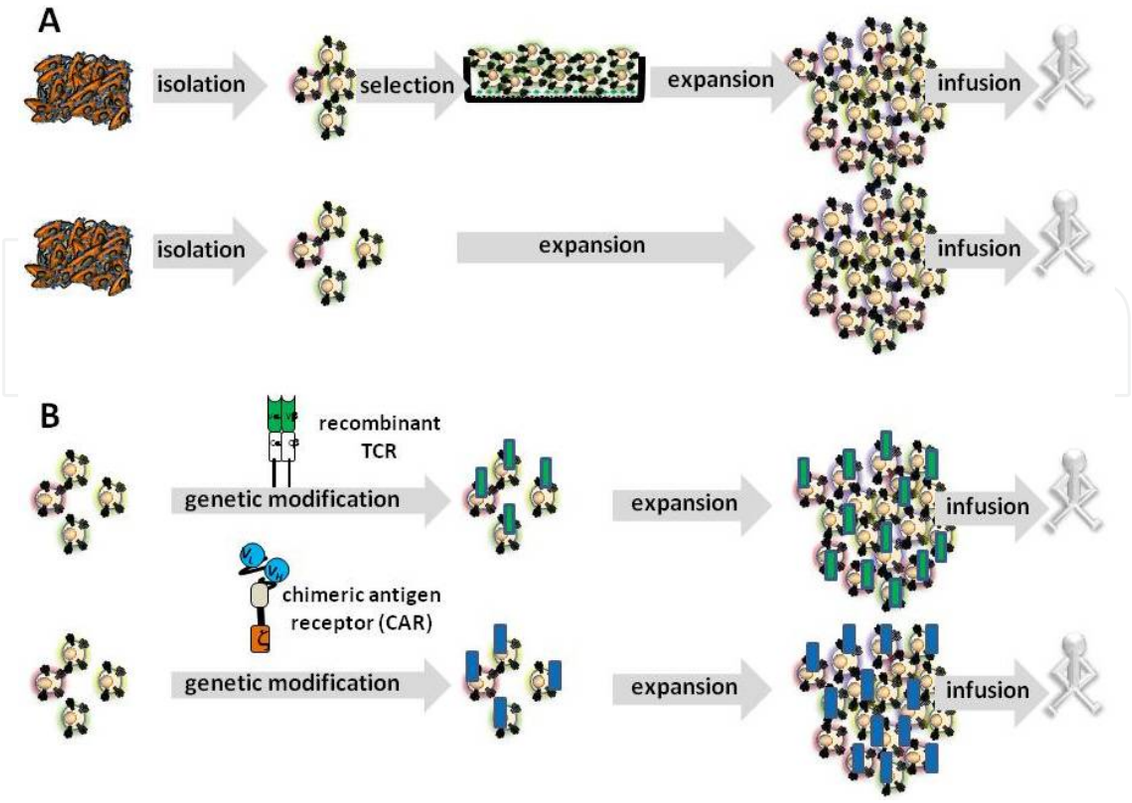

Figure 1. T cells used in adoptive cell therapy of melanoma. (A) Tumor infiltrating T cells (TILs) are isolated from melanoma biopsies, selected for reactivity towards melanoma cells, amplified in the presence of IL-2 to clinically relevant numbers and infused to the patient. Alternatively, TILs are expanded without prior selection for melanoma reactivity using a short-term amplification protocol ("young TILs"). (B) T cells from the peripheral blood of melanoma patients are genetically modified by retro- or lentivirus transduction to express a recombinant T cell receptor (TCR) or a chimeric antigen receptor (CAR), specific for a melanoma associated antigen, amplified and administered to the patient.

Target antigen	Adoptively transferred T cells	NCT ID	Center
MAGE-1 or MAGE-3	melanoma specific CD8+ T cells	NCT00045149	FHCRC
Tyrosinase	tyrosinase specific CD8+ T cells	NCT00002786	FHCRC
MART-1	MART-1 specific CD8+ T cells with or without high dose IL-2	NCT01495572	NIH
MART-1	MART-1 specific CD8+ T cells	NCT00512889	DFCI
MART-1	MART-1 specific T cells	NCT00720031	NUH
MART-1	MART-1 specific CD8+ T cells	NCT00324623	CHUV
MART-1	MART-1 specific TILs, high dose IL-2	NCT00924001	NIH
MART-1	MART-1 specific CD8+ T cells, low dose IL-2	NCT01106235	FHCRC
NY-ESO-1	NY-ESO-1 specific CD8+ T cells, low dose IL-2, anti-CTLA-4 antibody	NCT00871481	FHCRC

CHUV, Centre Hospitalier Universitaire Vaudois; **DFCI**, Dana-Farber Cancer Institute; **FHCRC**, Fred Hutchinson Cancer Research Center; **NIH**, National Institutes of Health; **NUH**, Nantes University Hospital

Table 2. Adoptive cell therapy with autologous, antigen specific T cells in patients with melanoma

3. T cells with engineered anti-melanoma specificity

The success of melanoma antigen specific T cells from peripheral blood strengthened efforts to obtain melanoma-specific T cell clones by genetic engineering of patient's T cells from the peripheral blood. In particular, the molecular cloning of the TCR from melanoma-reactive T cells enabled the engraftment of melanoma specificity to any T cell (Figure 1) [46-49]. A TCR with specificity for gp100 was cloned from melanoma reactive TILs and transferred by retrovirus-mediated gene transfer into blood T cells which thus obtained redirected specificity for $gp100^{+}$cells in addition to their parental specificity. TCR engineered T cells recognized $gp100^{+}$melanoma cells, secreted pro-inflammatory cytokines including IFN-γ and lysed $gp100^{+}$melanoma cells [50, 51]. By the same strategy, blood T cells were modified with the TCR specific for other melanoma associated antigens (Table 3). Using T cells modified with a gp100 specific TCR objective response was induced in 19% of patients, most responses were persistent [49]. Melanoma regression was also obtained in 5/11 melanoma patients after transfer of T cells modified with a TCR that recognizes NY-ESO-1, a protein encoded by a member of the cancer/germline family of genes [52, 53].

Melanoma regression was obtained in about 30% of patients after cell therapy with MART-1 specific T cells [49, 52, 54-56]. As a side effect, patients suffered from vitiligo and destruction of melanocytes in the eye and ear indicating that T cells with engineered specificity can target rare and healthy cells even with the cognate antigen at low levels. In a recent trial, patients were treated with T cells engineered with an anti-MAGE-A3 TCR [57]. While 5/9 patients experienced melanoma regression, three of them had mental status changes and two lapsed into coma and died. Histology revealed necrotizing leukoencephalopathy which is likely due to the recognition of previously unknown epitopes of MAGE-A9/A12, the latter expressed in the brain.

Prolonged clinical remission was observed when engineered T cells persisted in the circulation for longer times; TCR modified T cells were recorded in the blood for more than a year after initiation of treatment [56, 58]. Moreover, TCR engineered T cells were capable to penetrate the blood-brain barrier and to induce regression of brain metastases [57] giving hope that patients with metastases at otherwise incurable sites may benefit from adoptive cell therapy. However, tumor cells may become invisible to TCR modified T cells due to repression of the MHC complex [60], β2 microglobulin mutation [61], and deficiencies in the antigen processing machinery [60, 62], all of them resulting in diminished antigen presentation and less TCR-mediated T cell activation.

Engineering T cells with a recombinant TCR may produce a safety hazard when the transgenic $\alpha\beta$ TCR forms hetero-dimers with the respective α and β TCR chains of the endogenous TCR. Such mis-pairing of TCR chains can induce severe auto-reactivity as a result in gain of an unpredictable specificity [63, 64]. The situation was technically solved by different means including replacing the human by the homologous murine constant moieties of the TCR [65] and by inserting additional cysteine bridges [66] to facilitate preferential pairing of the recombinant TCR $\alpha\beta$ chains in the presence of the physiologic $\alpha\beta$ TCR. These and other

technical difficulties of the TCR strategy promoted the development of an artificial “all-in-one" receptor molecule to redirect T cells in an antigen-restricted fashion as summarized below.

Target antigen	Adoptively transferred T cells	NCT ID	Center
	IL-12 engineered TILs	NCT01236573	NIH
	IL-2 engineered TILs	NCT00062036	NIH
	CXCR2 and NGFR transduced TILs, high dose IL-2	NCT01740557	MDACC
	TGF-Beta resistant (DNRII) and NGFR transduced TILs, high dose IL-2	NCT01955460	MDACC
gp-100	anti-gp-100 TCR engineered CD8+ cells, anti-gp-100 TCR engineered TILs, high dose IL-2	NCT00085462	NIH
gp-100	anti-gp-100 TCR engineered T cells, high dose IL-2 plus gp-100 vaccination	NCT00610311	NIH
gp-100 & MART-1	anti-gp-100 TCR & anti-MART-1 TCR engineered T cells high dose IL-2 Peptide Immunization	NCT00923195	NIH
MAGE-A3	anti-MAGE-A3/12 TCR engineered T cells, high dose IL-2	NCT01273181	NIH
MAGE-A3	anti-MAGE-A3 TCR engineered T cells, high dose IL-2	NCT02153905	NIH
MAGE-A3	anti-MAGE-A3-DP4 TCR engineered CD4+ cells, high dose IL-2	NCT02111850	NIH
MAGE-A4	anti-MAGE-A4 TCR engineered T cells	NCT02096614	MUH
MAGE-A4	anti-MAGE-A4 TCR engineered T cells	NCT01694472	TMUCIH
MART-1	anti-MART-1 TCR engineered T cells, IL-2, peptide immunization	NCT00091104	NIH
MART-1	anti-MART-1 TCR engineered T cells, IL-2, MART-1 peptide pulsed dendritic cells	NCT00910650	UC
MART-1	anti-MART-1 TCR engineered T cells, high dose IL-2, peptide immunization	NCT00612222	NIH
MART-1	anti-MART-1 TCR T cells vs. anti-MART-1 TCR TILs, high dose IL-2	NCT00509288	NIH
MART-1	anti-MART-1 TCR engineered T cells, low dose IL-2, peptide immunization	NCT00706992	NIH
NY-ESO-1	anti-NY-ESO-1 TCR engineered T cells, high dose IL-2	NCT00670748	NIH
NY-ESO-1	anti-NY ESO-1 mTCR engineered T cells, high dose IL-2	NCT01967823	NIH
NY-ESO-1	anti-NY ESO-1 TCR CD62L+ T cells, high dose IL-2	NCT02062359	NIH
NY-ESO-1	anti-NY-ESO-1 TCR engineered T cells	NCT01350401	Adaptimmune
NY-ESO-1	anti-NY-ESO-1 TCR engineered T cells, cotransduced with IL-12 cDNA	NCT01457131	NIH
p53	anti-p53 TCR engineered T cells, high dose IL-2, p53 peptide pulsed dendritic cells	NCT00704938	NIH
p53	anti-p53 TCR engineered T cells, high dose IL-2	NCT00393029	NIH
tyrosinase	anti-tyrosinase(368-376) TCR engineered T cells	NCT01586403	LU
GD2	3rd generation anti-GD2 CAR engineered T cells	NCT02107963	NIH
VEGFR2	anti-VEGFR2 CAR engineered CD8+ T cells, low dose IL-2	NCT01218867	NIH

LU, Loyola University (Chicago); MDACC, M.D. Anderson Cancer Center; MUH, Mie University Hospital; NIH, National Institutes of Health; TMUCIH, Tianjin Medical University Cancer Institute and Hospital; UC, University of California;

Table 3. Adoptive cell therapy with engineered antigen specific T cells in patients with melanoma

4. CAR T cells with engineered specificity for melanoma

In order to link antigen recognition with the downstream signaling machinery of the TCR, Zelig Eshhar (Weizmann Institute of Science) reported a chimeric antigen receptor (CAR) molecule, also named immunoreceptor, which is composed in the extracellular moiety of a single chain fragment of variable region (scFv) antibody for binding and in the intracellular moiety of the CD3ζ endodomain to initiate T cell activation [67]. The coding sequence of such recombinant receptor molecule is transferred by retro- or lentiviral transduction into T cells in vitro (Figure 1) [68]. CAR engineered T cells, also nick-named "T-bodies", recognize their new target by CAR binding and become activated to secrete pro-inflammatory cytokines, to amplify and to lyse the cognate target cells. Since the binding domain is derived from an antibody the CAR recognizes the target in a MHC-independent fashion which makes major differences to TCR mediated T cell recognition. For instance, the CAR recognizes its target independently of the individual HLA subtype and CAR T cells are not affected by MHC repression and loss of HLA molecules on target cells which frequently occurs during tumor progression. However, recognition by CARs is restricted to target antigens on the cell surface; intracellular antigens like transcription factors are not visible to CAR T cells. Despite that limitation, a nearly infinite variety of targets can be recognized including those which are not classical T cell targets like carbohydrates and gangliosides [69].

Full and lasting T cell activation requires two complementary signals, one provided by the TCR/CD3 and the other by co-receptors the prototype of which is CD28. Prolonged T cell activation, however, requires costimulation and autocrine factors, in particular IL-2 which is only secreted upon TCR and simultaneous CD28 signaling. The lack of appropriate costimulation in the tumor lesion provides the rationale for combining the intracellular CD3ζ with the CD28 signaling domain in one polypeptide chain of a "second generation" CAR (Figure 2). A CAR with combined CD28-CD3ζ signaling domain provides both the primary CD3ζ and the required costimlatory signal when engaging the cognate target. CARs with a costimulatory domain clearly provide clinical benefit and improved T cell persistence compared to CARs with the CD3ζ domain only [70-72]. Other costimulatory moieties, such as 4-1BB (CD137) and OX40 (CD134), also provide full T cell activation when linked to CD3ζ in a CAR; the individual costimulory domains have different impact on T cell effector functions [73]. These and other costimulatory domains were furthermore combined in so-called "3rd generation" CARs which provide advantage for matured effector T cells in terminal differentiation but less in younger stages of T cell development [74]. A number of additional modifications of the CAR design were explored in order to improve T cell persistence and activation and finally the anti-tumor response [75, 76].

While the antibody domain defines the target specificity of the CAR, a plethora of antigens can potentially be used as target for the adoptive cell therapy of melanoma. T cells engineered for targeting melanoma-associated antigens include CARs with specificity for HMW-MAA, also known as MCSP [77, 78], melanotransferrin [79], and the gangliosides GD2 [80] and GD3 [81]. Trials are currently recruiting; to our best knowledge no published data are so far available.

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During the last years spectacular efficacy was achieved with CAR T cells in phase I trials for the treatment of lymphoma/leukemia [82, 83]. Clinical response and prolonged T cell activation was accompanied by a "cytokine storm", which occurred even weeks after initial T cell administration; the side effect can clinically be managed by treatment with a neutralizing anti-IL-6 antibody without affecting the anti-tumor efficacy.

The enthusiasm in CAR T cell therapy, however, was dampened by reports on serious adverse events and fatalities after CAR T cell administration [84, 85]. Targeting ErbB2 produced respiratory failure which is thought to be due to low levels of antigen on a number of healthy cells which are sufficient to trigger "on-target-off-organ" T cell activation [86]. This and other serious adverse events emphasize a careful evaluation of potential targets and the necessity for T cell dose escalation studies to balance anti-tumor efficacy and auto-immunity [75, 87, 88].

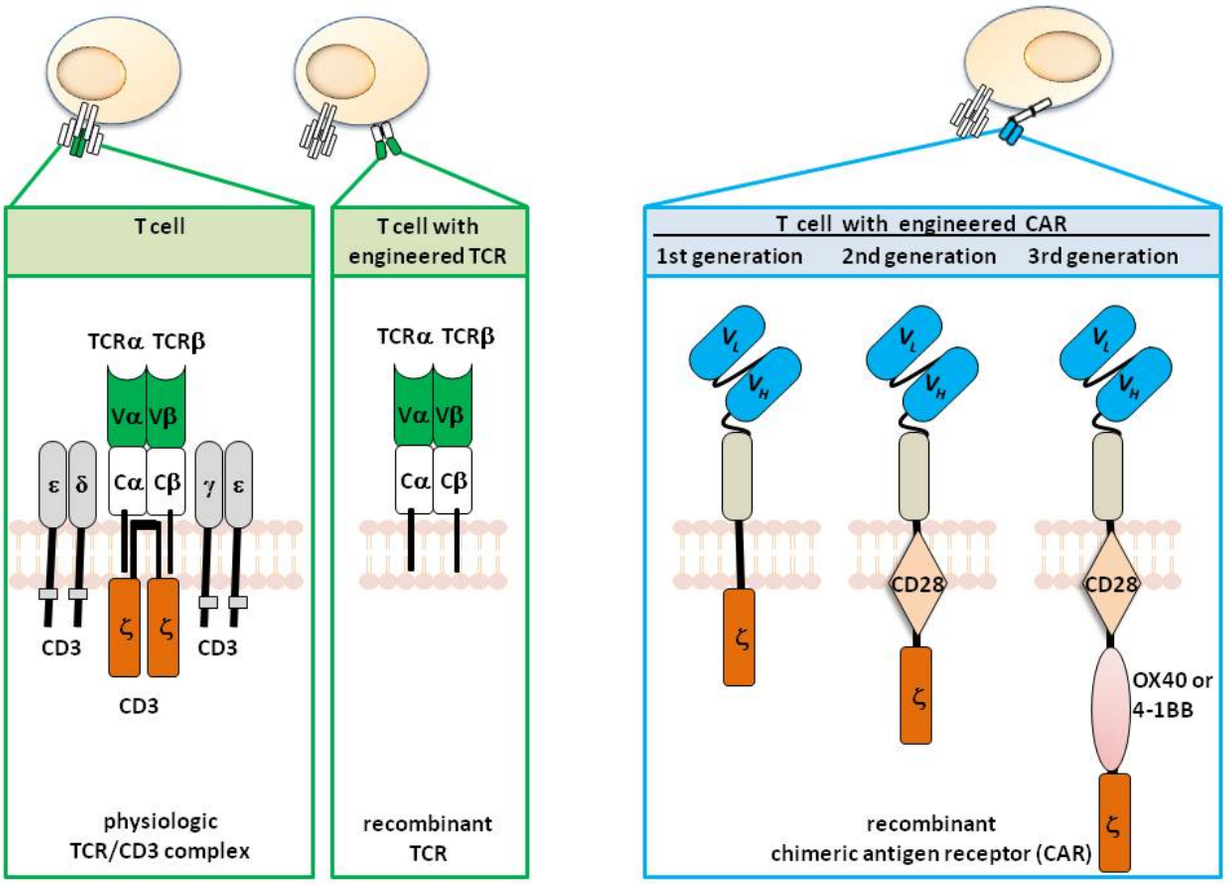

Figure 2. T cells with engineered specificity. T cells physiologically recognize their target by the T cell receptor (TCR) complex which is composed of the TCR α and β chain for recognition and the CD3 chains for signaling. The variable regions of each TCR chain (Vα and Vβ) together bind to the MHC presented antigen, Cα and Cβ represent the constant domains. T cells can be genetically engineered with defined specificity by expression of recombinant TCR $\alpha\beta$ chains of known specificity. In contrast to the TCR, the chimeric antigen receptor (CAR) is one polypeptide chain composed of a single chain fragment of variable region (scFv) antibody for antigen recognition, the extracellular spacer domain, a trans-membrane domain and the intracellular CD3ζ ("first generation" CAR), the CD28-CD3ζ ("second generation" CAR), or the CD28-OX40-CD3ζ ("third generation" CAR) signaling chain.

5. "Melanoma stem cells": Target cells to achieve long-term remission?

Despite the tremendous cellular and phenotypic heterogeneity in tumor lesions, cancer is thought to be initiated and maintained by so-called cancer stem cells (CSCs). Such pluripotent stem cells are of low abundance, induce tumors upon transplantation under limiting dilution conditions, resist radiation and chemotherapy, and drive self-renewal and a-symmetric differentiation into a variety of cell types. Residual CSCs are thought to initiate cancer relapse even after years of "dormancy", which can be more than a decade after surgical treatment of the primary lesion [89]. While the concept of the hierarchical organization in driving tumor progression was initially drawn upon deciphering hematological malignancies, basically the same organization was subsequently reported for other solid cancers including mammary, prostate, pancreatic, colon carcinoma and glioma [90-94].

Transplantation of melanoma cell subsets into recipient mice under limiting dilution conditions also revealed that a defined subset of cancer cells, and not every cell from the same biopsy, can induce tumors of same histology as the parental tumor [90, 95-97]. One conclusion is that melanoma is organized in a hierarchical manner originating from a particular initiator cell, the cancer stem cell, which gives rise to the described diversity of cells in an established lesion. Melanoma initiating cells were described by various, but not common markers, including the transporter protein ABCB5 [95], CD20 [97], or the nerve growth factor receptor CD271 [98]. While $CD271^+$melanoma cells are present in a frequency of approximately 1/2000 cells [98], transplantation under more rigorous conditions, i.e., ideally of one single melanoma cell, revealed that nearly every fourth randomly taken melanoma cell (1/2-1/15) can induce tumors in the host animal. This observation, however, questioned the validity of the stem cell paradigm for melanoma [99, 100]. Subsequent studies made clear that the potential to induce melanoma is not closely associated with a particular phenotype and that the number of potential CSCs in melanoma may not necessarily be low. If nearly every melanoma cell is capable to re-program to a tumor initiating cell under certain conditions, blocking stem cell properties in melanoma will reduce tumor initiation and growth in a transplantation model finally resulting in melanoma ablation [101].

Once the tumor lesion is established, a minor subset of cancer cells seems to take over to control malignant progression. Evidence for this hypothesis was provided from a pre-clinical model [79] which asked whether all or a defined subset of melanoma cells in an established xeno-transplanted lesion need to be eliminated to cause tumor regression. Such melanoma sustaining cell may be, but not must be identical to melanoma stem cells identified by transplantation assays.

Evidence for a particular targetable melanoma cell subset which sustains tumor progression was provided by the observation that elimination of $CD20^+$melanoma cells by adoptive transfer of CAR T cells completely eradicated xeno-transplanted melanoma. Those human melanoma biopsies contained a subset of $CD20^+$melanoma cells which constituted about 1-2% of melanoma cells and which are present in different histological melanoma types and tumor stages. A caveat is that in approximately 20% of melanoma samples analyzed so far, no $CD20^+$melanoma cells were detected by histological screening; CD20-specific CAR T cells did

not induce regression of those transplanted tumor lesions. Interestingly, CD20 re-expression in a random subpopulation of those tumor cells by genetic modification did not render the tumor lesion sensitive for eradication indicating that CD20 expression per se is not sufficient but requires additional capabilities to sustain melanoma progression [79].

There are additionally clinical observations that sustain the notion of $CD20^+$cells in promoting melanoma progression. Firstly, a patient with stage III/IV metastatic, refractory melanoma and 2% $CD20^+$melanoma cells who received intra-lesional injections of the anti-CD20 therapeutic antibody rituximab experienced lasting remission accompanied by a decline of the melanoma serum marker S-100 to physiological levels and a switch of a T helper-2 to a more pro-inflammatory T helper-1 response [102]. Although anecdotic, data provide the first clinical evidence that targeted elimination of $CD20^+$melanoma cells can produce regression of chemotherapy-refractory melanoma. Secondly, in a small pilot trial, stage IV melanoma patients without evidence of disease by way of surgery, chemo-and/or radiation therapy received the anti-CD20 antibody systemically for a 2 year period [103]. Data suggest a benefit of anti-CD20 therapy in overall and recurrence-free survival; a caveat being that the number of patients is still small for definitive conclusions.

Currently, the hierarchical stem cell model in the maintenance of an established melanoma is supported by some experimental evidence [79], whereas a body of information on melanoma initiation by transplantation of single melanoma cells sustains the stochastic model [99, 100], although not confirmed by others [98]. The most determining proof of the stem cell hypothesis, however, will be the successful melanoma elimination by targeting stem cells or stem cell properties. For the development of such therapeutic strategies several aspects need to be taken into account.

First, standard therapy will rapidly de-bulk the tumor lesion and the remaining melanoma stem cells, which are more chemo-and radiation resistant, will drive relapse of the disease. Since those melanoma initiating cells are merely in a "dormant" state and replicate less frequently than the majority of melanoma cells in the same lesion, anti-proliferative strategies by classical chemotherapeutic drugs are unlikely efficient. Transporter systems including ABCB5, which is highly expressed by melanoma stem cells [95], additionally contribute to chemotherapy resistance; the chemotherapy and/or radiation itself may promote expression of those transporter systems and survival of those resistant cells which finally contributes to relapse of the disease.

Second, if clinical progression correlates with the prevalence of $CD20^+$melanoma cells, targeted elimination of those melanoma cells requires to meet the fact that those target cells are a small minority. Targeted elimination, e.g., by CD20 redirected cytotoxic T cells or by CD20-specific therapeutic antibodies like Rituxan™ (rituximab) or Arzerra™ (ofatumumab), will be required to obtain substantial efficacy.

Third, the extraordinary functional and phenotypic plasticity of melanoma cells may make it necessary to have the therapeutic agent in place for a longer time. In their pre-clinical model, Schmidt and colleagues [79] used CAR T cells which persist for long-term acting as an antigen-specific guardian as long as target cells are present. Since repetitive re-stimulation sustains the

persistence and amplification of CAR T cells, cellular therapy has a major advantage compared to pharmaceutical drugs with a comparable short half-life. CAR T cells can moreover provide antigen-specific memory with defined specificity [104], potentially contributing to control melanoma in the long-term.

6. Production of engineered T cells for clinical application

Application of adoptive cell therapy to clinical use requires efficient production of cells according to good manufacturing practice (GMP). This particularly applies to patient's T cells which are ex vivo genetically modified. The vector used for T cell modification is of major relevance with respect to the efficiency and stability in modification. Crucial steps in this process are the stable integration of the genetic vector, the site of integration to avoid insertion mutagenesis, and the resistance of the vector to genetic repression. To date, most clinical trials were performed employing retroviral or lentiviral vectors which fulfill some but not all of these requirements. Recently, other vector systems including RNA modification are alternatively utilized and it is expected that these systems will be explored in parallel in the near future.

The way of stimulating the T cells ex vivo for genetic modification and subsequent amplification is crucial for both the success in transduction and the functional capacities of modified cells. T cells are commonly activated by TCR/CD3 stimulation in addition to IL-2 [105]; most protocols use anti-CD3 and anti-CD28 magnetic beads [83, 106] which can be easily eliminated during the production process. IL-2 is replaced by other cytokines such as IL-7 and IL-15 to obtain a T cell population with a more naive and central memory phenotype [107]. Alternatively, cell lines were engineered, so-called "artificial APCs", which are modified with the various co-stimulating molecules to mimic the physiological stimulation and to provide the required signals [108]. However, difficulties in adopting those cells to GMP standard prevent their broad application in large scale production processes.

For the production itself, static culture systems in flasks or gas permeable bags are traditionally used. Due to their amplification at low cell densities (0.25-1x10^6 cells per ml), high culture volumes are required to obtain clinically relevant T cell numbers which is more easily achieved by non-static systems including the WAVE-Bioreactor or the G-Rex100 device [83, 106, 109]. In order to produce engineered T cells for a large number of patients it will be required to manufacture cells in a closed system and to produce multiple batches in parallel in the same clean room facility without the risk of batch contamination.

7. Challenges and promise in the adoptive cell therapy of melanoma

To date, approximately half of the melanoma patients benefit from adoptive cell therapy with TILs. Specifically targeted T cells may further improve the therapeutic response. Despite substantial success, the strategy still has major challenges which need to be addressed in the near future.

Significant numbers of effector T cells have to accumulate in the targeted tumor lesion which is mediated by a network of chemokines. Adoptively transferred T cells use these networks to accumulate at the tumor site; melanoma cells secrete a number of chemokines including CXCL1 to attract lymphocytes. However, early imaging studies revealed that melanoma-specific T cells massively infiltrate the lungs, spleen and liver with only some accumulation at the tumor site before the cells decline to undetectable levels in circulation [110-112]. To improve tumor targeting TILs were engineered with CXCR2, the receptor for melanoma secreted CXCL1, which resulted in improved anti-tumor activity in a mouse model [113]. The strategy is currently being explored in an early phase I trial (Table 3) [113].

Since tumor eradication requires a beneficial T cell-to-target cell ratio, higher numbers of tumor-specific T cells applied per dose likely increase the clinical efficacy. The optimal dose of T cells, however, is still a matter of discussion and requires empiric evaluation. A number of trials, in particular applying TILs, administered up to 10^{10} cells per dose [27]. Such high doses in turn require extended expansions of T cells ex vivo with the risk of loss of the "young" phenotype and gain of more matured T cells. Highly expanded T cells become hypo-responsive to CD28 costimulation and rapidly enter activation induced cell death, in particular upon IL-2 driven expansion [114]. With respect to more potent effector functions short-term amplification protocols are envisioned for both TILs and engineered T cells. This may be achieved by T cell amplification in the presence of IL-15 and IL-21 and/or by 4-1BB co-stimulation [115].

On the other hand, administration of about 10^5 engineered T cells induced remarkable therapeutic efficacy in recent trials targeting $CD19^+$ leukemia [83]. Since the T cells substantially amplify in vivo upon antigen encounter, the capacity of cells to amplify under appropriate conditions is more relevant than the applied cell number.

Once targeted in sufficient numbers to the tumor tissue, a major challenge is the tumor selectivity of redirected T cells. While the TCR and the CAR is specific for a particular target, in most cases the targeted antigen is not exclusively expressed by cancer but also by healthy cells, although sometimes at lower levels [116, 117]. MART-1, frequently expressed by the majority of melanoma cells, is also expressed by melanocytes. Targeting such type of antigen frequently produces vitiligo, sometimes also inner ear toxicity with a certain degree of deafness [49]. Since nearly all "tumor-associated antigens" which are frequently used as targets for adoptive cell therapy are self-antigens, strategies are needed to minimize such off-target toxicities. Among these, low-avidity TCRs or CARs or combinatorial antigen recognition by two CARs are currently explored.

Melanoma cells may become invisible to TILs or TCR modified T cells due to down-regulation of their MHC components or due to deficiencies in antigen processing. However, melanoma cells may still be visible to CAR T cells which recognized their target by their antibody-derived binding domain in a MHC independent fashion. On the other hand, TCR T cells are capable to recognize cross-presented antigen, for instance tumor antigen presented by stroma cells, which is invisible to CAR T cells but helps to destroy the tumor lesion in the long-term [118, 119].

Consequently, a TCR-like CAR aims at combining the benefits of TCR and CAR redirected T cells. This is performed by using a single chain antibody with TCR-like specificity for recognizing MHC presented antigen. T cells with such a TCR-like CAR were successfully redirected in a MHC restricted fashion towards NY-ESO-1 and MAGE-A1, respectively [120, 121].

The redirected T cell activation depends on the amount of target antigen and binding affinity. Compared to TILs and TCR modified T cells, CAR T cells bind with extraordinary high affinity by their antibody-derived CAR binding domain. A furthermore increase in affinity by affinity maturation does not necessarily improve CAR redirected T cell activation [120, 122]; CD28 costimulation does not add to the affinity dependent activation threshold, however, prolongs T cell persistence and resistance to apoptosis [123]. Targeting cancer cells also depends on the amount of target antigen in addition to the binding affinity. Low affinity CARs require abundant antigen levels for efficient activation of engineered T cells while high affinity CARs are likewise effective against low antigen levels on target cells. In this context, the selectivity in targeting melanoma cells versus healthy cells needs to be discussed not only with respect to the targeted antigen itself but also to antigen amount and binding affinity.

Amplification and persistence of adoptively transferred cells correlates with clinical outcome in some trials [124]. T cells will persist in detectable numbers as long as targeted antigen is present, however, will contract to undetectable levels and disappear from circulation when no target is furthermore present. To enable survival of CAR T cells in the long-term, Epstein-Barr virus (EBV)-specific T cells were used as effector cells and modified with a tumor-specific CAR. The rationale is that EBV specific T cells are maintained in a sizable population in circulation by recognizing EBV antigens by their physiological TCR. The strategy is sustained by the first clinical observation that EBV-specific T cells engineered with an anti-GD2 CAR showed benefit over non-virus-specific, CAR engineered T cells in the treatment of neuroblastoma (NCT00085930) [124]. Other trials use EBV or CMV specific, autologous T cells engineered with a first or second generation CAR, for instance directed against HER2/neu (ErbB2) (NCT01109095), CD30 (NCT01192464), or CD19 (NCT00709033; NCT01475058; NCT01430390; NCT00840853; NCT01195480).

The T cell subset matters, adoptively transferred $CD8^+$T cell clones poorly persist [125] and need help of $CD4^+$cells. Prolonged T cell anti-tumor response also requires resistance to repression in the tumor tissue. A number of efforts are currently undertaken to counteract tumor associated T cell repression, in particular mediated by Treg cells and checkpoint mediators. In animal models, CD28 costimulation without induction of IL-2 secretion protects a CAR redirected T cell response from Treg cell repression [126]. On the other hand, repetitive T cell stimulation upregulates CTLA-4 which acts as negative regulator to return the T cell to a resting stage. Administration of a CTLA-4 blocker, e.g., ipilimumab antibody, may prolong the anti-tumor activation of transferred T cells, although it is not locally restricted and will likewise affect all T cells [127, 128]. Expression profiling of TCR-engineered T cells demonstrates overexpression of multiple inhibitory receptors in persisting lymphocytes, including PD-1 and CD160, the latter associated with decreased reactivity of TCR T cells in a ligand independent manner [129]. Essentially the same was observed for CAR T cells [130]. These

analyses point to a multi-factorial T cell repression in the tumor tissue; there is more than one uni-directional strategy needed to sustain the T cell anti-tumor response in the long-term.

A major hurdle of specific immunotherapy in general is the tremendous heterogeneity of cancer cells within the same lesion. Low or loss of target antigen expression negatively affects the long-term therapeutic efficacy of an antigen-redirected approach. This is supported by several reports which document a relapse of antigen-loss tumor metastases after adoptive therapy with melanoma-reactive T cell clones [39,131, 132]. A solution may be the use of polyclonal T cells with specificities for various melanoma antigens or T cells modified with different CARs recognizing different antigens; however, target-negative tumor cells will not be recognized. On the other hand, pro-inflammatory cytokines secreted by redirected T cells upon activation can attract a second wave of innate immune cells which in turn may eradiate the antigen-negative tumor cells. At least in an animal model, antigen-negative melanoma cells are indeed eliminated when co-inoculated with antibody-targeted cytokines [133]. T cells engineered with induced expression of transgenic IL-12 can attract innate immune cells including macrophages into the tumor tissue which eliminate antigen-negative tumor cells in the same lesion, at least in an immune competent animal model [134]. Such "TRUCK" cells ("T cells redirected for unrestricted cytokine killing") may pave a novel way to deliver transgenic cell products to pre-defined, target lesions.

Combination of adoptive cell therapy with pathway inhibitors may improve the efficacy in melanoma cell elimination, in particular in disseminated stages of the disease. Metastatic melanoma patients with the B-raf activating mutation V600E benefit from a small molecule drug, PLX4032 or vemurafenib, which inhibits the mitogen-activated protein kinase (MAPK) pathway. Treatment with vemurafenib is accompanied by increased T cell infiltrations in the melanoma lesions [135, 136] which may contribute to the therapeutic effect and may be improved by co-administration of melanoma-specific T cells.

While adoptive cell therapy is mostly performed with modified or non-modified T cells, other cells like monocytes, macrophages as well as NK cells can also be redirected by CARs in an antigen-specific fashion [137-141, 144]. In contrast to T cells, NK cells can be rapidly activated and exhibit high cytotoxic potential and continuously growing NK cell lines such as NK-92 can be used for adoptive cancer immunotherapy [142]. CD3ζ chain CARs trigger cytolytic activities of NK cells which has been shown for CARs with various specificities [138, 141, 143-147]. Similar to T cells, the anti-tumor activity was improved by adding 4-1BB or 2B4 (CD244) costimulatory domains [148, 149]. Since NK cells cannot provide IL-2 or IL-15 required for amplification, CAR modified NK cells were additionally engineered to release IL-15 which sustains NK cell expansion and CAR-mediated cytotoxicity in the absence of IL-2 [150]. Despite these and other advances during the last years, experience with CAR engineered primary NK cells in clinical trials is still limited; whether redirected cells of the innate immune system are more advantageous in melanoma elimination than modified T cells has moreover to be explored in clinical trials.

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