Chapter 13

Adoptive Cell Therapy of Melanoma: The Challenges of Targeting the Beating Heart

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Additional information is available at the end of the chapter

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1. Introduction

The identification of melanoma-associated antigens, the isolation of tumor infiltrating T cells from melanoma lesions, and the significant progress in engineering redirected T cells has favored the development of various strategies in the adoptive immunotherapy of melanoma. Recent trials in adoptive cell therapy (ACT) have achieved spectacular results in inducing remission in advanced stages of the disease, although produced on-target off-tumor toxicities, emphasizing the tremendous potential benefit of harnessing the immune system for fighting the disease. Moreover, the identification of so-called melanoma stem cells along with strategies for selectively eliminating subsets of melanoma cells implies that there is a need for redefining therapeutic targets in melanoma. This review discusses current challenges in the rational design of adoptive cell therapy to target "the beating heart" of melanoma.

1.1. Advanced stages of melanoma resist conventional therapeutic regimens

Surgical resection of tumor lesions in early stages of the disease is the curative option for combating melanoma; a 10-year-survival rate of 75 - 85% can be achieved for melanoma in stage I or II. However, melanoma in stage III or IV is still associated with low survival rates of less than 1 year upon diagnosis [1]. Despite the development of novel drugs and major improvements in therapeutic regimens, significant responses were only achieved in predefined groups and of short duration. Treatment with the chemotherapeutic drug dacarbazine (DTIC) and vemurafenib, an inhibitor of mutated BRAF, produced a median progression-free survival of 64% with dacarbazine, respectively 84% with vemurafenib of approximately 6 months [2-4]. The biology of melanoma and the heterogeneity of malignant cells are thought to be responsible for this unsatisfactory situation. First, melanoma cells can persist

for long periods of time in a "dormant" stage without any progression in tumor formation [5]. Second, melanoma cells can disseminate early into distant organs including the brain forming micro-metastases, which are small in cell numbers and frequently beyond the detection limit of current imaging procedures [6, 7]. Third, many melanoma cells are notoriously resistant to chemo- and radiation therapy [8-10], making alternative strategies in tumor cell elimination necessary.

Therefore, in more progressed stages of the disease the recruitment of the cellular immune defense to eliminate cancer cells is thought to be an alternative. Administration 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 prolongs the disease-free survival although at a relatively low response rate and without being curative over time [13, 14]. However, these and other observations imply that activation or modulation of the patient's immune response may be effective in the treatment of melanoma. A number of approaches for enhancing the immune cell response against melanoma are currently explored with some success. In particular, the adoptive transfer of autologous T cells isolated from melanoma lesions and expanded to large numbers ex vivo has produced encouraging phase II results [15, 16]. The administration of patient's blood T cells engineered with defined specificity for melanoma-associated antigens are additionally being explored in a number of trials. In this review, we summarize evidence for the potency of adoptive T cell therapy in the treatment of melanoma and discuss current challenges in achieving long-term remission. Upcoming strategies in selective targeting cancer stem cells are also discussed.

2. Adoptive cell therapy can successfully fight melanoma

Melanoma can trigger a curative immune response; this conclusion is drawn from the clinical observation of spontaneous and complete melanoma regressions and of the higher frequency of melanomas among immune compromised patients [17, 18]. More direct evidence for the immune cell control of melanoma growth was obtained by the treatment with high dose IL-2, which produces an objective response rate of 16%. Indeed, some of the patients receiving thus treatment exhibit a long-term complete response for years [11, 19]. These observations are remarkable in light of the low and short-lived response rates after chemotherapy and currently drive the development of adoptive T cell therapy for treatment of late stage melanoma.

The development of adoptive cell therapy (ACT) was further strengthened by upcoming technologies in isolating tumor infiltrating lymphocytes (TIL's) from melanoma biopsies (Figure 1). First described in 1969 [20], TIL's from melanoma lesions consisted of both effector and helper T cell subsets and can be expanded *ex vivo* in the presence of IL-2. The expanded cells are then selected for melanoma reactivity. A strong rationale for using these T cells in adoptive therapy is provided by the observation that the infusion of high TIL numbers correlates with better clinical outcome [21, 22] although

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the prevalence of TIL's in primary melanoma lesions and metastases is not a prognostic factor itself.

Protocols according to GMP standards have been established in several centers to isolate and amplify TIL's to numbers appropriate for adoptive therapy. Melanoma reactive T cells are expanded in the presence of IL-2 by culture on feeder cells expressing melanoma antigens [23]. Subsequent to TIL re-infusions, metastases regressed in the majority of patients and a stable disease phase followed. However, only few patients remained in complete remission [21]. The disappointing therapeutic efficacy, despite high numbers of infused TIL's is thought to be due to low responsiveness of highly expanded T cells which are unable to execute a productive anti-melanoma attack after administration to the patient. Current TIL protocols therefore attempt to administer so-called "young TIL's" (Figure 1), i.e. melanoma infiltrating T cells which underwent short-term culture expansions and therefore passed through fewer cell division cycles prior to re-infusion and thereby exhibit a less differentiated phenotype [24]. Another change in protocols is that TIL's are not selected for melanoma reactivity; the rationale behind this is that re-infusion of *ex vivo* IFN-γ secreting TIL's exhibited no major benefit compared to non-responding TIL's [16]. Early phase I trials showed improved persistence of young TIL's [25] and 50% response rates in a cohort of 20 patients [26], which is just as effective as traditionally grown TIL's [27]. Different non-randomized phase II trials at the NCI and at Sheba Medical Center confirmed these early observations (Table 1) [28, 29]. A roadmap describing critical steps for comparative testing the TIL strategy in a randomized multi-center setting was recently published in a White Paper on adoptive cell therapy [30].

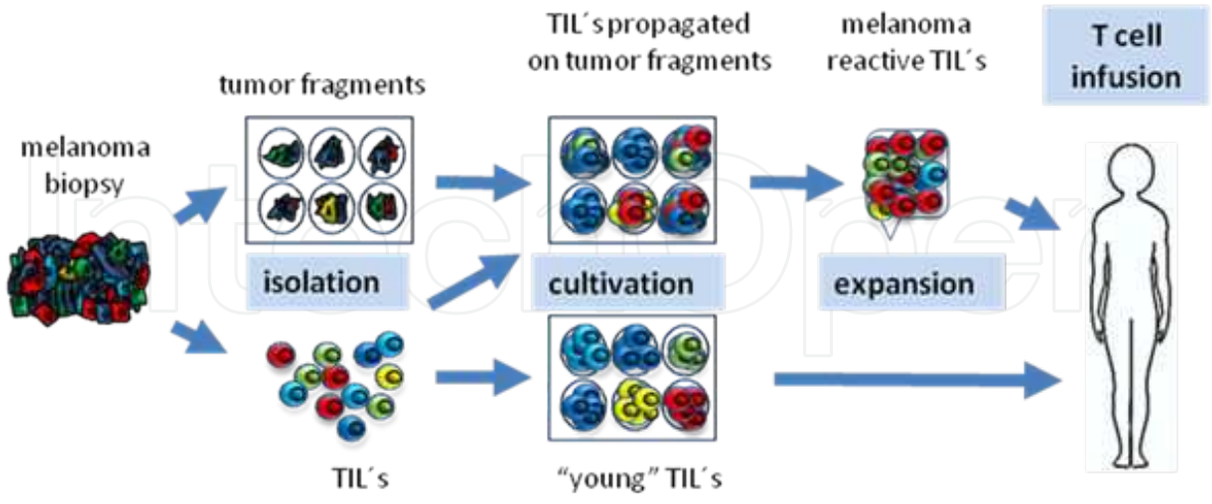

Figure 1. Adoptive cell therapy for metastatic melanoma. Adoptive cell therapy with tumor infiltrating lymphocytes (TIL´s) makes use of melanoma-specific TIL´s which are isolated from a melanoma biopsy, amplified *ex vivo* by stimulation with melanoma biopsy cells and propagated to high numbers in the presence of IL-2. In more recent trials, TIL´s are propagated short-term ex vivo without stimulation by melanoma cells and administered as "young" TIL´s.

Target antigen	Adoptively transferred T cells	NCT ID / Reference	Center
	melanoma specific $CD8^+$ T cells	[118]	FHCRC
	melanoma specific T cells	[119]	LUMC
MART-1	MART-1 specific $CD8^+$ T cells	[113]	DFCI
MART-1	MART-1 specific $CD8^+$ T cells	NCT00512889	DFCI
MART-1	MART-1 specific $CD8^+$ T cells	[87]	UR
MART-1	MART-1 specific $CD8^+$ T cells	[33]	UNH
MART-1	MART-1 specific $CD8^+$ T cells	NCT00324623	CHUV
MART-1	MART-1 specific $CD8^+$ T cells	NCT01106235	FHCRC
NY-ESO-1	NY-ESO-1 specific $CD8^+$ T cells and anti-CTLA-4 antibody	NCT00871481	FHCRC
	TILs	[114]	NIH
	TIL	[120]	NIH
	TILs	[27]	NIH
	TILs	[29]	NIH
	TILs	[115]	NIH
	TILs	NCT00287131	SMC
	TILs	NCT000604136	HMO
	TILs	NCT01005745	MOFFITT
	TILs and IFN-γ	NCT01082887	NUH
	"young" TILs	[116]	NIH
	"young" TILs	[28]	SMC
	"young" TILs	NCT01118091	NIH
	"young" TILs	NCT01319565	NIH
	"young" TILs	NCT01369888	MIH
	"young" TILs	NCT01468818	NIH
	"young" TILs	NCT00513604	NIH
MART-1	MART-1 specific TILs	NCT00720031	NUH
MART-1	MART-1 specific TILs (DMF5)	NCT00924001	CC
	IL-2 engineered TILs	[117]	NIH
	IL-2 engineered TIL	NCT00062036	NIH
	IL-12 engineered TIL	NCT01236573	NIH
	CXCR2 engineered TIL	[86]	MDACC
NY-ESO-1	anti-NY-ESO-1 TCR	[121]	NIH
NY-ESO-1	anti-NY-ESO-1 TCR	NCT00670748	NIH
MART-1	anti-MART-1 TCR (low-affinity)	[49]	NIH
MART-1	anti-MART-1 TCR	NCT00910650	UC
MART-1	anti-MART-1 TCR (high-affinity)	[38]	NIH
gp-100	anti-gp-100 TCR	[38]	NIH
MART-1	anti-MART-1 TCR	[114]	NIH
gp-100	anti-gp-100 TCR	[114]	NIH
MART-1	anti-MART-1 TCR	NCT00612222	NIH
gp-100	anti-gp-100 TCR	NCT00610311	NIH
MART-1	anti-MART-1 TCR plus MART-1 vaccination	NCT00923195	NIH
gp-100	anti-gp-100 TCR plus gp-100 vaccination	NCT00923195	NIH
p53	anti-p53 TCR	NCT00393029	NIH
VEGFR2	anti-VEGFR2 CAR engineered $CD8^+$ T cells	NCT01218867	NIH
Ganglioside GD-3	anti-GD-3 CAR	PI: M. Davies	MDACC

CHUV, Centre Hospitalier Universitaire Vaudois; **DFCI**, Dana-Farber Cancer Institute; **FHCRC**, Fred Hutchinson Cancer Research Center; **HMO**, Hadassah Medical Organization; **LUMC**, Leiden University Medical Center; **MDACC**, M.D. Anderson Cancer Center; **MOFFITT**, H. Lee Moffitt Cancer Center and Research Institute; **NIH**, National Institutes of Health; **NUH**, Nantes University Hospital; **PI**, principal investigator; **SMC**, Sheba Medical Center; **UC**, University of California; **UR**, University of Regensburg

Table 1. Adoptive cell therapy trials in patients with metastatic melanoma

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3. Adoptive cell therapy with antigen-specific T cells

The rationale for using melanoma antigen-specific T cells is based on the observation that the success of TIL therapy in some patients correlates with the presence of melanoma-reactive T cells, in particular with those cells specific for Melan-A, MART-1 or gp100 [23, 31]. The median survival of patients treated with Melan-A specific TIL's was 53.5 months compared to 3.5 months for patients who received TIL's without Melan-A specificity [32]. These observations together with a number of technical obstacles in obtaining TIL's from biopsies strengthened efforts to derive melanoma-specific T cell clones from peripheral blood lymphocytes for the use in adoptive cell therapy. The strategy was corroborated by a 50% response rate obtained after transfer of MART-1 or gp100 specific T cell clones isolated and propagated *ex vivo* from peripheral blood lymphocytes (Table 1) [33]. Melanoma reactive T cell clones in peripheral blood are rare, TIL therapy increases the otherwise low magnitude of the tumor-reactive T cell compartment *in vivo*, which matches the reactivity in the TIL product [34]. Interestingly, individual TIL products from different patients contain unique patterns of reactivity against shared melanoma-associated antigens [34]. TIL isolation and expansion *in vitro*, however, is extremely laborious. This limit leads to attempts to engineer patient's blood T cells with pre-defined specificity for more specifically redirecting the cytotoxic response toward melanoma. It is therefore assumed that the clinical efficacy of TIL therapy can be improved by application of T cells with more defined tumor-reactivity.

To engineer specificity for melanoma, T cell receptors (TCR's) were cloned from TIL's of responding melanoma patients and transferred to peripheral blood T cells of the same patient (Figure 2) [35-38]. The gp100 specific TCR was one of the first TCR's, cloned from melanoma TIL's and introduced *ex vivo* by retrovirus-mediated gene transfer into blood T cells, which thus obtained redirected specificity for gp100 positive cells. In contrast to their non-modified counterparts, TCR engineered T cells responded to $gp100^{+}$ melanoma cells by secreting pro-inflammatory cytokines including IFN-γ and by lysing the target cells [45, 46]. Similarly, blood T cells were engineered with recombinant TCR's with specificity for MART-1 or MAGE-A1. The functional avidity of cloned TCR's was improved and engineered T cells were successfully used in subsequent trials [47, 48]. About 30% of patients receiving ACT with MART-1 specific T cells responded with melanoma regression; 19% of patients treated with gp100 specific TCR T cells exhibited objective response, most responses were persistent [38]. TCR engineered T cells also showed efficacy towards brain metastases, which indicates that patients with otherwise incurable metastatic sites may benefit from ACT (Table 1) [115]. In patients with prolonged clinical remission, engineered T cells were present in the circulation for more than a year after initiation of treatment; this indicates that therapeutic efficacy and long-term anti-melanoma immunity may correlate with T cell persistence [49, 50].

However, the enthusiasm for adoptive cell therapy with TCR modified T cells has been dampened by several limitations. Tumor cells including those of the melanoma undergo clonal evolution, and some of these evolved cells evade T cell recognition, for instance, as a result of repression of their MHC complex [51], of mutations in their $\beta 2$ microglobulin chain [52], and of deficiencies in their antigen processing machinery [51, 53]. Each of these altera-

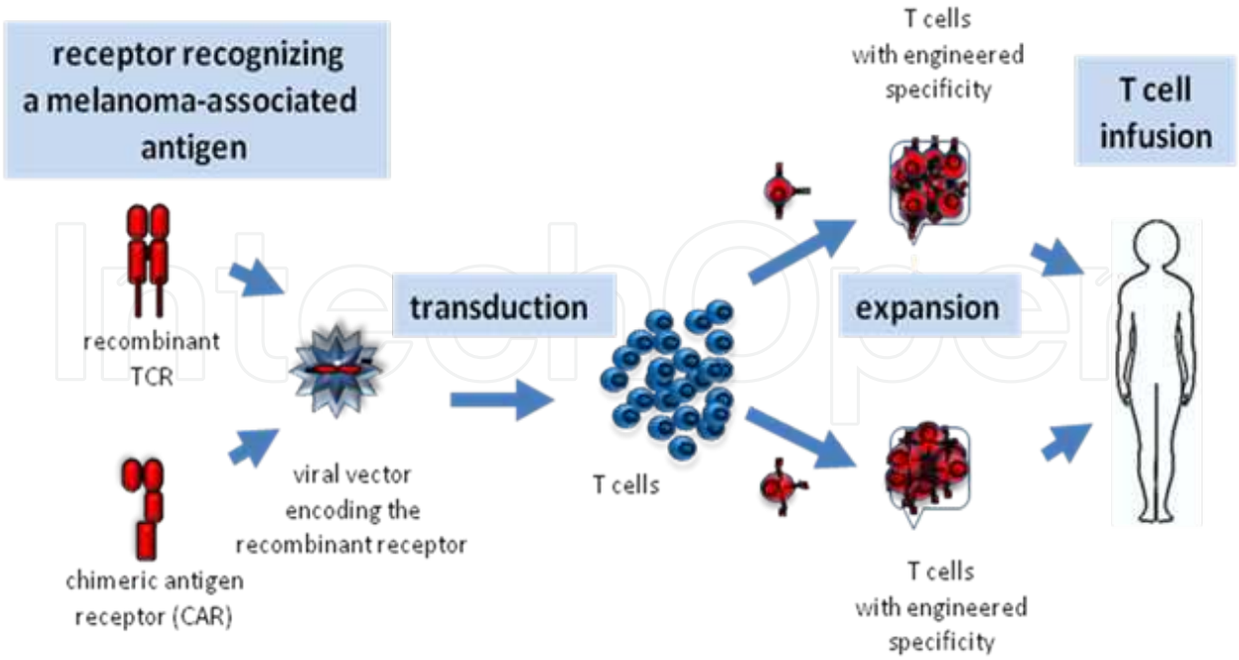

Figure 2. Adoptive cell therapy with redirected T cells. T cells from the peripheral blood of the patient are engineered *ex vivo* by retro- or lentiviral gene transfer with cDNA coding for a T cell receptor (TCR) with specificity for a melanoma-associated antigen. Alternatively, T cells are engineered with a chimeric antigen receptor (CAR) which recognizes a melanoma-associated antigen by an antibody-derived binding domain. Engineered T cells are expanded *ex vivo* prior to administration to the patient.

tions renders the melanoma cell invisible to a TCR-mediated T cell attack. A possible safety hazard moreover became apparent when analyzing in more detail the transgenic TCR, which is co-expressed with the physiological TCR in the same T cell. The transgenic TCR turned out to create new but unpredictable specificities by forming hetero-dimers of the recombinant α and β TCR chains with the respective chains of the physiological TCR. Undesirable mispairing of TCR chains may result in loss of specificity and may induce severe auto-reactivity [54, 55]. Tremendous efforts were subsequently made to solve the problem including replacement of TCR constant moieties by the homologous murine domains [56] and creation of additional cysteine bridges [57] to enforce preferential pairing of the recombinant $\alpha\beta$ TCR chains in the presence of the physiological TCR.

These and other technical difficulties promoted the development of an artificial "one-chain-receptor" molecule to redirect T cells in an antigen-restricted manner (Figure 3). In a seminal paper Zelig Eshhar of the Weizmann Institute of Science described a chimeric antigen receptor (CAR), also named immunoreceptor, which is composed in the extracellular part of a single chain antibody for antigen binding and in the intracellular part of the TCR/CD3ζ endodomain for provision of T cell activation [58]. The CAR modified T cell, also known as "T-body", becomes activated by binding to antigen, and secretes pro-inflammatory cytokines, amplifies and lyses target cells expressing the respective antigen. By using an antibody for binding, the CAR recognizes the target in a MHC-independent fashion and is therefore not affected by loss of HLA molecules, which frequently occurs during neoplastic

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progression. An additional advantage over transgenic TCR's is that CAR's can be used independently of the individual HLA subtype. However, the T-body strategy is restricted to antigens expressed on the surface of the target cell; intracellular antigens are not visible to CAR's. Due to the broad variety of antibodies available, a nearly unlimited panel of antigens can be targeted with high affinity and specificity, including those which are not classical T cell antigens, e.g. carbohydrates. High affinity CAR's activate engineered T cells even after binding to low amounts of target antigen; this not only makes the approach highly sensitive, but also makes the choice of the appropriate melanoma-selective antigen difficult.

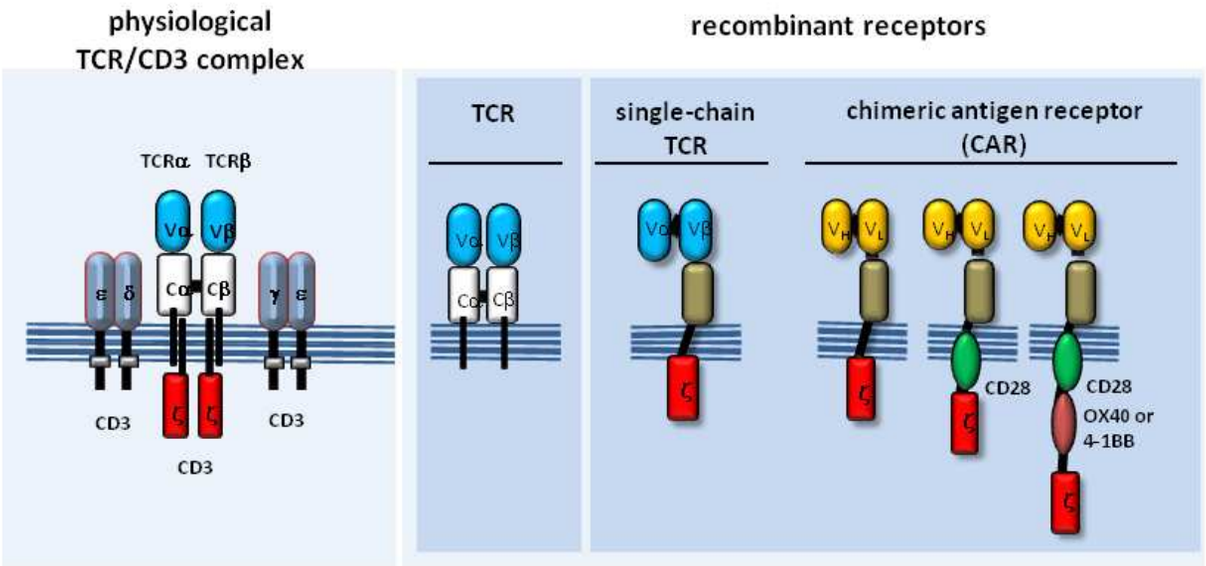

Figure 3. Recombinant receptors to redirect T cells for use in antigen-specific cell therapy. The physiologic T cell receptor (TCR)/CD3 complex consists of the α and β TCR chains, which recognize major histocompatibility complex (MHC)-presented antigen by binding through both variable regions Vα Vβ, and of the CD3 chains. Antigen engagement induces clustering of the TCR complex and the primary signal for T cell activation is generated by the intracellular CD3ζ chain. Recombinant TCR α and β chains can be engineered to T cells in order to provide a new specificity. Alternatively, the V regions of the TCR chains can be combined and fused to the intracellular CD3ζ chain to produce a T cell activation signal upon binding to antigen. The chimeric antigen receptor (CAR) makes use of an antibody binding domain for antigen recognition which is enigneered by fusing the variable (V) regions of the immunoglobulin heavy (H) and light (L) chain. The V_H-V_L single chain antibody is linked via a spacer to the intracellular CD3ζ chain to produce the primary T cell activation signal upon antigen binding. Intracellular signaling domains of costimulatory molecules like CD28 can be added to provide appropriate costimulation in addition to the primary CD3ζ signal.

T cells require two signals for full and lasting activation, one provided by the TCR and the other by costimulatory co-receptors; the prototype of which is CD28. The corresponding ligands are usually not present in the tumor micro-environment. Some effector functions including IL-2 secretion require CD28 costimulation along with the primary TCR/CD3ζ signal; this provides a rationale for combining the intracellular CD3ζ with the CD28 signaling domain in one polypeptide chain (Figure 3) [59]. Other costimulatory domains, such as 4-1BB (CD137) and OX40 (CD134), were also linked to CD3ζ; each domain has a different impact on T cell effector functions [60]. Costimulatory domains were furthermore combined in so-called 3rd generation CAR's, and a number of additional modifications have been intro-

duced in the last years to improve T cell persistence and activation [61, 62]. CAR's with a costimulatory domain clearly demonstrated clinical benefit and improved T cell persistence compared to CAR's targeting the same antigen but with only the CD3ζ domain [63-65].

Various CARs were engineered for targeting melanoma-associated antigens, including HMW-MAA, also known as MCSP [67, 68], melanotransferrin [69], the ganglioside GD2 [70] and GD3 [71]. A clinical trial targeting melanoma cells with CAR engineered T cells is currently recruiting participants [66]. Recent phase I trials using CAR redirected T cells in the treatment of lymphoma/leukemia exhibited spectacular efficacy [72, 73]. However, the enthusiasm was dampened by reports on serious adverse events and even fatalities after CAR T cell therapy [74, 75]. Targeting ErbB2 produced a cytokine storm and respiratory failure in one case [76] which is thought to be due to low levels of antigen on a number of healthy cells which can trigger CAR T cell activation. On the one hand, this event points out that ACT with CAR modified T cells may be a powerful therapy; but, on the other hand, emphasizes the necessity for careful T cell dose escalation studies to balance anti-tumor efficacy and auto-immunity[61, 77, 78].

4. Challenges and premises in the adoptive cell therapy of melanoma

To date, approximately half of the melanoma patients treated with TIL ACT benefit from this therapy; genetic modification of T cells may further improve clinical response to melanoma, but this will have to be proven in upcoming trials. However, the strategy has potential challenges which need to be addressed.

A major challenge of redirected T cells is the tumor selectivity for the target antigen itself, which in most cases is not exclusively expressed on tumor cells but also on healthy cells [79], although almost always at lower levels: for instance MART-1, which is also expressed by melanocytes. When targeting these antigens, vitiligo and inner ear toxicity resulting in a certain degree of deafness are frequently observed side effects [38]. From this perspective it is reasonable to assume that off-target toxicities may be adverse reactions for clinical efficacy in an anti-melanoma response [80]. Since nearly all tumor-associated antigens are self-antigens, strategies will have to be developed to ensure that off-target toxicities are kept to a minimum. Whether T cells with low-avidity TCR or CAR are less prone to induce such undesirable side effects is currently under investigation.

Melanoma cells, like other cancer cells, down-regulate components of the MHC and become increasingly deficient in antigen processing. As a consequence, TCR engineered T cells can no longer bind to and destroy those melanoma cells. However, they may be visible to a CAR recognizing surface antigens in a MHC independent manner, because of the antibody-derived binding domain (Figure 3). TCR redirected T cells, on the one hand, may also recognize cross-presented targeted antigen, for instance by stroma cells, but this is not the case for CAR engineered T cells. Cross-presented antigen, on the other hand, may help to destroy stroma, which is required to eliminate large tumor lesions [39, 40].

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To avoid mispairing of the recombinant TCR with the physiological TCR chains and the resulting unpredictable auto-immunity, TCR-like single chain antibodies were used as targeting domain in a CAR. Thus combining the MHC-restricted recognition of antigen with the T-body strategy. T cells with TCR-like CAR were redirected towards NY-ESO-1 and MAGE-A1, respectively [41, 42]. The possible advantages of these MHC restricted CAR's compared to the use of recombinant TCR's still has to be determined in trials.

The antibody-derived binding domain of a CAR displays extraordinary high affinity compared to a TCR. However, an increase in affinity, for instance, by affinity maturation, does not necessarily improve CAR redirected T cell activation above threshold [41, 43], which is not additionally modulated by CD28 costimulation [44]. A similar effect is also assumed for TCR mediated T cell activation. The TCR or CAR binding avidity probably affects the persistence of engineered T cells at the targeted tumor site. Strong binding to a target antigen may cause the T cells to be trapped and to become fully activated for a cytolytic attack, whereas low avidity interactions may not provide sufficiently long T cell – melanoma cell contacts. In addition to the binding avidity, the amount of target antigen on the cell surface also impacts on the selectivity of redirected T cell activation. In essence, low affinity binding directs the activity of engineered T cells preferentially toward target cells with abundant antigen levels; high affinity binding is likewise effective against low antigen levels on target cells. The optimized affinity to sustain a more selective T cell trafficking to the tumor and activation while avoiding targeting healthy cells that are expressing low quantities of the same antigen, however, still has to be determined.

A beneficial T cell-to-target cell ratio at the tumor site seems to be required for efficient tumor elimination. Higher numbers of engineered T cells applied per dose will probably increase clinical efficacy; the majority of recent trials have applied up to 10^{10} cells per dose [27]. These and higher numbers of engineered T cells can be generated by extended expansion protocols; however, cells with a "young" phenotype may not be generated for adoptive transfer under these conditions. Short-term amplification protocols are therefore envisioned for both TIL's and engineered blood T cells. However, the majority of recent trials targeting $CD19^+$ leukemia provided evidence for therapeutic efficacy at numbers less than or equal to 10^5 engineered T cells [73]. This once again raises the question of whether high T cell doses are required for a therapeutic effect.

The clinical outcome of adoptive cell therapy correlates with the persistence of adoptively transferred T cells [81]. As long as T cells engage their cognate antigen, T cells will expand and persist in detectable numbers; but when the antigen is no longer present, the T cell population will contract to potentially undetectable levels and disappear from circulation. To improve survival of CAR T cells, Epstein-Barr virus (EBV)-specific T cells were engineered with a tumor-specific CAR based on the rationale that T cells recognizing the low amounts of EBV antigens by their physiological TCR will be maintained in a sizable population in circulation and in the process providing enough CAR T cells to recognize and kill melanoma cells in the surrounding tissues. A clinical trial with EBV-specific T cells engineered with an anti-GD2 CAR thus showed benefit over non-virus-specific, CAR engineered T cells in the treatment of neuroblastoma [81].

Adoptively transferred $CD8^+$ T cell clones may be less persistent than $CD4^+$ T cell clones due to T cell exhaustion after extensive *ex vivo* amplification and multiple rounds of activation. In addition, $CD4^+$ T cell help is essential for $CD8^+$ T cell persistence *in vivo*; adoptively transferred pure $CD8^+$ T cell clones may fail to persist [82]. T cell therapy may be combined with antibody therapy to prolong the initiated immune response. For instance, CTLA-4 is upregulated on the surface of activated T cells, where it acts as negative regulator to return the T cell to a resting stage. Co-application of the anti-CTLA-4 blocking antibody, ipilimumab, may prolong the anti-tumor activation of transferred T cells, although it would also affect all the other T cells.

Besides maintaining a high number of T cells in circulation, another challenge is to accumulate significant numbers of effector T cells in the tumor lesion. A tightly controlled network of chemokines controls the migration of cells in the body; adoptively transferred T cells use these networks to accumulate at the tumor site. The expression of specific chemokine receptors controls how cells will migrate against the chemokine gradient into the targeted lesion. Melanoma cells secrete a number of chemokines including CXCL1. However, early imaging studies revealed that melanoma-specific T cells massively infiltrate the lungs, spleen and liver with some accumulation at the tumor site, which clearly represents a minority of the transferred cells, before the cells decline to undetectable levels in circulation [83-85]. Since those T cells do not express CXCR2, the receptor for melanoma secreted CXCL1, TIL's were engineered with CXCR2 which generated improved melanoma accumulation and anti-tumor activity in a mouse model [86]. The strategy is currently being explored in an early phase I trial (Table 1) [86].

One of the major hurdles of redirected immunotherapy of cancer in general is the tremendous heterogeneity of cancer cells with respect to the expression of the targeted antigen. Low or lack of antigen expression within the malignant lesions will negatively affect the long-term therapeutic efficacy of the approach. Several reports document relapse of antigen-loss tumor metastases after adoptive therapy with melanoma-reactive T cell clones [87-89] and argue for the use of polyclonal T cells with various melanoma specificities. Melanoma cells expressing the target antigen may successfully be eliminated by redirected T cells, whereas antigen-negative tumor cells will not be recognized. T cell populations modified with different CAR's recognizing different antigens expressed by the same tumor may be able to overcome these limitations. However, pro-inflammatory cytokines secreted by redirected T cells into the tumor micro-environment upon activation may attract a second wave of non-antigen restricted effector cells, which in turn may eradiate antigen-negative tumor cells. At least in an animal model, antigen-negative melanoma cells are indeed eliminated when co-inoculated with antibody-targeted cytokines [90]. Moreover, T cells engineered with induced expression of transgenic IL-12 attract innate immune cells including macrophages into the tumor tissue; they eliminate antigen-negative tumor cells in the same lesion [91].

Highly expanded T cells, such as TIL's, become hypo-responsive to CD28 costimulation and rapidly enter activation induced cell death, in particular upon IL-2 driven expansion [92].

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This may be counteracted by expansion in the presence of IL-15 and IL-21 and/or by co-stimulation via 4-1BB by an agonistic antibody [93].

Metastatic melanoma patients with the B-raf activating mutation V600E transiently 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 [94, 95]. Combination of B-raf inhibition with melanoma-specific ACT may provide an option to prolong the clinical response.

Although the TCR downstream signaling machinery is used by the prototype CAR, monocytes, macrophages as well as NK cells can also be redirected by CAR's in an antigen-specific fashion [96, 97]. Whether redirected non-T cells are advantageous in tumor elimination to cancer patients in general and to melanoma patients in particular has to be explored in clinical trials.

5. Does targeting "melanoma stem cells" provide hope for long-term remission from melanoma?

Observations that a number of malignant lesions display a tremendous cellular and phenotypic heterogeneity and contain pluripotent stem cells led to the hypothesis that cancer is initiated and maintained by so-called cancer stem cells (CSC's). Low abundance, induction of tumors upon transplantation under limiting conditions, radiation and chemo-resistance, self-renewal and a-symmetric differentiation into a variety of cell types are properties postulated for CSC's. The concept was sustained by deciphering the hierarchical organization in hematological malignancies [98], and subsequently in solid cancers including mammary, prostate, pancreatic, colon carcinoma and glioma [99-103]. Transplantation of melanoma cell subsets under limiting dilution conditions showed that a subset of cancer cells can induce tumors of the same histological phenotype as the parental tumor [99, 104, 105]. A first study using the limiting dilution transplantation assay identified a melanoma cell subset which exhibits stem-like capacities and expresses CD20 [106]. A conclusion drawn from these and other experiments was that melanoma is organized in a hierarchical manner originating from an initiator cell. In this context, several phenomena in melanoma biology which have been clinically observed but not well understood are described by the CSC model, for instance, metastatic relapse more than a decade after surgical treatment of the primary lesion. Residual CSC's are thought to drive cancer relapse even after years of "dormancy" [107]. Moreover, melanoma initiating cells were identified as expressing either the transporter protein ABCB5 [104] or the nerve growth factor receptor CD271; the latter occurs in melanoma in a frequency of approximately 1/2000 cells [108].

However, transplantation under more rigorous conditions, i.e., ideally of one isolated melanoma cell, revealed that nearly every fourth randomly taken melanoma cell (1/2 - 1/15) can induce tumors and raising the question of the validity the stem cell paradigm for melanoma [109, 110]. From these and subsequent studies, it has been concluded that the potential of melanoma induction is not closely associated with a particular phenotype and that the num-

ber of potential CSC's in melanoma may not necessarily be low. This resulted in a further conclusion that nearly every melanoma cell is capable to re-program to a tumor initiating cell under certain experimental conditions of xeno-transplantation irrespectively which particular marker phenotype the cell expressed at the time of isolation from a melanoma lesion.

Once the tumor is established, a minor subset seems to take over control of melanoma progression. Evidence is provided by recent observations from a pre-clinical model [69], which addressed the question of whether specific elimination of defined melanoma cells from an established xeno-transplanted lesion causes tumor regression by adoptive transfer of antigen-specific cytotoxic T cell. The rationale is that, if there is a clearly defined hierarchy of cancer cells in an established tumor, specific ablation of the melanoma sustaining cells from the established tumor tissue must inevitably lead to a decay of the tumor lesion independently of targeting the cancer cell mass. However, the melanoma sustaining cell may, but must not, be identical to CSC's identified by the transplantation assay. Targeted elimination of a minor subset of $CD20^+$ melanoma cells completely eradicated transplanted melanoma lesions, whereas targeted elimination of any random melanoma cell population in the same lesion did not. $CD20^+$ melanoma cells are rare, i.e. approximately 1-2%, in melanoma, independently of the histological type and the transplanted tumor tissue. A caveat is that in approximately 20% of melanoma samples, no $CD20^+$ melanoma cells could be detected by histological screening. When these tumors were transplanted, adoptive transfer of CD20-specific CAR T cells did not induce tumor regression. Interestingly, CD20 re-expression in a random subpopulation of those tumor cells did not render the tumor lesion sensitive for complete eradication with CD20-specific T cells. This indicates that CD20 expression *per se* is not dominant in maintaining melanoma progression. However, the phenotype of $CD20^+$ melanoma cells may be flexible and associated with additional capabilities which mediate the dominant effect.

The first clinical evidence confirming this concept was recently provided by a case report [111]. A patient with stage III/IV metastatic melanoma, which harbored $CD20^+$ melanoma cells at a frequency of 2%, received intra-lesional injections of the anti-CD20 therapeutic antibody rituximab and concomitant dacarbazine treatment. Dacarbazine as mono-therapy had already proved to be ineffective. This treatment produced lasting complete and partial remission accompanied by a decline of the melanoma serum marker S-100 to physiological levels, a switch of a T helper-2 to a more pro-inflammatory T helper-1 response, all without treatment related grade 3/4 toxicity. Although anecdotic, this data provides the first clinical evidence that targeting the subset of $CD20^+$ melanoma sustaining cells can produce regression of chemotherapy-refractory melanoma. Moreover, the report highlights the potency of selective cancer cell targeting in the treatment of melanoma.

These observations although so far based on a pre-clinical model and a clinical observation which will have to be reproduced in larger cohorts have major impact on the future development of melanoma therapy.

First, the melanoma maintaining cells may be more resistant to current therapy regimens than the bulk of melanoma cells. Standard therapy strategies attempt to eliminated all cancer cells in a tumor lesion; elimination of any other cancer cells than the tumor progressing cells will rapidly de-bulk the tumor lesion. The melanoma will inevitably relapse, driven by

the remaining melanoma sustaining cells, which are extraordinary resistant to chemotherapeutics. This resistance is probably due to transporter molecules like ABCB5, which are highly expressed by a number of CSC's including melanoma [104] and therefore efficiently counteract chemotherapy. Melanoma maintaining cells like other CSC's are merely in a "dormant" state and replicate less frequently than the majority of cancer cells in the same lesion, which reduces the efficacy of anti-proliferative drugs. Low proliferative capacities together with the efficient export of chemotherapeutics contribute to CSC resistance toward a variety of therapeutic drugs. As a consequence, alternative strategies that specifically induce cell death of those cells are required. Moreover, the situation is exacerbated by the fact that the melanoma maintaining cells in the lesion are rare and unlikely to be eliminated by the random targeting provided by most therapeutic agents. Specific targeting by cytotoxic T cells redirected towards CD20 or by CD20-specific therapeutic antibodies like Rituxan™ (rituximab) or Arzerra™ (ofatumumab), probably as adjunct to a tumor de-bulking strategy, may improve the situation.

Second, whether the prevalence of $CD20^+$ melanoma maintaining cells in a tumor lesion may correlate with clinical progression or relapse has to be addressed. If so, the frequency of $CD20^+$ melanoma cells may serve as a surrogate marker for therapeutic efficacy and/or prognosis. Chemotherapy and/or radiation may induce amplification of these cells thus contributing to their accumulation during tumor progression and metastasis.

Third, melanoma maintaining cells may exhibit an extraordinary functional and phenotypic plasticity. As a consequence, continuous presence of targeting therapeutic agents will be required to eliminate those cells, which exhibit newly acquired melanoma initiating and/or maintaining capacities. In their pre-clinical model, Schmidt and colleagues [69] used CAR engineered T cells which penetrate tissues, scan for targets and persist for long-term acting as an antigen-specific guardian. These T cells are present in the targeted lesion as long as cells expressing the target antigen appear. Repetitive restimulation of these T cells, for instance by engaging their TCR with EBV-specific antigens [63, 81], may sustain persistence of CAR T cells in sufficient numbers over long periods of time. In this constellation, cellular therapy has a major advantage compared to pharmaceutical drugs, which are present in therapeutic levels for short periods; in the case of melanoma the required period for screening for re-appearance of such melanoma initiating cells may be many years. The development of an antigen-specific memory by adoptively transferred CAR T cells, as recently shown in a pre-clinical model [112], may be of benefit to patients in preventing a melanoma relapse.

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Abbreviations

ACT, adoptive cell therapy; **CAR**, chimeric antigen receptor; **CTLA-4**, anti-cytotoxic T-lymphocyte-associated antigen-4; **CSC**, cancer stem cell; **EBV**, Epstein-Barr virus; **GMP**, Good Manufacturing Practice; **IFN**, interferon; **IL**, interleukin; **TCR**, T cell receptor; **TIL**, tumor infiltrating lymphocyte

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