Engineering T cell immunity by TCR gene transfer
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T CELL RECEPTOR GENE THERAPY: CRITICAL PARAMETERS FOR CLINICAL SUCCESS

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TCR gene therapy aims to induce immune reactivity against tumors by introducing the genes encoding a tumor-reactive T cell receptor into patient T cells. This approach has been extensively tested in pre-clinical mouse models and initial clinical trials have demonstrated the feasibility and potential of TCR gene therapy as a cancer treatment. However, data from pre-clinical and clinical studies suggest that both the therapeutic efficacy and safety of TCR gene therapy can be and needs to be further enhanced. This review highlights those strategies that can be followed to develop TCR gene therapy into a clinically relevant treatment option for cancer patients.

| INTRODUCTION

Over the past few decades, great efforts have been made to enhance endogenous T cell reactivity against human tumors, and in recent years two approaches have started to show a significant clinical effect. First, non-antigen-specific immunomodulation in the form of the administration of monoclonal antibodies that block Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) or other T cell checkpoint molecules have successfully been utilized in patients with metastatic melanoma and renal cell carcinoma[1,2]. Second, administration of autologous tumor-infiltrating lymphocytes (TIL) that have been expanded *ex vivo* has been utilized to treat patients with metastatic melanoma. When TIL therapy was given in combination with non-myeloablative lymphodepletion, an impressive 50% objective response rate has been observed in clinical trials at two different centers[3-6].

Although these studies demonstrate the potential value of T cell based immunotherapies, there are a number of limitations associated with these approaches. First, the success of these therapies is – at least thus far – restricted to melanoma and renal cell carcinoma, two tumor types that are generally assumed to be more immunogenic than other tumors (although we note that the molecular basis for such a difference in immunogenicity is at present unclear). Thus, it is possible that the tumor-reactive T cell repertoire for other human tumors is too small to mobilize by T cell checkpoint blockade or TIL therapy[7]. Second, immunity induced by these therapies is not specifically steered towards defined tumor-associated antigens, and it is plausible that T cell therapies could be more effective and/or less toxic if the immune response was specifically directed towards defined tumor-associated antigens.

In contrast to these two approaches that aim to enhance an undefined tumor-specific T cell response, T cell receptor (TCR) gene therapy does not rely on the pre-existing presence of tumor-reactive T cells, and does allow one to target defined tumor-associated antigens of choice. This approach is based on the observation that antigen specificities can be transferred between T cells by introducing the genes encoding the TCRα- and β-chain that together form the αβ TCR heterodimer[8]. Thus, the introduction of the genes encoding a tumor-reactive TCR can be utilized to re-direct patient-derived T cells towards an antigen of interest, thereby establishing a tumor-reactive T cell compartment that would be otherwise absent.

The concept of genetic engineering of T cell immunity has developed from a

somewhat futuristic plan into a realistic clinical possibility over the last 15 years (**Fig. 1**). Initial studies in the late nineties showing that human T cells could be redirected towards antigen-expressing cells by TCR gene transfer[9,10] were followed by work that showed that both $CD4^+$ and $CD8^+$ T cells transduced with TCR can function in *vivo* in mouse models[11-15]. Furthermore, subsequent studies in mice[16,17] demonstrated that the central underlying rationale for TCR gene therapy is valid; it is possible to create a defined tumor-reactive T cell compartment towards antigens of choice, irrespective of self-tolerance. In the above pre-clinical studies and the clinical studies carried out thus far gamma-retroviral vectors were used to transfer TCR genes into T cells. This approach leads to the long-term redirection of T cell specificity as transferred TCR genes are stably integrated in the genome of redirected T cells. Recently lentiviral vectors have also been used in a number of pre-clinical studies[18-21] and a proof-of-principle study has shown that a non-viral transposase-mediated gene transfer system can also be used to achieve stable transfer of TCR genes in T cells[22].

Two phase I clinical trials involving TCR gene therapy have been performed in melanoma patients thus far, both by the group of Rosenberg at the National Cancer Institute (NCI) surgery branch[23,24]. In the first trial, patients with metastatic melanoma were treated with autologous T cells modified with a TCR (termed DMF4) specific for the melanocyte differentiation antigen MART-1 and the gene-modified cells were infused after a lymphodepleting regimen[23]. While no treatment-related toxicity was observed, the objective response rate of 17% (2 out of 17 patients) was low compared to that observed in TIL therapy trials performed by the same group[3-5]. This discrepancy may at least in part have been related to the low level of TCR expression on the gene-modified T cells, as well as to the poor persistence of TCR-modified T cells after infusion in this study.

In the second clinical trial, patients with metastatic melanoma were treated with T cells modified with either a MART-1 specific TCR (DMF5) that exhibits a higher affinity than the previously used DMF4 TCR, or a TCR (154) specific for the melanocyte differentiation antigen gp100[24]. The expression of the introduced TCR and the persistence of modified T cells were markedly increased compared to the first trial, which may have been a result of the

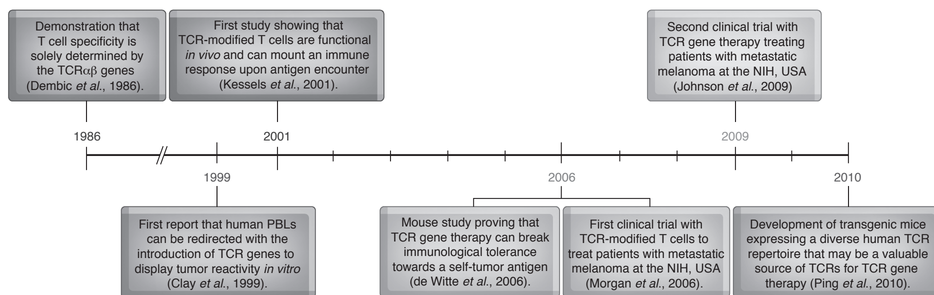

Figure 1. Milestones in the development of TCR gene therapy. TCR, T cell receptor.

intrinsic properties of the TCRs[25,26] and the format of the gene expression cassette used in this second clinical trial (Internal ribosome entry site (IRES) vs. P2A). Despite this, objective clinical response rates remained relatively low, with 30% (6/20 patients) for the DMF5 TCR and 19% (3/16 patients) for the gp100 (154) TCR, respectively. Thus, the clinical experience with TCR gene therapy in these studies can be summarized as followed: while there is now clear evidence for the clinical feasibility of TCR gene therapy, there is only limited proof for efficacy, and this issue needs to be addressed.

During the time in which these clinical trials have been performed, a number of preclinical studies have been carried out that provide leads for the development of more effective TCR gene therapy protocols. In addition, preclinical work has also revealed some potential toxicities of TCR gene therapy that need to be addressed. Here we combine data from the preclinical and clinical studies over the past years to discuss how the further development of TCR gene therapy may take place, focusing on 3 main questions:

I) What tumor-antigens represent effective and safe targets for TCR gene therapy and how can suitable TCRs that target these antigens be generated?
II) Which of the potential toxicities associated with TCR gene therapy represent 'real' risks and what strategies can best be used to prevent or control such toxicities?
III) Which 'adjuvant strategies' can best be used to enhance the clinical efficacy of TCR gene therapy of cancer?

| CHOICE OF SUITABLE TARGET ANTIGENS AND ISOLATION OF POTENT T CELL RECEPTORS FOR TCR GENE THERAPY

Which antigens to pick? General considerations with regard to efficacy

Conceptually there are a number of criteria that can be used to judge the potential suitability of a tumor antigen as a target for TCR gene therapy. With regards to safety, a high degree of tumor-specific expression is desirable to limit the chances of damage to normal tissues, and this important issue is discussed in greater detail below. In terms of efficacy, the following factors need to be taken into consideration. First, the heterogeneity of expression of the target antigen within the population of cancer cells is likely to influence therapeutic efficacy. For example, tumor-initiating cancer stem cells have been identified in a number of different cancers[27-30] and if expression of the target antigen is not found on these cells, treatment is unlikely to be successful unless the cancer stem cells would be eradicated through bystander killing. A second factor expected to influence therapeutic efficacy is the likelihood of down-regulation of target antigen expression. The risk of tumor escape via down-regulation of target antigen expression may potentially be minimized by targeting proteins that play an essential role in maintaining the malignant phenotype. However, the number of antigens that fulfill this criterion – and are still sufficiently tumor-specific (see below)- is likely to be low. A third factor that is likely to influence therapeutic efficacy relates to the expression profile of the target antigen in normal tissues, since efficacy may be compromised by dose limiting toxicity as a result of the destruction on normal tissues expressing the target antigen[31].

Which antigens to pick: safety concerns

Tumor-associated antigens (TAA) can be subdivided into discrete categories based upon their expression pattern in normal tissues and on whether these antigens are genetically 'self' or arise as a consequence of mutations.

The cancer/testis (C/T) antigens are expressed in a variety of human cancers, and also in the human germline[32-34]. Expression of C/T antigens in other healthy tissues is generally presumed to be absent[35-37] which marks them as the class of shared TAA with the most restricted expression pattern in untransformed cells. There is evidence though that at least certain C/T antigens can be expressed by thymic epithelial cells, suggesting that there may be some level of T cell tolerance towards these antigens. A second class of shared TAA is formed by the tissue-specific differentiation antigens, a group of antigens that is typically only expressed by the tumor and its tissue of origin. Examples of tissue-specific differentiation antigens include the MART-1/Melan-A[38] and gp100[39] antigens that are expressed in both melanocytes and in melanoma cells, and which were targeted in the two first clinical trials of TCR gene therapy[23,24]. Tissue-specific differentiation antigens have also been described for cell lineages in other organs, such as the prostate[40]. It is noted though that – in spite of their name – these lineage differentiation antigens are often also expressed to some extent in other (developmentally related) cell types[24], a potential cause of on-target toxicity that will be discussed further below. A third class of TAA consists of proteins which are frequently expressed at elevated levels in tumors but that are also present in lower levels in a variety of normal tissues. Examples of this class of TAA include p53[41], Her2/neu[42], MDM2[43,44] and cyclin-D1[45].

A final class of TAAs is formed by mutated self-proteins that can potentially form targets for T cell based immunotherapy of cancer[46-49]. When the mutation involved first occurred within the cancer-initiating cell (or one of its daughters), this class of tumor antigens represent the safest possible target for TCR gene therapy, with a maximal degree of tumor specificity. It is important to realize though that certain driver mutations in cancer development are hereditary. As an example, the CDK4 mutation that results in a novel HLA-A2 restricted T cell epitope[49] may seem a perfect target for TCR gene therapy but is sometimes observed in familial melanoma[50]. For these patients, the targeting of this antigen would certainly not result in selective tumor cell recognition. Nevertheless, the majority of mutations within each tumor genome are likely to be tumor-specific, and can therefore be considered potential MHC-class I restricted neo-antigens.

While some of the neo-antigens that are formed by mutations can be shared by patients (something further discussed below), the majority of these neo-antigens is likely to be patient specific. It is currently unknown to what extent recognition of such patient-specific neo-antigens contribute to the clinical responses upon TIL therapy or anti-CTLA4 treatment, and this will be an important issue to address. Specifically, if recognition of patient-specific neo-antigens would turn out to be predominantly responsible for the observed clinical responses this would represent a significant set-back for the TCR gene therapy field, as the targeting of patient-specific antigens by TCR gene therapy is clearly a much more demanding task than the targeting of shared antigens.

A prevailing view among tumor immunologists has been that, despite a lack of tumor-specific expression, even TAA that are widely expressed in normal tissues (e.g p53, MDM2. Her2/neu) may represent safe targets for T cell based immunotherapeutic approaches. This view has been based on the fact that for many of these TAAs, expression is increased in tumor cells relative to normal cells and this could provide a 'window of opportunity', allowing tumor cell destruction without destruction of normal cells. The lack of toxicity observed in clinical trials of cancer vaccines that aim to induce T cell responses against antigens such as p53 and CEA has sometimes been taken as evidence for the safety of targeting these antigens in adoptive T cell therapy trials. However, this reasoning is flawed: vaccination against TAAs aims to mobilize an endogenous T cell response that for most self antigens will be small in size and quality/ affinity as a result of immunological tolerance[17,51-54]. In contrast, TCR gene transfer can be used to break tolerance and induce robust responses to TAAs, using TCRs that are as potent as any anti-viral TCR. Therefore, the fact that a TAA has been shown a safe target in vaccination studies is not informative with respect to its use as a target for TCR gene therapy.

Strong experimental support for this notion comes from a number of recent studies that have demonstrated that the introduction of a high avidity T cell repertoire can result in the destruction of normal tissues that express this target antigen. For example, while the targeting of p53 by vaccination in both pre-clinical and clinical studies has not resulted in any significant toxicities[55], it has been demonstrated that mice that are treated with T cells transduced with a high affinity p53-specific TCR die as a result of the destruction of the hematopoietic compartment, a toxicity that is dependent on p53 expression by the hematopoietic compartment (Lauwen *et al*., manuscript submitted). The potential danger of targeting p53 by TCR gene therapy has been underscored by an *in vitro* study in which human T cells transduced with a high affinity p53-specific TCR were observed to recognize some normal cells expressing p53[56]. Notably, in the experiments by Offringa and colleagues, toxicity was only observed when the T cell populations used for TCR gene modification were unable to present the p53 epitope themselves. In cases in which T cells also do express the antigen that is being targeted, fratricide of these cells is likely to explain the absence of pathology upon cell administration. Although such fratricide prevents ablation of the host hematopoietic system, it will obviously also compromise the antitumor effect of these cells.

In line with the data on the targeting of p53, the targeting of CEA with a high affinity CEA-specific T cell compartment has been shown to lead to fatal colitis in mice as a result of CEA expression on intestinal tissue[52]. Severe colitis was likewise observed in a recent trial in 3 out of 3 patients that received T cells modified with a CEA-specific TCR[31], an observation that underscores that data obtained in well-chosen mouse models can be useful to assess safety risks. Finally, the fact that even a low level of antigen expression in vital tissues can form a safety risk is emphasized by two recent clinical studies that utilized T cells transduced with Chimeric Antigen Receptors (CAR). First, Lamers *et al.* have demonstrated that treatment of renal cell carcinoma patients

with T cells transduced with a CAR specific for Carbonic Anhydrase IX leads to liver toxicity[57]. Likewise, Rosenberg *et al.* have recently observed severe lung toxicity that resulted in the death of a patient following the infusion of T cells transduced with a Her2/neu-specific CAR[58]. In both cases, a low level of target antigen expression was observed in the organ involved.

Are there cases in which toxicity due to on-target recognition is acceptable? In the second clinical trial of TCR gene therapy in which the MART-I and gp100 melanocyte differentiation antigens were targeted with high affinity TCRs, a significant number of patients experienced treatment-induced toxicities that can be explained by the destruction of normal MART-I/ gp100 expressing cells in the skin, eye and ear[24]. In this case, these toxicities could be successfully treated by the application of topical steroids and therefore may be viewed as clinically acceptable. These data demonstrate that expression by non-transformed cells can be acceptable, provided that antigen-expression is restricted to non-essential tissues. To evaluate whether the toxicity that can be expected is likely to be acceptable, a rigorous assessment of the expression pattern of any new antigen that is targeted by TCR gene therapy is critical prior to the start of clinical trials. This assessment should at least involve the analysis of gene expression data in different human tissues. However, it is important to realize that gene expression analyses will likely fail to detect expression of an antigen in a small subset of (perhaps critical) cells within an organ. As an example, the toxicity seen in the trial by Lamers and colleagues would not be predicted by the sole analysis of microarray data of human liver[57]. Because of this concern, analysis of protein levels at the cellular level (i.e. by immunohistochemistry) clearly seems a preferred approach. Furthermore, since the most important question is whether immunologically relevant levels of the target antigen of interest are expressed in normal tissues, it also seems valuable to directly assess recognition of a large panel of human cell types by the TCR-modified T cells (M. Heemskerk (Leids Universitair Medisch Centrum) personal communication). These type of preclinical studies on antigen expression will provide a certain degree of confidence about the safety of targeting a given antigen and thereby aid in rational antigen choice. However, for many target antigens, expression will not be fully tumor-specific, and the safety profile of TCRs that target such an antigen can only be definitively determined by clinical testing. Initial clinical studies targeting such antigens should therefore assess the consequences of escalating levels of the TCR-modified T cell response. If designed in analogy with safety studies for other pharmaceuticals, such a phase I study would involve the infusion of increasing numbers of TCR-modified T cells. However, when T cell administration is performed subsequent to lymphodepletion, this concept of classical dose escalation becomes problematic, as the administration of a low TCR-modified T cell dose will be accompanied by an increased ability to undergo *in vivo* homeostatic proliferation. To address this issue, we would propose to replace dose escalation by 'frequency escalation' in such safety studies, in which a constant number of T cells is infused of which an increasingly high percentage is modified with the TCR of interest.

Of the three commonly considered classes of target antigens for TCR gene

therapy (the differentiation antigens, C/T antigens and overexpressed antigens), the C/T antigens probably represent the most promising targets for TCR gene therapy of cancer, taking current technology and data into account, First, of these three classes of TAA, C/T antigens represent the safest set of targets for TCR gene therapy, since expression in all normal tissues that can be accessed by the immune system appears to be absent[35-37]. Second, for many C/T antigens expression is observed in a variety of human cancers[32], which means that relatively large groups of patients can potentially be treated. As a downside, while C/T antigens are expressed in diverse human cancers, the frequency of expression is often relatively low, making clinical trial enrollment slow. Furthermore, expression of these antigens is often heterogeneous[32,59], and we lack data that show whether the targeting of C/T antigens with heterogeneous expression can lead to sustained cancer regression. Future clinical trials with TCRs specific for C/T antigens will reveal whether the current optimism about these antigens is justified[60,61].

A class of tumor antigens that is not commonly considered as targets for TCR gene therapy is formed by mutations that are shared between patients and that are sometimes also observed in different tumors types[62]. Conceptually, these shared mutated antigens are very attractive targets for TCR gene therapy for the following reasons. First, as discussed above, most of these antigens are likely to be safe targets owing to their exclusive expression in tumor cells. Second, targeting these mutations should be clinically feasible in terms of cost, time taken to generate the appropriate TCRs and clinical trial enrollment. Third, the fact that these mutations are shared suggests that they may be 'driver mutations' that play an essential role in maintaining the malignant phenotype[63], and escape of T cell recognition by down-regulating expression of the mutated antigen is therefore unlikely. However, it is likely that many of the mutations that are shared between individuals are effectively 'invisible' to T cells. This is because the combined probability of a peptide encoding a mutation 1) being processed by the HLA class I processing pathway, 2) being presented by a HLA allele and 3) being immunogenic is very small. Nevertheless, given that these antigens are conceptually very attractive targets for TCR gene therapy, it does seem worthwhile to assess whether any of these shared mutations encode immunogenic peptide epitopes that are presented by common HLA alleles.

While shared mutated antigens represent a conceptually more attractive target for TCR gene therapy, the majority of neo-antigens within each tumor are likely to be patient-specific. The targeting of such unique patient-specific mutated antigens would require patient-specific TCR gene therapy, an approach that even five years ago would have been viewed as impossible from both a technological and financial point of view. However, the rapid development of next generation sequencing technologies[64] means that the routine sequencing of individual tumor genomes is becoming a reality, thereby enabling the identification of potential neo-antigens on a per patient basis. Furthermore, approaches for the identification and isolation of antigen-specific T cells have also gained substantially in throughput over the past years[65-67]. If the time required for TCR generation and validation can also be reduced substantially in the coming years, it does seem possible

that, tailor-made TCR gene therapy can at some point be tested in the clinic.

How to get the TCR?

Having decided which antigen to target, the essential next step is to obtain a TCR that recognizes this peptide-MHC complex, preferably with high affinity. One source of TCRs for TCR gene therapy that has already been exploited clinically is patient material. Both MART-1 specific TCRs used in the clinic thus far were isolated from a melanoma patient that experienced tumor regression after TIL therapy[23,24]. While it clearly seems preferable to isolate TCRs from patients that experience tumor regression after therapy than from patients that progress, it is important to point out that the mere presence of a given antigen-specific T cell population does not inform us of its role in cancer regression. If more data were available on the relationship between specific T cell reactivities and clinical course, such data could perhaps be used to make a more informed choice of TCRs for use in gene therapy trials. Towards this goal, we have recently established a research line in collaboration with the NIH Surgery Branch (Bethesda, USA) and the Chaim Sheba Medical Center (Tel Aviv, Israel) that aims to gain insight into the composition of the shared TAA-reactive T cell compartment in melanoma patients treated with TIL therapy. Using a high-throughput MHC tetramer screening platform[66] based on some 150 HLA-A2 restricted melanoma-associated peptides, we have established that TIL therapy induces a demonstrable increase in the tumor-reactive T cell compartment (Kvistborg *et al.*, manuscript in preparation). Future experiments will aim to establish whether the presence of certain T cell reactivities can perhaps be correlated to clinical course.

While patient material will likely remain a source for TCRs used in TCR gene therapy, some limitations should be noted. First, it will not be possible to obtain TCRs against any given antigen because of immunological self-tolerance. Second, for those TCRs that can be identified in patient material, their affinity for cognate peptide may be suboptimal, again as a result of self-tolerance. As it has been shown that high-avidity T cells mediate better tumor control than their low-avidity counterparts[68-70], and as tumors may present low amounts of antigen or MHC[71], the clinical efficacy of TCRs obtained from low avidity T cells may not be sufficient to mediate cancer regression.

To allow the isolation of TCR genes without the limitations of self-tolerance, a variety of technological platforms have been developed in the past 15 years. These platforms that include allo-CTL systems[45,72,73], HLA-transgenic mice[44] and phage/yeast/T cell display systems[74-76] have been extensively described in a recent review[77] and will not be discussed here. However, a major step towards the straight-forward generation of TCRs against human antigens was recently achieved by the Blankenstein group[78]. In a heroic effort, this group created a mouse model in which the entire human TCR loci have been introduced and their murine counterparts have been inactivated. As these hTCR mice also express human MHC molecules (HLA-A2 in the recent paper, but others sure to come), this mouse model allows one to generate fully human TCRs against epitopes of interest. The hTCR mice from Blankenstein display a diverse TCR repertoire with marked similarities to the human TCR repertoire. Furthermore, these mice were shown to be capable of mounting a T cell response against a series

of different antigens, suggesting that these mice can form a very valuable source of TCRs for use in TCR gene therapy. In addition to its value for the generation of a collection of TCRs for clinical use, the model should also be useful to address fundamental questions with regard to T cell tolerance against different classes of tumor-associated antigens. For instance, is the affinity of human TCRs specific for human C/T antigens similar for T cells isolated from mice and men, or is there an imprint of tolerance, even for antigens with such a restricted tissue expression?

| OFF-TARGET SAFETY RISKS OF TCR GENE THERAPY

In addition to the potential for on-target toxicity described above, there are a number of potential off-target safety risks associated with TCR gene therapy that have been known for years[79,80]. However, recent studies have highlighted that one of these risks is more than just a theoretical concern. Specifically, the pairing of endogenous and introduced TCR chains in TCR-modified T cells is known to lead to the formation of so called 'mixed TCR dimers' (**Fig. 2**). This repertoire of newly formed T cell receptors has obviously not been screened against self-reactivity, and it has been argued that the TCR-modified T cell pool may therefore be reactive against undefined self antigens[79]. Only recently, experimental evidence has been obtained that demonstrates that the self-reactive T cell repertoire that is created upon the formation of mixed TCR dimers can indeed result in autoimmune destruction[81,82]. Specifically, the Heemskerk group utilized primary human T cells to show that mixed TCR dimers that display auto-reactivity in *in vitro* assays are readily formed on human TCR-modified T cells[81]. Furthermore, our work showed the potential *in vivo* consequences of such *de novo* generated self-reactivity, by demonstrating that mixed TCR dimer formation can lead to lethal cytokine driven autoimmune pathology in mouse models of TCR gene therapy. It is important to point out that this pathology, termed TCR gene therapy-induced graft-versus-host-disease (TI-GVHD), only becomes apparent under

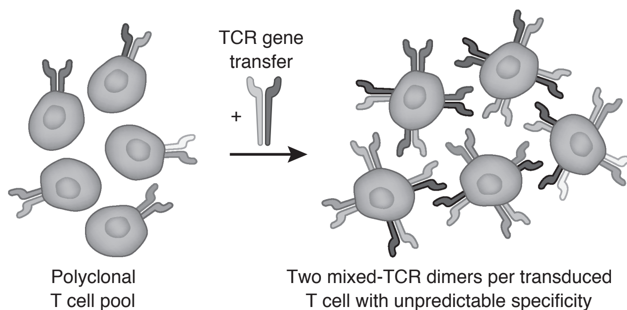

Figure 2. Formation of mixed-TCR dimers upon TCR gene transfer. The introduction of a new TCR into a T cell can lead to pairing of endogenous and introduced TCR chains. Theoretically, two new TCRs consisting of one endogenous and one introduced TCR chain can be formed. Problematically, the specificity of these mixed-TCR dimers cannot be predicted and may lead to autoreactivity. TCR, T cell receptor.

conditions in which the TCR-modified T cell response is vigorous. It is however observed for 5 out of 5 TCRs tested and under different *in vivo* conditions[82].

If the formation of mixed TCR dimers can lead to graft-versus-host-disease, one would predict that it would be valuable to limit the formation of mixed TCR dimer expression on TCR-modified T cells. To this end we have shown that a combination of two TCR engineering strategies can be used to ameliorate the observed mixed TCR dimer dependent autoimmunity in mice. Specifically, the use of TCR engineered with an additional inter-chain disulphide bond – an approach first developed by Greenberg and colleagues[83] – in a gene expression cassette that utilizes a virus-derived P2A element[77] to link the TCR-α and TCR-β genes can limit or prevent autoimmunity in mice after TCR gene transfer[82]. Importantly in addition to enhancing the safety of TCR gene therapy, this combination of TCR engineering strategies also enhances the anti-tumor efficacy of TCR gene therapy in mice (our unpublished observation).

While mixed TCR dimer dependent toxicity has been seen in mice, no such toxicity has been observed in the clinical trials of TCR gene therapy carried out to date[23,24,31]. As a result of this it has been argued that TCR gene therapy-induced GVHD is a problem unique to mice, and that such toxicity does not form a significant risk for future clinical trials[84]. However, it took almost a decade of optimizing conditions for TCR modified T cell therapy for mixed dimer dependent toxicity to be observed in mouse models, and in early mouse experiments it was in fact also perceived as a non-issue[16]. Since increasing the *in vivo* function of TCR-modified T cells in humans is also desirable, and as autoreactive mixed TCR dimers are observed *in vitro* on human TCR-modified T cells[81], we disagree with the viewpoint that mixed TCR dimer-dependent toxicity can not occur in the human situation. Indeed, there is no conceptual framework that would explain why mixed dimer expressing cells would be toxic to mice but not men. Thus, there is a very strong rationale for employing strategies to limit autoreactive mixed TCR dimer formation in future clinical trials of TCR gene therapy, especially as such strategies do not have to reduce TCR-modified T cell function.

| IDENTIFYING STRATEGIES THAT ENHANCE THE ANTI-TUMOR EFFICACY OF TCR GENE THERAPY

While the toxicity issues described above are clearly a concern, the disappointing clinical responses observed to date indicate that the most important issue at present is to understand what it takes to induce durable clinical responses by TCR gene therapy. The low response rate in the two trials by Rosenberg and colleagues may at least in part be explained by the nature of the antigens that were targeted: the identified epitopes from melanocyte differentiation antigens are those to which T cell tolerance is not strict, and this may imply that their presentation by tumor cells is also inefficient. In addition, contrary to T cell responses in TIL, TCR gene-modified T cell responses are mono-specific, and efficacy may possibly be enhanced by simply targeting multiple antigens simultaneously,

something that will undoubtedly be tested in the near future (**Fig. 3**).

It is also likely though that in addition to the nature of the target and the number of targets chosen, the anti-tumor efficacy of TCR gene therapy can also be increased by other alterations. Support for this notion is provided by studies showing that there are a number of parameters that can be manipulated to enhance the therapeutic efficacy of TCR-modified T cells in mice. First, adoptive T cell transfer studies have demonstrated that depletion of the endogenous T cell pool of the host with lymphodepleting chemotherapy or total body irradiation greatly increases anti-tumor immunity[85,86]. Second, enhancing the expression of the introduced TCR can greatly improve the anti-tumor efficacy of TCR-modified T cells[87]. Third, the composition of the cell graft affects the anti-tumor potential of TCR-modified T cells, with a high precursor frequency of TCR-modified T cells in the cell graft leading to an enhanced anti-tumor efficacy even if the absolute number of TCR-modified T cells that is given is unchanged[87]. While these strategies all enhance the anti-tumor efficacy of TCR-modified T cells, the clinical trials of TCR gene therapy performed to date already utilized lymphodepletion, optimized TCR transgene cassettes and reached a high frequency of TCR-modified T cells. Nevertheless, the number of clinical responses observed was relatively low. This suggests that additional parameters may also need to be manipulated to achieve durable clinical responses with TCR gene therapy, the most promising of which are discussed below.

Manipulation of the cytokine milieu in the form of systemic administration of IL-2

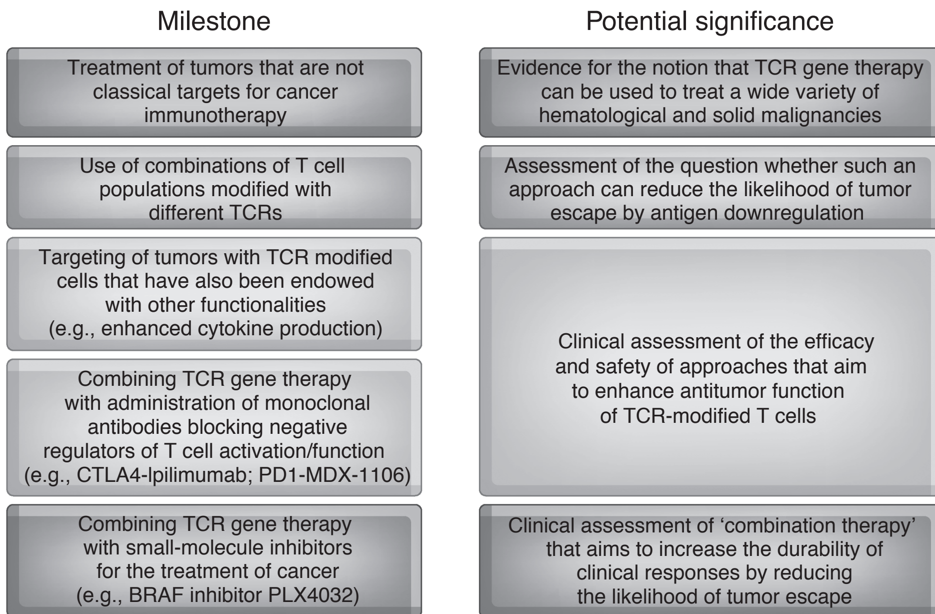

Figure 3. Potential future milestones in the clinical development of TCR gene therapy. TCR, T cell receptor.

to patients was used in the clinical trials of TCR gene therapy to support the survival/expansion of the adoptively transferred TCR-modified T cells. However, it is likely that further refinement of this approach with regards to both the cytokines used and the way in which they are provided (systemic vs. local production) will lead to improvements in the therapeutic efficacy of TCR gene therapy. In terms of which cytokines are manipulated, data from pre-clinical models suggests that supplementation with alternative cytokines to IL-2, such as IL-7[88], IL-12[89], IL-15[90] or IL-21[91] may be better able to promote the anti-tumor efficacy of TCR-modified T cells.

Second, systemic delivery of cytokines is associated with significant side effects for cytokines such as (high dose) IL-2 and IL-12[89,92]. To avoid this toxicity, two different approaches that enable more localized production of cytokines have been developed. A first approach involves the conjugation of cytokine-loaded nanoparticles to the surface of T cells prior to adoptive cell transfer[93]. This approach is relatively straightforward and enables localized delivery of not only cytokines but also a range of other potentially useful small molecules. However, the duration of cytokine supply will ultimately be time-limited, and whether this is a pro or con remains to be established. A second approach involves the genetic engineering of TCR-modified T cells, thereby enabling the cells to produce the cytokine themselves long-term [89]. In this case, it may be preferable to have cytokine production regulated in an inducible fashion (either by TCR triggering or by a pharmacological agent) to limit the chances of treatment related toxicities like those observed with IL-12 engineered TCR-modified T cells[89]. In addition to localized production of cytokines, localized inhibition of the effect of certain cytokines may also enhance TCR gene therapy efficacy. For example, tumor-derived production of TGF-β has a marked suppressive effects on anti-tumor T cell responses[94,95] and blockade of TGF-β signaling in TCR-modified T cells by engineering them with a dominant-negative TGF-β receptor-II[96] enhances anti-tumor efficacy in a pre-clinical spontaneous tumor model (our unpublished data).

In addition to the manipulation of the cytokine milieu, manipulation of the cell population used for gene transfer may also be used to enhance therapeutic efficacy. Initial studies in mice[97] and more recent clinical studies[98,99] have demonstrated that the acquisition of a fully differentiated effector phenotype in T cells prior to adoptive transfer leads to diminished in vivo function after adoptive transfer. In contrast, naïve T cells[100], central-memory T cells[101,102] and stem-cell like memory T cells[103] have been shown to have superior *in vivo* function in pre-clinical models on a per cell basis. At present, there is no evidence that the presence of 'older' cells with limited potential for clonal expansion within cell grafts containing 'younger' cells is detrimental. Therefore, the identification of more optimized *in vitro* T cell activation and growth regimens that produce enhanced numbers of 'young' cells seems likely to be of greater value than the development of technology to purify less differentiated cells from a heterogeneous cell population.

Finally, manipulation of some of the pathways in T cells that act as negative regulators of T cell function may prove to be a key factor in enhancing the therapeutic efficacy of TCR gene therapy. T cell checkpoint blockade, for instance in the form of CTLA-4 or PD-1 blockade,

has been demonstrated to enhance anti-tumor T cell responses in pre-clinical models[104-106]. Furthermore CTLA-4 blockade has recently been demonstrated to enhance overall survival in a phase III clinical trial in metastatic melanoma patients[1], showing that the efficacy of T cell checkpoint blockade is not restricted to (sometimes contrived) mouse model systems. Which T cell checkpoint molecules would form the most interesting candidate targets in the context of TCR gene therapy? The success of CTLA-4 blockade seems at least in part due to enhanced priming of antigen-specific T cell responses. However, in the setting of TCR gene therapy, the priming phase is really not much of an issue since large numbers of recently activated TCR-modified T cells are transferred into a lymphodepleted host. Because of this, PD-1 blockade, or blockade of other molecules that primarily regulate T cell activity in the effector phase of the anti-tumor response, may form a more attractive approach to enhance the efficacy of TCR gene therapy. A note of caution regarding systemic immune modulation with monoclonal antibodies is that it may also lead to an increase in autoimmune side-effects[1]. Therefore, the specific targeting of immune modulation to TCR-modified T cells may prove a preferable approach to systemic immune modulation[107].

| CONCLUSIONS

Much progress has been made in the development of TCR gene therapy in recent years, a fact highlighted by how we have progressed from the first demonstration of the *in vivo* function of TCR modified T cells in mice[11] to the clinical testing of TCR gene therapy in cancer patients[23,24,31] in less than a decade. However, as discussed in this review there are a number of issues that need to be addressed if TCR gene therapy is to realize its considerable promise. Two chief issues among these are to identify which tumor antigens can be effectively and safely targeted with TCR gene therapy, and to move to clinical trials in which not only T cell specificity but also T cell functionality is manipulated. The latter may be achieved either by changing the environment in which the cells reside or through genetic engineering of the cells themselves (**Fig. 3**).

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