RECENT PROGRESS AND FUTURE DIRECTIONS USING ENGINEERED T CELLS FOR THE TREATMENT OF CANCER*

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Received 22 June 2010

Accepted 5 July 2010

Adoptive immunotherapy with tumor infiltrating lymphocytes (TILs) is a potent therapy for metastatic melanoma. In this process, naturally occurring tumor reactive TILs that bear T-cell receptors (TCR) targeted against tumor cells are generated *ex vivo* and administrated into the patients. The generation of tumor-reactive T cells is not always possible in all of the patients. To overcome this limitation, we can now insert highly avid TCRs into T cells that can recognize tumor antigens. Genetic engineering of TCR genes into normal T cells is a powerful new strategy to generate large numbers of defined antigen-specific cells for therapeutic application. This approach has evolved beyond experimental stage into a clinical reality. The feasibility of TCR-engineered T cells has been shown to be an effective clinical strategy resulting in the regression of established tumors in recent clinical trials. In this review, we discuss the progress and prospects of TCR-engineered T cells as a therapeutic strategy for treating patients with melanoma and other cancers.

Keywords: T cell; T-cell receptor; tumor antigen; adoptive immunotherapy; melanoma; gene therapy.

Introduction

Stage IV metastatic melanoma is mostly incurable with median survival period of 8 to 9 months and a 3-year survival rate of only 10 to 15% (Balch *et al.*, 2001). Metastatic melanoma is generally resistant to both chemotherapy and radiotherapy. Currently there are two US FDA approved therapies for patients with metastatic melanoma, Interleukin-2 and dacarbazine resulting in objective response rates of around 15% (Tsao *et al.*, 2004). Spontaneous regression of melanoma has been observed in some cases for many years suggesting the immunogenic nature of this tumor in comparison with other tumors (Chong *et al.*, 1989; King *et al.*, 2001).

*Invited review article.

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The possibility that we might be able to use this immunogenicity as a foundation for the development of an alternative therapy has generated interest in the use of immunotherapy for the treatment of melanoma. In addition, the identification of a large number of tumor-associated antigens opened up the possibility of specifically targeting candidate tumor antigens to selectively eliminate tumor cells by immunotherapy (Rosenberg, 1999).

Current immune-based therapies can be classified into three main categories: (1) Non-specific immune stimulation, this is mediated by the administration IL-2 or anti-CTLA4 antibody leading to the activation of endogenous tumor-reactive cells *in vivo* and has been shown to cause tumor regression in melanoma and renal cell carcinoma (Sarnaik and Weber, 2009; Rosenberg *et al.*, 1998); (2) Active immunization or vaccination of patients against tumor antigens have yielded some encouraging results, but the regression of solid tumors are rare and reported only in a very small number of patients (Rosenberg *et al.*, 2004); (3) Adoptive cell therapy involving the generation of lymphocytes with anti-tumor activity, which are then infused into cancer patients with appropriate T-cell growth factor IL-2 to support their *in vivo* survival and expansion (Rosenberg and Dudley, 2009).

Adoptive Immunotherapy

Adoptive cellular immunotherapy involves the administration of autologous or allogenic tumor-reactive T cells into patients to achieve tumor regression and has been successful in transplant-related malignancies, leukemia and melanoma. In melanoma, this process involves the identification of lymphocytes with high affinity for tumor antigens that can be selected *in vitro* and expanded and administered to patients. When autologous anti-tumor lymphocyte cultures are administered to patients with high-dose interleukin IL-2 following a lymphodepleting-conditioning regimen, the cells can expand *in vivo*, traffic to tumor, and mediate tumor regression resulting in durable objective clinical responses. The administration of naturally occurring TILs has been shown to have an objective response rate ranging from 50–70% in metastatic melanoma patients, including bulky invasive tumors at multiple sites including liver, lung, soft tissue and brain (Dudley *et al.*, 2008; Rosenberg *et al.*, 2008). To be a candidate for TIL therapy, a patient must meet several requirements. Tumor must be accessible for surgical resection or biopsy and viable tumor-reactive TILs must be generated from the tumor in adequate numbers. A major limitation to the widespread application of TIL therapy is the difficulty in generating human T cells with anti-tumor reactivity. It has been reported that approximately only one half of melanomas reproducibly give rise to anti-tumor TILs (Dudley *et al.*, 2003). As an alternative approach, TCR genes from tumor-reactive T cell clones can be isolated and transferred to normal T cells. TCR gene transfer would allow the generation of large numbers of defined antigen-specific T cells for therapeutic application. Therefore, TCR-based immunotherapy has the advantage that we can administer very large numbers of genetically engineered T cells with

high avidity for tumor antigens. Ultimately it may be possible to establish a collection of defined TCR genes that could be used to treat a diverse set of patients' cancer tailored to each patient depending on the expression pattern of tumor antigens.

TCR as a Therapeutic Tool

The success of TIL therapy for the treatment of metastatic melanoma formed a strong foundation for TCR-engineered T cells. The first step in TCR gene therapy is to isolate a T-cell clone with high affinity for a defined target antigen. Genes encoding the TCR can be isolated from patients with rare reactive high-avidity T-cell clones that recognize and lyse target tumor cells. The TCR α and β chains are identified, isolated and cloned into either retroviral or lentiviral vectors using recombinant DNA techniques (Fig. 1). The genetic transfer of TCR α and β chains directed against specific tumor antigens can create generic antigen-specific T-cells. The first successful transfer of a melanoma-reactive TCR to human peripheral blood lymphocytes (PBLs) conferring anti-tumor reactivity was reported in 1999 (Clay *et al.*, 1999). Since then several reports demonstrated that transfer of a tumor antigen-specific TCR into T cells results in an antigen-specific T-cell population (Schaft *et al.*, 2003; Morgan *et al.*, 2003; Zhao *et al.*, 2005). This approach bypasses the need to isolate the tumor-specific effector cells from each patient. TCR-engineered T cells upon encounter with antigen-positive tumor cells secrete immunostimulatory cytokines, exert antigen-specific cytotoxicity and are able to expand in response to the antigen stimulation. It has been shown that the specificity of the TCR-transduced T cells is not altered compared to the parental T-cell clone (Morgan *et al.*, 2003). Recent advances in gene transfer technology opened up the possibilities of creating very large numbers of genetically modified lymphocytes with appropriate therapeutic properties (Schaft *et al.*, 2003; Morgan *et al.*, 2003 and 2006). High-affinity T-cell receptors can be introduced into normal T cells of cancer patients and the adoptive transfer of these cells into the lymphodepleted patients has been shown to mediate cancer regression (Johnson *et al.*, 2009; Morgan *et al.*, 2006). TCR-engineered T cells complement the clinical success of adoptive cell transfer therapy of TILs by offering the benefit of this therapy to patients who fail to generate TILs.

Choice of Tumor Antigen for TCR Therapy

In the past two decades, fundamental advances in immunology and tumor biology combined with the identification of a large number of tumor antigens have lead to significant progress in the field of cell-based immunotherapy (Blattman and Greenberg, 2004; Rosenberg, 1999). The majority of human tumor-associated antigens are non-mutated self-antigens shared between individuals (Rosenberg, 2001). Many antigens recognized by autologous T lymphocytes have been identified on human melanoma (Romero *et al.*, 2006). The first step in TCR-based therapy is the choice of appropriate melanoma tumor-antigens to target the T cells. The TCR

Fig. 1. TCR Gene Therapy. Shown are the steps involved in the development of TCR gene therapy. Starting with a tumor-reactive T cell, the TCR alpha and beta genes are cloned. These genes are then inserted into a retroviral vector using either an internal ribosome entry site (IRES) or a picornavirus 2A ribosomal skip peptide (2A) in order to synthesize both TCR chains. Retroviral vector supernatant can then be produced and used to transduce primary human T cells, which results in the expression of the new TCR gene in the T cell.

should target a protein that is widely and selectively expressed in tumor cells, and the TCR should have high affinity for its antigen, and be strongly expressed in T cells. Several TCRs have been identified targeting a variety of melanocyte differentiation antigens like gp100 (Morgan *et al.*, 2003), MART-1 (Hughes *et al.*, 2005) and tyrosinase (Roszkowski *et al.*, 2005; Frankel *et al.*, 2010).

In addition to targeting melanocyte differentiation antigens, many investigators have focused on cancer testis (CT) antigens. More than 110 CT antigens have been identified that are normally expressed only in the human germ line, but are also expressed in various tumor types, including melanoma, and carcinomas of the bladder, lung and liver (Suri, 2006; Simpson *et al.*, 2005). These immunogenic proteins are being vigorously pursued as targets for therapeutic cancer vaccines and TCR-based adoptive immunotherapy. CT antigen NY-ESO-1 has been chosen as the initial target for TCR-based therapy at the NCI (Zhao *et al.*, 2005; Robbins *et al.*, 2008), and the preliminary results are encouraging. Significant efforts are underway to identify TCRs for a large number of other cancer testis antigens that are expressed in melanomas. Selection of appropriate target tumor-antigens is crucial for ultimate long-term tumor regression and to reduce the severity of associated toxicity to the normal tissue. Targeting T cells against tumor-associated CT antigens might selectively eliminate tumor cells and avoid or reduce toxicity to normal tissue.

Challenges Facing TCR Gene Therapy

Recent TCR-based clinical trials in melanoma patients have highlighted the promise as well as the challenges facing this therapy. Various factors will contribute towards the clinical efficacy of TCR gene therapy including, the affinity of the transgenic TCR, the maintenance of the TCR gene expression over time, and the *in vivo* persistence of the TCR-engineered T cells. Significant progress has been made towards improving the clinical-scale transduction and expression of TCRs on T cells. In this review, we will discuss recent advances towards these hurdles and the issues that remain to be resolved (Table 1).

Engineering to Improve TCR Activity

TCRs used for engineering T cells for clinical application have to exhibit high affinity for their specific peptide-HLA complex, as the transgenic TCR has to compete for cell-surface expression with the endogenous TCR. Numerous strategies have been developed to generate high-affinity TCRs directed against self-antigens. One of the anticipated potential problems in ectopic expression of tumor-specific TCRs is the formation of mixed dimers between endogenous and introduced TCR chains. However no evidence of toxicity or autoimmunity was observed in the clinical trials. Several strategies have been developed to prevent mixed dimer formation while increasing TCR expression. Development of murine-human hybrid TCRs are reported in which constant region of human receptors are replaced by the murine counterparts. Hybrid TCRs exhibited enhanced expression leading to superior function of the engineered T cells including high levels of cytokine release and cytolytic activity (Cohen *et al.*, 2006). In another approach, introduction of an additional cysteine residue at the constant region of the TCR α and β was used to increase the pairing of TCR chains (Cohen *et al.*, 2007; Kuball *et al.*, 2007). Single or dual

Table 1. Potential Hurdles Facing the Success of TCR Gene Therapy.

Potential Problem	Possible Solution
T cells	
Transduced T cells fail to expand <i>in vivo</i>	<ul style="list-style-type: none">• Vaccination after adoptive cell transfer• Lymphodepletion of the host• Cytokine administration• Cytokine engineering (Hsu <i>et al.</i>, 2007; Liu and Rosenberg 2001)
Loss of transferred T cells	<ul style="list-style-type: none">• Anti-apoptotic gene transfer (Charo <i>et al.</i>, 2005)
Selection of ideal T cell population for transduction	<ul style="list-style-type: none">• Introduction of TCR into T_{CM} cells for long-term survival and expansion <i>in vivo</i> (Gattinoni <i>et al.</i>, 2005a)
T cell trafficking to tumor site	<ul style="list-style-type: none">• Select highly expressed tumor antigen• Induction of tumor antigen expression (Wargo <i>et al.</i>, 2009)
TCR	
Low affinity TCR	<ul style="list-style-type: none">• Isolate high affinity TCR (Johnson <i>et al.</i>, 2006 and 2009)• Isolate TCR from the non-tolerant allogenic T cell repertoire (Wilde <i>et al.</i>, 2009)• Modify TCR by genetic engineering (Robbins <i>et al.</i>, 2008)• Use transgenic mouse to isolate high affinity TCR (Johnson <i>et al.</i>, 2009; Parkhurst <i>et al.</i>, 2009)
Sub-optimal TCR expression	<ul style="list-style-type: none">• Additional cysteine residues at the constant region of the TCR α and β chains (Cohen <i>et al.</i>, 2007)• Removing N-glycosylation residues in the TCR (Kuball <i>et al.</i>, 2009)• Codon optimization of the TCR to increase expression (Scholten <i>et al.</i>, 2006)• Chimeric TCR with human/mouse TCR α and β constant region (Cohen <i>et al.</i>, 2006)• Improved vector design (Wargo <i>et al.</i>, 2009; Yang <i>et al.</i>, 2008)
Host factors	
T cell suppression by TGF-beta	<ul style="list-style-type: none">• Expression of dominant negative TGF receptor on T cells (Gorelik and Flavell, 2001)
Cell-mediated immune suppression	<ul style="list-style-type: none">• Lymphodepletion conditioning-radiation or chemotherapy
e.g. T-reg, myeloid-derived suppressor cells	<ul style="list-style-type: none">• Depletion by antibodies
Low level of tumor antigen expression	<ul style="list-style-type: none">• Induction of tumor antigen expression (Wargo <i>et al.</i>, 2009)• Local irradiation
Toxicity to normal tissue	<ul style="list-style-type: none">• Targeting tumor-specific antigens e.g. cancer testis antigens (Zhao <i>et al.</i>, 2005)
Negative regulatory receptors e.g. CTLA-4, PD-L1	<ul style="list-style-type: none">• Blocking by antibody or gene silencing by RNA interference
Loss of antigen expression or tumor escape	<ul style="list-style-type: none">• Employ multiple TCRs against a variety of tumor antigens

amino acid substitutions in the CDR α or β chain has been shown to provide modest increase in TCR affinity that can enhance the antigen-specific reactivity in T cells (Robbins *et al.*, 2008). Removal of defined N-glycosylation motifs in the constant domains of TCR chains resulted in increased functional avidity and enhanced recognition of tumor cells (Kuball *et al.*, 2009). Some reports described improved expression of TCRs on the cell surface by codon optimization. In this approach, rare codons used by the wild type or murine-derived TCR are replaced by those codons most frequently distributed in highly expressed human genes. During the optimization process, cis-acting AT- or GC-rich sequence stretches, cryptic splicing and RNA instability motifs are removed (Scholten *et al.*, 2006).

The possibility of using HLA-transgenic mice to generate TCRs against human tumor antigens offers a valuable strategy to overcome the tolerance of patients to self-tumor antigens and thus allows us to generate high avidity TCRs. High affinity TCRs can be obtained by immunization of transgenic mice expressing human HLA with human tumor antigenic peptides. Murine cytotoxic T lymphocytes (CTLs) generated by this method are used to isolate murine TCRs specific for HLA-restricted human peptides. This strategy has been shown feasible for peptides from the human gp100 (Johnson *et al.*, 2009), p. 53 (Cohen *et al.*, 2005), CEA (Parkhurst *et al.*, 2009) and MAGE-A3. Expression of mouse TCRs in human T cells could generate an immune response against the transgene resulting in the rejection of transduced T cells, but we have not observed such an event in humans.

TCR Delivery

Majority of TCR transfer studies reported have employed murine leukemia virus-based gene delivery systems. Although these vectors are preferred system for gene delivery to date in the clinical trials, they have limitations. Lentiviral vectors are being developed to engineer human T cells with TCRs for tumor immunotherapy (Yang *et al.*, 2008; Tsuji *et al.*, 2005). Lentiviral systems may have the advantage of their ability to transduce the T cells in the absence of TCR-mediated activation. This may be useful in keeping the transduced cell population in a less differentiated state that is superior for adoptive immunotherapy (Gattinoni *et al.*, 2006). It is also reported that lentiviral vectors are less prone to insertional mutagenesis due to their random integration unlike gamma retroviral vectors that have preferential integration at the transcription start sites of the genes (Wu *et al.*, 2003).

TCR α and β chains are assembled in different configurations in retroviral vectors either alone or together. Initial bicistronic vectors were generated with dual promoters or linked with IRES sequences (Morgan *et al.*, 2003). The gene inserted downstream of the IRES is expressed poorly compared to the gene positioned upstream (Mizuguchi *et al.*, 2000). This might lead to sub-optimal level of expression of TCR and poor T cell response. More recent vectors are constructed with the virus-derived 2A cleavage peptide element that allows optimal stoichiometric expression of both α and β chains (Yang *et al.*, 2008; Johnson *et al.*, 2009).

Clinical Trials

The first human clinical trial of melanoma TCR gene therapy was reported in 2006 from our group (Morgan *et al.*, 2006). In this phase I trial, HLA-A2-positive metastatic melanoma patients were treated with retrovirally-transduced autologous PBLs expressing a TCR against MART-1. The TCR was cloned from the TILs of a resected melanoma lesion that recognized the MART-1: 27–35 epitope. Gene transfer efficiency in this trial was in the range of 21–72% as assessed by the staining for the Vb12 protein in this TCR. Gene transfer was achieved equally between CD4 and CD8 cells. Gene-modified T cells were detected as long as 1 year after adoptive cell transfer. Following T cell infusion, tumor regression was observed in 2 out of 17 patients. In addition to the initial report, a total of 31 patients were treated in this protocol and 4 patients (13%) experienced an objective regression of metastatic melanoma. Response rate in this first human TCR clinical trial is less than that of the TIL trials that are reported to be in the range of 50–70%. However, this trial provided the first proof of principle for the novel genetic immunotherapy involving TCR-modified T cells.

In an effort to improve the efficacy of TCR-based therapy, a high-avidity TCR that recognized the MART-1: 27–35 epitope was generated (Johnson *et al.*, 2009). It was anticipated that a more highly reactive TCR might yield more effective tumor response in patients. In this second trial, objective cancer regression was observed in 30% of patients. However, patients exhibited destruction of normal melanocytes in the skin, eye, and ear. Local steroid administration was employed to treat hearing loss and uveitis. This trial revealed that T cells expressing highly reactive TCRs mediate cancer regression and also target cognate-antigen-expressing cells throughout the body. In another trial, a highly reactive TCR against the human melanocyte differentiation antigen gp100: 154–162 epitope raised by immunizing HLA-A0201 transgenic mice was used to treat melanoma patients (Johnson *et al.*, 2009). Sixteen melanoma patients were treated in this protocol. Cells expressing the murine TCR in this trial persisted at similar levels as the cells expressing the human TCR against MART-1 and mediated tumor regression (Johnson *et al.*, 2009). An objective clinical response rate of 19% was reported in this trial.

The transfer of T cells engineered with TCRs directed against melanoma differentiation antigens MART-1 and gp100 resulted in a 13–30% objective response rate, which was lower than the 50–70% rate seen in TIL therapy. Several reasons could be attributed to this lower response rates including the fact that shared melanoma differentiation proteins may not be the ideal target antigens. The phenotype of the PBLs used to engineer TCR may be different from that of the TILs in terms of the expression of homing and other molecules necessary for the efficient trafficking and effector function of the T cells. Alternatively the immune response by the TILs against the tumor may be of polyclonal nature targeting a variety of normal as well as mutated tumor antigens.

Overcoming Tumor Microenvironment Inhibition of T Cell Function

Highly effective T cells can be generated against the desired tumor antigens and yet have no impact at all on tumor growth. Both the T cell phenotype used for TCR gene modification and the condition these cells encounter at the tumor site can be major determining factors on the outcome of the therapy. It has been shown in some cases that normal tumor growth proceeds despite of having as much as 30% of all circulating CD8 cells with anti-tumor reactivity (Rosenberg *et al.*, 2005 and 2008). T cells in the tumor microenvironment can be suppressed, whereas similar cells at other sites or peripheral blood can exhibit profound effector function (Bai *et al.*, 2008). The mechanism of the local suppression of effector T cell function at the tumor is not completely understood. Several factors responsible for this phenomenon are proposed, including the local presence of inhibitory cytokines such as IL-10 (O'Garra *et al.*, 2008), TGF- β (Wrzesinski *et al.*, 2007), the presence of other cell types capable of actively suppressing immune reactions such as CD4⁺ regulatory T cells (Colombo and Piconese, 2007) or the stimulation of inhibitory molecules on T cells such as PD-1 (Barber *et al.*, 2006; Ahmadzadeh *et al.*, 2009) and CTLA-4 (Sarnaik and Weber, 2009). Strategies to address some of these issues are already incorporated in the current clinical trials involving TCR-modified T cells, for example, lymphodepletion of the host increases the effectiveness of cell transfer therapy by eliminating cellular elements like regulatory T cells and myeloid-derived suppressor cells (Gattinoni *et al.*, 2005b) and also by removing the lymphocyte pool in the body that might compete for the available growth factors.

The programmed death 1 receptor (PD-1) is an inhibitory receptor that is expressed by chronically activated CD4 and CD8 T cells (Sharpe *et al.*, 2007; Greenwald *et al.*, 2005). PD-1 is expressed by a larger number of T cells infiltrating metastatic melanoma lesions than T cells in normal tissues and peripheral blood in melanoma patients. The PD-1⁺ TILs exhibit an exhausted phenotype with impaired effector function compared with PD-1⁻ TILs and PBLs (Ahmadzadeh *et al.*, 2009). This observation indicates that the PD-1 pathway has a suppressive role on T-cell effector function. To overcome this suppression, current TCR-engineered T cells could be improved by incorporating potential anti-PD-1 strategies like inhibitory RNA to suppress the expression of PD-1. Similar strategies to prevent the expression of CTLA-4 can potentially enhance the activity of transferred cells *in vivo*. T cells can undergo apoptosis after infusion due to the lack of growth factors or co-stimulation. Survival of tumor-specific T cells may be enhanced by engineering them to co-express anti-apoptotic genes like *Bcl-2* (Charo *et al.*, 2005).

Concluding Remarks

Within a decade of the first demonstration of TCR gene transfer into T cells, significant progress has been made leading to a small number of initial clinical trials to treat melanoma. With these encouraging results, several efforts are underway to

advance this therapy to identify TCRs to treat a variety of cancers. It is likely that progress in this field will evolve rapidly and that TCR gene therapy will become a reality for the treatment of many diverse human malignancies.

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