

## REVIEW ARTICLE

# Adoptive immunotherapy for cancer: the next generation of gene-engineered immune cells

L. J. Berry<sup>1\*</sup>, M. Moeller<sup>1\*</sup> & P. K. Darcy<sup>1,2</sup>

1 Cancer Immunology Program, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia

2 Department of Pathology, University of Melbourne, Melbourne, Victoria, Australia

## Key words

cancer; effector cells; genetic modification; single-chain variable fragment receptor; T cell receptor; transduction

## Correspondence

Dr Phillip K. Darcy  
Cancer Immunology Research  
Peter MacCallum Cancer Centre  
Locked Bag 1 A'Beckett St  
Melbourne, VIC 8006  
Australia  
Tel: +61 3 9656 3749  
Fax: +61 3 9656 1411  
e-mail: phil.darcy@petermac.org

Received 1 July 2009; accepted 1 July 2009

doi:10.1111/j.1399-0039.2009.01336.x

## Abstract

Adoptive cellular immunotherapy involving transfer of tumor-reactive T cells has shown some notable antitumor responses in a minority of cancer patients. In particular, transfer of tumor-infiltrating lymphocytes has resulted in long-term objective responses in patients with advanced melanoma. However, the inability to isolate sufficient numbers of tumor-specific T cells from most malignancies has restricted the broad utility of this approach. An emerging approach to circumvent this limitation involves the genetic modification of effector cells with T cell receptor (TCR) transgenes or chimeric single-chain variable fragment (scFv) receptors that can specifically redirect T cells to tumor. There has been much progress in the design of TCR and scFv receptors to enhance the antigen-specific activation of effector cells and their trafficking and persistence *in vivo*. Considerable effort has been directed toward improving the safety of this approach and reducing the immunogenicity of the receptor. This review discusses the latest developments in the field of adoptive immunotherapy using genetically modified immune cells that have been transduced with either TCR or scFv receptor transgenes and used in preclinical and clinical settings as anticancer agents.

## Introduction: adoptive cellular immunotherapy

Cellular immunotherapies for the treatment of cancer have used both active and passive approaches such as vaccines, tumor-specific antibodies or adoptive transfer of tumor-specific T cells. Tumor vaccines aimed at actively stimulating a patient's adaptive immune system have been developed as both preventative and therapeutic anticancer strategies. Current vaccination approaches in patients have used vaccines comprising peptide antigen, whole tumor cells, dendritic cells, viral and DNA vectors or idio-type vaccines in combination with immunostimulatory adjuvants (1). In general, these vaccines have been relatively successful in animals; however, these results have not translated into human trials (2). Given the poor results of vaccines in cancer patients to date, in particular, patients with established disease, other cellular therapies have emerged. One such approach includes adoptive immunotherapy, which involves *ex vivo* manipulation and expansion of autologous T cells, followed by their re-infusion into tumor-bearing hosts (2).

\*These authors contributed equally to this manuscript

The *ex vivo* expansion of lymphokine-activated killer (LAK) cells or tumor-infiltrating lymphocytes (TIL) has achieved some remarkable response rates in cancer patients. Up to 70% objective response rates have recently been reported observed in patients with advanced melanoma following transfer of interleukin (IL)-2-activated TIL cells combined with lymphodepletion (3). However, despite encouraging responses in patients with melanoma, response rates for other common cancers including breast, prostate and ovarian have remained low. This is in part because of the difficulty in isolating and expanding endogenous tumor-reactive cells from these tumor types, poor persistence of T cells following transfer and the presence of immunosuppressive factors in the tumor microenvironment (4, 5). To overcome these limitations and broaden the therapeutic scope of immune-based strategies, the genetic modification of T cells has been explored as an alternative approach. The focus of this review will be to outline current understanding and developments in the field of adoptive immunotherapy using genetically modified T lymphocytes and other immune cell subsets.

### Redirection of T lymphocytes by genetic modification

Genetic engineering of patient T cells offers a means to potentially enhance the cytotoxic, tumor-targeting properties of naturally occurring T cells while overcoming the reliance on components of the endogenous immune system that restrict current active immunization strategies. The development of viral transduction methodology to stably express T cell receptor (TCR) transgenes or chimeric single-chain variable fragment (scFv) receptors on the surface of T cells has significantly expanded the targeting capacity of T cells. Numerous genetic approaches are currently focusing on enhancing the tumor recognition, efficacy, persistence and trafficking of genetically modified T cells to give rise to T cells potent against a range of cancers. However, as these cells acquire enhanced functional capacities, the new challenge faced by tumor immunologists is to find the optimal balance between evoking antitumor responses while controlling potential autoimmune pathology.

### Genetic modification using TCR genes

TCR genes isolated from antigen-specific tumor-reactive T cells can be exploited as therapeutic molecules by transfer of genes encoding the TCR- $\alpha$  and - $\beta$  chains from a donor T cell to a recipient T cell of any specificity. Retroviral vectors containing TCR- $\alpha$  and - $\beta$  genes have been extensively studied in experimental mouse models and showed to be safe, feasible and capable of mediating tumor regression in patients (3). However, the ability to isolate endogenous, high-affinity T cells specific to tumor antigens is limited to a minority of malignancies. Therefore, an alternative approach for isolating tumor-specific TCR genes has involved the use of mice expressing human major histocompatibility complex (MHC) molecules capable of presenting tumor antigens to the murine immune system that recognizes them as foreign. Success of this strategy would require the murine TCR genes to have some level of 'humanization' prior to use in human patients to prevent immunogenicity against the mouse transgenes. Encouragingly, such progress has been achieved for several TCR genes (6). Nevertheless, a major problem associated with TCR transgene modification of T cells is the potential for the transgenes to mispair with endogenous TCR- $\alpha/\beta$  chains, which would result in the development of TCR's with undefined specificity. Several approaches have been developed to overcome this problem that include murinization of TCR genes, structural changes to the TCR genes such as codon optimization, introduction of an additional cysteine pair into the constant region of the TCR and restriction of TCR- $\alpha/\beta$  transgene transduction to oligoclonal or  $\gamma\delta$  T cells (7). Nevertheless, given the potential for TCR transgenes to mispair with endogenous TCR, and the potential for downregulation of MHC-peptide antigen complexes on tumors, alternative gene-engineering strategies using scFv receptors are currently under investigation.

### Redirection of T cells by chimeric single-chain receptors

Initial approaches to generate chimeric receptors capable of mimicking the signaling power of the TCR involved the fusion of immunoglobulin-derived variable domains with the separate constant regions of the TCR- $\alpha$  and - $\beta$  chains, thus conferring antibody-type specificity. Such 'T-body' receptors have been shown to signal appropriately for T cell activation and release of cytokines and cytolytic molecules that result in tumor cell lysis following receptor ligation (8). However, the difficulty associated with transduction of two separate  $\alpha$ - and  $\beta$ -chain transgenes led to the development of chimeric scFv receptors. First-generation scFv chimeric receptors comprised an extracellular antigen recognition domain of a single-chain antibody fused via a hinge region to a transmembrane and cytoplasmic signaling domain containing either TCR- $\zeta$  or Fc $\epsilon$ RI- $\gamma$ . As the recognition domain of the receptor is usually derived from a mouse monoclonal antibody, antigen recognition is not MHC dependent, unlike the physiological TCR, but rather directed to native cell surface molecules. Among the first tumor-associated antigens (TAAs) to be targeted with the scFv approach was erbB2 (HER2/neu), a proto-oncogene product of the epidermal growth factor receptor family that is upregulated on a number of cancers including breast and ovarian carcinomas and associated with poor prognosis. A significant advantage of the scFv-modified T cell approach relative to TCR transgene modification is its versatility to be adapted to targets of various classes including glycolipids and carbohydrates that have a lower frequency of mutation compared with antigen of protein origin. Carbohydrate residues also present as effective immunotherapeutic antigens because of their aberrantly high expression on tumors (9). One carbohydrate antigen that has generated much attention is Lewis-Y ( $Le^Y$ ), a carbohydrate residue that is expressed on a large proportion of small-lung cancers and carcinomas of the ovary and breast. Recent studies in our laboratory have shown the ability of primary human T cells transduced with an anti- $Le^Y$  chimeric scFv receptor to specifically delay the growth of a  $Le^Y$ -positive human ovarian carcinoma cells *in vivo* and that these gene-modified T cells were not inhibited by soluble  $Le^Y$  antigen present in patient serum (10). Over the past decade, single-chain antibody receptors targeting a wide range of TAAs have been developed, enabling the scFv approach to be applied to the treatment of several types of malignancies (Table 1).

Although scFv chimeric receptors can be targeted to a broad range of antigens on tumors, the monospecific nature of scFv receptors restricts their targeting to a single antigen. As such, heterogeneous expression of antigen targets on a tumor creates a potential problem. Therefore, constructs targeting two or more antigens have been generated. A bispecific chimeric scFv receptor comprising an extracellular binding domain of two fused scFv antibody fragments showed reactivity against both carcinoembryonic antigen (CEA) and mucin pan-adenocarcinoma tumor antigen (TAG-72). The coupling of the bispecific scFv receptor to a TCR- $\zeta$  signaling domain resulted in specific lysis of target cells expressing

**Table 1** Redirection of T cells to tumor by chimeric scFv receptors

Cancer type	Target antigen	Chimeric receptor	Effector cell	Study type	References
B cell	CD19	scFv- $\zeta$	Human	<i>In vivo</i>	(11)
		scFv-CD28-CD137- $\zeta$	Human	<i>In vivo</i>	(12)
	CD20	scFv-4-1BB- $\zeta$	Human	<i>In vitro</i>	(13)
		scFv- $\zeta$	Human	<i>In vitro</i>	(14)
		scFv-CD28- $\zeta$	Human	<i>In vitro</i>	(15)
		scFv-SP163-CD28-CD137- $\zeta$	Human	<i>In vivo</i>	(16)
Colon	B lymphoma idiotype	scFv- $\gamma$	Mouse	<i>In vitro</i>	(17)
	CEA	scFv- $\zeta$	Mouse, human	<i>In vivo</i>	(18)
		scFv- $\zeta$	Human	<i>In vitro</i>	(19)
		scFv-CD28- $\zeta$	Mouse, human	<i>In vivo</i>	(20)
		scFv-CD28- $\zeta$	Human	<i>In vitro</i>	(21)
		scFv- $\zeta$	Human	Clinical	(22)
Ovarian	EGP40	scFv- $\gamma$	Human	<i>In vitro</i>	(23)
	FBP	scFv- $\gamma$	Mouse	<i>In vivo</i>	(24)
Breast and associated	erbB2,3,4	scFv- $\gamma$	Human	<i>In vitro</i>	(25)
		scFv- $\zeta$	Mouse, human	<i>In vivo</i>	(26)
		scFv- $\gamma$	Mouse	<i>In vivo</i>	(27)
		scFv-CD28- $\zeta$	Mouse, human	<i>In vivo</i>	(28)
		scFv-CD28- $\zeta$	Human	<i>In vivo</i>	(29)
Prostate	PSMA	heregulin- $\zeta$	Rat, mouse	<i>In vitro</i>	(30,31)
		scFv- $\zeta$	Human	<i>In vivo</i>	(32)
		scFv-CD28- $\zeta$	Human	<i>In vivo</i>	(33)
Adeno-Carcinoma	TAG-72	scFv- $\zeta$	Human	<i>In vivo</i>	(34)
Melanoma	GD3	scFv- $\zeta$	Human	<i>In vitro</i>	(35)
		scFv- $\zeta$	Human	<i>In vitro</i>	(36)
		scFv- $\gamma$	MD45	<i>In vitro</i>	(37)
Many (neovas-culature)	HLA-MAGE-A1	scFv- $\gamma$	Hybridoma	<i>In vitro</i>	(38)
	KDR	scFv- $\gamma$	Human	<i>In vitro</i>	(39)
Neuro-blastoma	VEGFR2	VEGF- $\zeta$	Mouse	<i>In vivo</i>	(40)
Many	GD2	scFv- $\zeta$	Human	<i>In vitro</i>	(41)
		scFv- $\zeta$	Human	<i>In vitro</i>	(42)
		scFv- $\gamma$	Human	<i>In vitro</i>	(43)
Renal cell carcinoma	CA9	scFv- $\gamma$	Human	<i>In vitro</i>	(44)
	CAIX	scFv-CD4- $\gamma$	Human	<i>In vitro</i>	(45)
		scFv-CD4- $\gamma$	Human	Clinical	(45)
		scFv- $\gamma$	Human	<i>In vitro</i>	(46)
Epithelia	Lewis-Y	scFv-CD28- $\zeta$	Human	<i>In vivo</i>	(47)
		scFv- $\gamma$	MD45 hybridoma	<i>In vitro</i>	(48)
Lymphoma	CD30	scFv- $\gamma$	Human	<i>In vitro</i>	(49)
Cervical	CD44 v7/8	scFv- $\zeta$	Mouse	<i>In vivo</i>	(50)
Leukemia	CD33	scFv- $\zeta$	Human	<i>In vitro</i>	(51)
		scFv-CD28- $\zeta$	Human	<i>In vitro</i>	(52)
		scFv-ICOS- $\zeta$	Human	<i>In vitro</i>	(52)
		scFv-4-1BB- $\zeta$	Human	<i>In vitro</i>	(52)
Multiple cancers	8H9	scFv-CD28- $\zeta$	Human	<i>In vivo</i>	(53)
	MUC1	scFv-CD28-OX40- $\zeta$	Human	<i>In vivo</i>	(54)
	Mesothelin	scFv-CD28-CD137- $\zeta$	Human	<i>In vivo</i>	(55)
Rhabdomyosarcomas	(fAChR)	scFv- $\zeta$	Human	<i>In vitro</i>	(56)

CAIX, carboxy-anhydrase-IX; CEA, carcinoembryonic antigen; EGP, epithelia glycoprotein; FBP, folate-binding protein; GD, gangliosides; HMW-MAA, high molecular weight melanoma associated antigen; ICOS, inducible T cell costimulator; KDR, kinase insert domain-containing receptor; PSMA, prostate-specific membrane antigen; scFv, single-chain variable fragment; TAG-72, mucin pan-adenocarcinoma tumor antigen; VEGF, vascular endothelial growth factor.

either antigens *in vitro* (57). This concept now requires further validation in preclinical animal models. Nevertheless, this study highlights the broad potential of the scFv receptor approach. Fundamentally, the spectrum of cell surface

antigens that can be targeted by scFv receptors is limited only by the capacity to produce a corresponding antibody.

The mechanism by which chimeric scFv receptors mediate the destruction of target cells following specific antigen

activation remains to be fully elucidated; however, several studies have shown that different cytolytic pathways may be involved depending on whether T cell lines or primary T cells were used and the type of target cells used. Previous mechanistic studies have shown that murine CD8<sup>+</sup> T cells transduced with a scFv anti-CEA receptor evoked cytolytic activity using a perforin-dependent pathway and were not reliant upon FasL or tumor necrosis factor (TNF) for rejection of colon carcinoma *in vitro* and *in vivo* (58). However, work in our laboratory has shown that the Fas/FasL pathway was important in the lysis of CEA-positive target cells by a MD45 T cell line transduced with an anti-CEA scFv receptor. Importantly, the target cells in the study were Fas sensitive (59). The functional release of cytokines, including IL-2, interferon (IFN)- $\gamma$ , granulocyte-macrophage colony-stimulating factor and TNF- $\alpha$ , in response to activation of scFv receptor-modified cells may also produce direct or indirect antitumor effects (55). Further experiments using human T cells transduced with chimeric scFv receptor constructs may further enhance our understanding of cytolytic pathways involved in target cell destruction of human cancers.

### Application of scFv receptor-modified T cells *in vivo*

The *in vivo* antitumor efficacy of scFv receptor-modified T cells was first evaluated in a Winn-type assay (60). In these studies, erbB2<sup>+</sup> NIH-3T3 tumor cells were combined with either nontransduced or scFv-anti-erbB2(FRP5)- $\zeta$  transduced mouse T cells (C196), prior to subcutaneous injection into athymic nude mice. Mice injected with gene-modified C196 cells showed inhibition of erbB2<sup>+</sup> tumor cell growth for up to 8 days compared with no inhibition of tumor cell growth by non-transduced C196 cells. A similar result was observed in a 4-day established subcutaneous NIH-3T3-erbB2 tumor model in nude mice. In this model, transduced C196 cells were shown to more effectively traffic to the tumor site than non-transduced T cells (60). Together, these results showed that genetic modification of T cells with a scFv-based chimeric receptor could confer antitumor reactivity *in vivo*. Following the optimization of retroviral transduction methods for primary T cells, further studies showed that scFv-anti-erbB2(FRP5)- $\zeta$  transduced primary mouse T cells could also mediate an effective and specific antitumor response *in vivo*. The consecutive intratumoral transfer of these gene-modified T cells for 5 days into BALB/c mice bearing subcutaneous erbB2<sup>+</sup> HC11 mouse mammary epithelial tumor cells resulted in total tumor regression (61). More recently, T cells transduced with scFv chimeric receptors have been shown to mediate antitumor effects in even more stringent mouse tumor models. Primary mouse T cells engrafted with scFv-anti-erbB2 or scFv-anti-CEA receptors have been shown to mediate specific rejection of established metastatic breast carcinoma or subcutaneous colon carcinoma, respectively, when administered intravenously post tumor inoculation (28). An important observation in these studies was the ability of engineered T cells to effectively localize to the site of tumor burden following systemic administration.

In other *in vivo* studies, the antitumor activity of receptor-modified murine TIL cells was evaluated in mouse models bearing either intraperitoneal or pulmonary metastases (62). In one of these studies, nude mice were intraperitoneally implanted with human IGROV-1 ovarian cancer cells and treated 3 days later with scFv-anti-folate-binding protein (FBP)- $\zeta$  transduced TIL cells derived from mouse MC38 colon adenocarcinoma cells. Mice treated with gene-engineered TIL cells showed significantly increased survival (~90 days) compared with mice treated with TIL cells expressing an irrelevant chimeric scFv receptor (~31 days) (62). These results were particularly encouraging given that dissemination of ovarian cancer to the surface of the peritoneal cavity is a likely occurrence in patients with this disease. In the second study, irradiated C57BL/6 mice were given intravenous injections of a murine 24JK sarcoma cell line transduced with the FBP gene (24JK-FBP). After 3 days, mice were treated with scFv-anti-FBP transduced TIL cells, which led to a significant decrease in the number of lung metastases compared with mice that received TIL cells transduced with an irrelevant scFv receptor (62). These studies showed that gene-modified T cells had the capacity to specifically react against tumor *in vivo*; however, high and consecutive doses of IL-2 were required for the therapeutic effect.

Further studies assessing the function of scFv receptor expressing T cells *in vivo* have included chimeric receptors reactive against the metastasis-associated variant of CD44, v6-exon, TAG-72 and vascular endothelial growth factor receptors (VEGFR). One study showed that engineered C196 mouse CD8<sup>+</sup> lymphocytes expressing a scFv-anti-CD44v6- $\zeta$  construct were able to specifically inhibit the growth of rat pancreatic carcinoma (AS14) xenografts in BALB/c nude mice. However, the ability of these receptor-engineered cells to prevent tumor metastases was not reported (63). In another study, human peripheral blood lymphocytes (PBL) expressing a humanized scFv-anti-TAG72(CC49)- $\zeta$  chimeric receptor were shown to be 75%–100% immunoprotective when coadministered subcutaneously with human colon adenocarcinoma cells (LS174T) or intraperitoneally with FasL-positive endometrial carcinoma-derived KLE-B cells into SCID-NOD mice (34). Interestingly, the expression of FasL by tumor cells did not affect the survival or function of engineered T cells *in vivo*. The antitumor activity of primary mouse T cells expressing a chimeric scFv receptor reactive against murine VEGFR Flk-1 has also been assessed *in vivo* (40). The transfer of CD8<sup>+</sup> T cells engineered with a scFv-anti-VEGFR- $\zeta$  receptor into tumor-bearing mice showed reduction in the growth of 7-day established subcutaneous implants of murine adenocarcinoma (CT26) in BALB/c mice, B16.F10 melanoma in C57BL/6 and nude mice, and LS174T human adenocarcinoma in nude mice (40). Importantly, the therapy showed no toxicity despite the fact that Flk-1 is expressed to some degree on normal tissue of the retina, kidney and pancreas. Despite the fact that IL-2 had no independent antitumor effect in these models, its coadministration with engineered T cells was critical to generate a significant therapeutic effect. In addition, response rates of greater than 70% tumor growth inhibition

were only achieved following multiple doses of engineered T cells.

The *in vivo* antitumor responses reported by gene-modified effector cells in these studies have proved promising; however, the effective treatment of large established tumors by engineered effector cells still remains a major therapeutic hurdle. To address this limitation and improve the effectiveness of the chimeric scFv receptor approach, considerable interest has been shown in the development of chimeric scFv receptors that provide additional stimulatory signals.

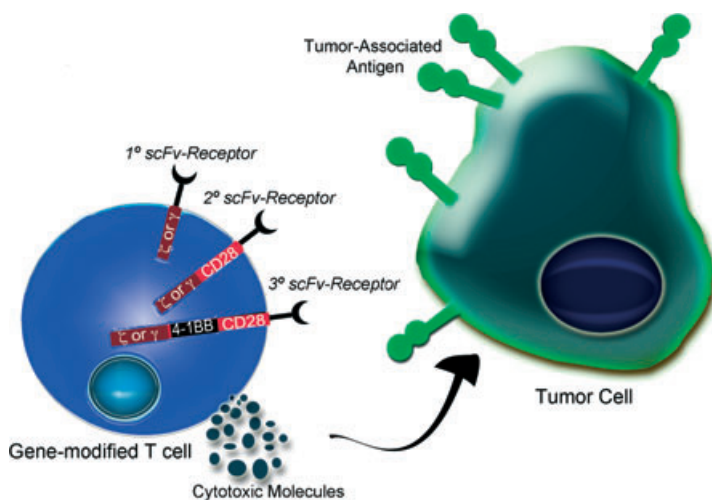
### Enhancing effector cell activity by modification of scFv receptor signaling components

Despite the ability of engineered T cells expressing these first-generation receptors to mediate antitumor activity against early tumor *in vivo*, the response was limited when dealing with established disease. This was thought to be because of the inability of these receptors to induce proliferation of resting T cells and/or trigger the production of optimal amounts of cytokine, thereby resulting in suboptimal T cell activity. Given that under normal circumstances resting T cells require both a primary TCR-derived signal and a secondary costimulatory signal for full activation, chimeric scFv receptors lacking one or both of these signals could result in apoptosis or unresponsiveness of the engineered cell. A number of strategies have been used to bypass this problem including the administration of exogenous IL-2, immunization with tumor cells transfected with B7-1 or B7-2 genes or the addition of CD28 mAbs; however, each of these approaches has had limited success.

An alternative and potentially more therapeutically feasible approach to circumvent the problem of T cell anergy in the absence of costimulation has been to engineer T cells with second-generation chimeric scFv receptors that incorporate a functional antigen-dependent costimulatory signal (Figure 1). A number of molecules with costimulatory activity in T cells have been identified, including CD2, CD4, CD8, CD5 and CD28 (64, 65). The CD28 receptor, in particular, has been

shown to play an important role in the activation of naïve T cells via interaction with members of the B7 family of molecules expressed on the surface of Antigen presenting cells (APCs). As such, the successful generation of a chimeric scFv receptor containing a CD28 intracellular domain, linked in tandem with either the TCR- $\zeta$  or Fc $\epsilon$ RI- $\gamma$  domains, has been a major development in this field and found to be required for the full activation of engineered T cells (66). Adoptive transfer experiments showed that mice administered T cells expressing the scFv-CD28- $\zeta$  receptor showed greater reduction in tumor burden, including established metastatic disease, compared with mice that received T cells expressing the first-generation scFv- $\zeta$  receptor (67).

Several studies have also assessed whether incorporation of other costimulatory domains into the chimeric receptor construct could enhance effector T cell function. Costimulatory domains evaluated have included TNF receptor family members such as 4-1BB, inducible T cell costimulator (ICOS) and OX40 (68). However, when compared *in vitro* with T cells expressing the scFv-CD28- $\zeta$  chimeric receptor, no enhanced effector cell function was reported for chimeras containing these different costimulatory domains (51). Further studies comparing the antitumor activity of T cells engineered with these various receptors needs to be performed *in vivo*. The latest development in the area has involved the functional assessment of third-generation scFv receptors expressing three stimulatory domains linked in series (Figure 1). One study showed that T cells transduced with a scFv-anti-CD19-CD28-4-1BB-TCR $\zeta$  receptor improved the antigen-specific activation, proliferation and cytolytic activity compared with T cells engineered with an anti-CD19-CD28-TCR $\zeta$  receptor (12). Another study by the same group reported enhanced persistence of T cells gene engineered with an anti-mesothelin scFv-CD28-4-1BB-TCR $\zeta$  receptor compared with T cells gene modified with chimeric receptors containing only two signaling domains (55). An additional study has involved incorporation of a src kinase, lck, into the receptor design. This kinase is postulated to promote CD8 or CD4 cross-linking, which in turn, can promote phosphorylation of



**Figure 1** Genetically modified T cells expressing first-, second- and third-generation scFv receptors. The single-chain variable fragment (scFv) of an antibody that specifically binds tumor-associated antigen (TAA) is linked via a hinge region to a transmembrane and cytoplasmic signaling tail. First-generation receptors comprise one signaling domain, such as T cell receptor (TCR)- $\zeta$  or Fc $\epsilon$ RI- $\gamma$ . Second-generation receptors contain two signaling domains such as the costimulatory CD28 domain linked in tandem with the TCR- $\zeta$  domain. Third-generation receptors comprise three stimulatory domains, such as CD28, 4-1BB and TCR- $\zeta$  linked in series. Upon specific recognition of TAA by the chimeric scFv receptor, activation of T cells results in release of cytotoxic molecules and destruction of tumor target cells.

immunoreceptor tyrosine-based activation motifs within the chimeric receptor, thus increasing chimeric receptor potency. Interestingly, T cells expressing the scFv-CD28-TCR $\zeta$ -lck receptor showed increased IL-2 production following stimulation *in vitro* (69). Thus, design of optimal scFv receptor constructs comprising multiple costimulatory domains remains to be fully elucidated in preclinical animal models.

### **Enhancing the proliferative capacity, survival, persistence and tumor localization of gene-modified T cells**

For gene-modified T cells, to mount an effective antitumor response, they must not only be able to specifically recognize tumor antigen via their scFv receptor but also have the ability to proliferate following activation, traffic to and persist at the tumor site. For conventional T cells, the proliferative response to tumor is often poor because of low tumor immunogenicity and early deletion of self-reactive T cells from the repertoire. In addition, costimulatory ligands may be absent or down-regulated on a tumor or APC (if not fully activated by poorly immunogenic tumor). The inclusion of a CD28 signaling component in scFv chimeras, as discussed in the previous section, has been shown to initiate a two- to four-fold increase in antigen-specific proliferation (67). However, the levels of scFv receptor-mediated proliferation do not currently match the proliferative responses observed for T cells in response to foreign antigen, but this may be improved with further optimization of intracellular scFv signaling domains.

Another approach to improve the proliferative capacity of engineered T cells involves the generation of dual-specific T cells that recognize both a specific tumor associated antigen (TAA) and a potent immunogen. Such dual-specific T cells have been shown to react to both TAA and immunogens including alloantigen, Epstein–Barr virus, cytomegalovirus and influenza virus (70, 71). *In vivo* expansion of alloreactive T cells modified with a chimeric scFv-anti-FBP receptor has been shown following immunization with allogeneic cells and, importantly, produced antitumor effects following adoptive transfer into mice bearing FBP<sup>+</sup> tumor (72). Thus, dual-specific T cells may enable the generation of an antitumor proliferative response equivalent to the potent response produced to infectious agents. In addition, as the endogenous specificity of dual-specific T cells is already known, there is reduced risk that these cells comprised autoreactive clonotypes and may therefore provide a safer alternative to the use of bulk T cell populations for adoptive immunotherapy strategies.

Several strategies are currently being investigated to enhance the long-term survival and persistence of engineered T cells *in vivo*. These include genetic over-expression of antiapoptotic molecules such as Bcl-2 and Bcl-X<sub>L</sub>, inhibition of molecules involved in the downregulation of immune responses such as PD1 and CTLA-4, and the use of pro-survival cytokines such as IL-2, IL-15 and IL-7 during culture or administration of T cells (68).

To further enhance the antitumor response by gene modification of T cells, investigations in our laboratory and others have shown enhanced therapeutic efficacy when both

CD4<sup>+</sup> and CD8<sup>+</sup> gene-modified T cells were coadministered, in contrast to mice administered with either subtype alone. Notably, the response rates observed correlated with localization and persistence of both gene-engineered CD4<sup>+</sup> and CD8<sup>+</sup> T cells at the tumor site (73). Critical for this effect was the requirement that the transferred CD4<sup>+</sup> T cells were antigen specific. Importantly, mice that survived primary tumor challenge were able to reject subsequent tumor challenge in an antigen-specific manner, showing the long-term persistence of functional engineered cells in these mice. Further analysis of T helper CD4<sup>+</sup> subtypes (Th<sub>1</sub> vs Th<sub>2</sub>) showed that help provided by engineered Th<sub>1</sub> CD4<sup>+</sup> cells, enabled a stronger recall response to rechallenge with antigen expressing tumor cells (73).

Crucial to a successful antitumor response is the ability of sufficient numbers of immune cells to traffic to and penetrate the site(s) of tumor challenge. In an antigen-mediated immune response, pro-inflammatory danger signals at the tumor site induce the upregulation of chemokines and vascular adhesion molecules in tumor tissue through pattern recognition receptors at the surface of dendritic cells and Natural killer (NK) cells. Such danger signals can attract T cells that express the appropriate chemokine receptors and adhesion molecules. The expression of chemokines by tumor cells makes them a plausible target for the redirection of specific T cells to the tumor site; however, tumor-reactive T cells often lack the necessary receptor for chemokines produced by the tumor. T cells engineered to constitutively express the chemokine receptor CXCR2 showed initiation of calcium ion mobilization (important for chemokine receptor signaling), migration toward tumor and secretion of IFN- $\gamma$  in response to the corresponding chemokine ligand CXCL1 *in vitro* (74). Furthermore, *in vivo* analysis is required to validate the potential application of T cells engineered to express chemokine receptors.

### **Genetic engineering of alternative immune cell types**

Genetic engineering of host immune cells is not limited to T cells alone and as such other effector cell types used in redirection strategies using single-chain receptors provide additional avenues for adoptive cellular therapies. NK cells can eradicate tumor or virally infected cells that possess defective, altered or absent MHC class I surface expression. However, the activity of NK cells is often inhibited by expression of ligands binding NK cell inhibitory receptors and/or downregulation of ligands for NK cell activation receptors. To address this, several antitumor scFv constructs have been engineered into NK cells to redirect their lysis toward TAA regardless of MHC expression status. For example, CD56<sup>+</sup>CD3<sup>-</sup> NK cells retrovirally transduced with a scFv- $\zeta$  chimeric receptor redirected against the CD19 molecule could enhance NK cell-specific lysis of leukemic cells. Furthermore, the addition of a 4-1BB costimulatory molecule (CD137) into the chimeric receptor (scFv-anti-CD19-4-1BB- $\zeta$ ) enhanced the activation, cytotoxicity and cytokine secretion of transduced NK cells *in*

*in vitro* compared with NK cells whose chimeric scFv receptor lacked the 4-1BB component (75). Studies undertaken in our laboratory have shown that mouse NK cells engineered to express a scFv-anti-erbB2-CD28- $\zeta$  construct could specifically eradicate tumors in a proportion of mice following adoptive transfer (76). Taken together, these results indicate that utilization of gene-engineered NK cells may broaden the scope of adoptive cellular immunotherapies used either alone or in combination with other immune effector cells.

The specificity of LAK cells can also be altered through the expression of chimeric scFv receptors. One study showed that LAK cells generated from mice transgenic for a scFv- $\zeta$  receptor gene construct could specifically and effectively lyse wild-type LAK cell resistant EL4 tumor targets transfected with the appropriate antigen (77). Transgenic LAK cells were unable to lyse antigen-negative EL4 tumor cells. Hence, this set of experiments showed that conferring LAK cells with an additional non-MHC-restricted specificity might improve the function of these cells for use as an effective anticancer treatment.

Neutrophils are another potential type of effector cell that may be harnessed for genetic modification with chimeric scFv receptors and redirected against disease. Bone marrow-derived neutrophils, however, are terminally differentiated and programmed to die within a few days, thus rendering these cells unfit for transduction. A more efficient method of generating gene-modified neutrophils has been to either expand and differentiate retrovirally transduced CD34<sup>+</sup> progenitor cells *in vitro* using the appropriate cytokines or propagate these cells *in vivo* following transplantation of hematopoietic stem cells (HSCs) into immunodeficient mice (78). Using such approaches, one study showed that neutrophils expressing either a scFv- $\gamma$  or scFv- $\zeta$  chimeric receptor, reactive against gp120, could mediate target-specific cytolysis *in vitro*. Interestingly, neutrophils transduced with the scFv- $\zeta$  construct showed greater cytolytic function than the scFv- $\gamma$  chimera (78).

Macrophages are phagocytic cells whose principal role is to engulf and digest cellular debris and foreign matter. These leukocytes also play a role in antigen processing and presentation, thereby stimulating lymphocytes and other immune cells to respond effectively to pathogen. Macrophages are capable of lysing target cells through the release of reactive oxygen intermediates and production of cytokines including IL-1 $\beta$  and TNF- $\alpha$  following receptor cross-linking. One strategy aimed at redirecting the lytic function of macrophages toward cancer cells involved the expression of a membrane anchored scFv fusion protein, reactive against the breast cancer-associated antigen MUC1. This involved using a recombinant nonreplicative modified vaccine Ankara virus-based gene-transfer vector (79). Macrophages expressing the scFv construct were shown to specifically produce IL-12 and lyse targets following exposure to MUC1-expressing tumor cells, despite the lack of a specific cytoplasmic signaling domain in the chimera. Although the mechanism by which the fusion protein triggered macrophage activity remains unclear, the enhanced binding of macrophages to tumor targets may have facilitated the interaction of other cell surface

receptors that in turn may have contributed to the antitumor activity.

Other studies have evaluated the feasibility of using genetically modified HSCs for human immunodeficiency virus (HIV) or cancer treatment. In two separate studies, mice were depleted of bone marrow and reconstituted with either retrovirally transduced HSC that expressed a chimeric receptor reactive against either gp120 (80) or FBP (24). Interestingly, in both models, T cell independent immunity was observed. Depletion of T cells in mice reconstituted with FBP-reactive HSC did not abrogate tumor inhibition, suggesting that transduced effector cells, other than T cells, could confer effective protection against tumor. This was also consistent with a study by Hege et al., in which transplantation of gene-modified HSC into immunodeficient SCID mice led to protective immunity by circulating myeloid cells and NK cells that expressed high levels of the chimeric receptor (80). Taken together, these results showed the ability of effector populations other than T cells to redirect systemic immunity. The design of chimeric scFv receptor strategies that harness multiple immune cell functions could be used as a progressive treatment of disease either independent of or combined with T cell therapy and other conventional therapies.

### Clinical application of gene-modified T cells

The successful generation and expansion of gene-engineered human T cells *ex vivo* in conjunction with encouraging pre-clinical results in animal models have justified the translation of this approach to the clinic. As the successful transduction of T cells by retroviruses required the cells to be actively cycling, patient T cells have been harvested from small blood donations or leukaphoresis samples and activated in culture using antibodies, lectins and/or IL-2 prior to retroviral transduction (81). The efficiency of this initial protocol has been improved by the use of polycations such as recombinant fibronectin fragments (retrofectin) that enhance virus and T cell interactions (82).

Initial clinical trials evaluated the function of engineered T cells using scFv-CD4- $\zeta$  transduced T cells targeting the HIV gp120 antigen in acquired immunodeficiency disease syndrome patients. A cohort of 24 patients received a single dose of  $2 \times 10^{10}$ – $3 \times 10^{10}$  gene-modified autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The transferred T cells were well tolerated in patients and circulating anti-gp120 CD4<sup>+</sup> and CD8<sup>+</sup> transduced T cells could be detected up to 42 weeks post infusion, with evidence of trafficking to mucosal HIV reservoirs. Gene-modified virus-specific T cells showed sustained cell survival in these patients, independent of exogenous IL-2 administration (83). In a subsequent Phase II trial involving a cohort of 40 patients, some reduction in levels of HIV burden and a trend toward reduced recurrent viremia was observed (84).

Clinical evaluation of human T cells transduced with an anti-MART-1 TCR transgene showed for the first time that gene-modified human T cells could elicit antitumor efficacy in melanoma patients. Two of the fifteen patients administered T cells cultured for only 6–9 days *ex vivo* prior

to re-infusion and together with lymphodepletion exhibited sustained objective responses that correlated with persistence of the gene-engineered T cells (3). Although this response was lower than that observed in previous TIL therapy trials, it provided landmark evidence that engineered T cells could produce antitumor efficacy and that unlike TIL cells, gene-modified T cells have the potential to target a broad range of tumor antigens present on a wide range of tumor types.

Another clinical trial involved the use of autologous T cells retrovirally transduced with an anti-carboxy-anhydrase-IX (CAIX) scFv receptor to patients with renal cell carcinoma (85). In this trial, several patients developed progressive liver toxicity related to increasing doses of gene-modified T cells, thought to be the result of autoimmunity produced against target antigen expressed on normal liver tissues. This indicates that careful choice of antigen for targeting is highly important for future clinical trials. Another recent trial assessed the feasibility of using gene-engineered autologous T cells for the treatment of ovarian cancer. Gene-engineered T cells expressing a scFv-anti-folate- $\gamma$  receptor chimeric receptor in conjunction with high-dose exogenous IL-2 were administered to an initial cohort of eight ovarian cancer patients. In this trial, no reduction in tumor burden was observed and over half the patients experienced toxic side effects, most likely related to IL-2 administration. In addition, despite the detection of gene-modified T cells early after transfer, there was no long-term persistence of transferred cells (52). The inclusion of murine components and lack of a costimulatory domain within the scFv receptor construct in these first-generation scFv-engineered T cells were thought to be responsible for inducing a human anti-mouse antibody effect and suboptimal effector cell activation, respectively, hindering the antitumor response. Despite lack of effective antitumor activity by scFv-transduced T cells in these clinical trials, they have provided important information for future trials. Upcoming trials will test second-generation scFv constructs comprising a costimulatory CD28 signaling domain linked in tandem with TCR- $\zeta$  (scFv-CD28- $\zeta$ ) that has been shown to enhance T cell proliferation and persistence *in vivo* and use a 'humanized' scFv antibody to avoid immunogenicity. Indeed, our group will be shortly commencing a Phase I trial using a fully humanized scFv-anti-Le<sup>Y</sup>-CD28- $\zeta$  receptor for the treatment of multiple myeloma patients.

### **Incorporation of safety measures for gene-engineered cells**

Retroviruses provide an efficient means of transducing cells with TCR or scFv transgenes. However, their use raises concerns regarding the possibility of inducing oncogenesis in the host cells caused by random insertion into the genome. Genomic integration of viral vectors may result in deregulation of growth control in immune cells, which could in turn, lead to lymphoma or leukemia. The introduction of transgenes into HSCs led to leukemia in some patients because of the unintentional activation of oncogenes (86). In addition, leukemia was also reported in mice that had

received mouse HSCs transduced with a nerve growth factor reporter gene (87). These studies highlighted that gene therapies involving the transduction of HSCs need to be treated with caution. Nevertheless, there have been no reported transformation events involving adoptive transfer of gene-modified T cells to date. Alternative means of gene transduction are also currently being investigated including the use of lentiviral vectors(88), nonviral transposon systems (89) and direct RNA electroporation techniques (90).

To safeguard against any adverse events, the introduction of a conditional 'suicide gene' into engineered T cells may selectively eliminate adoptively transferred cells should toxicity occur. Initially, genes derived from pathogens, such as the herpes simplex thymidine kinase (HSVtk) gene, were used. HSVtk has the ability to convert specific nucleoside analogs, including the antiviral drug gancyclovir, into lethal products that induce death of the dividing cell. However, limitations to this approach have been reported from clinical trials where slow dividing engineered T cells expressing the HSVtk were not effectively eliminated. Instead, T cell responses to the HSVtk protein led to selective and rapid deletion of transferred T cells (91). Therefore, other suicide gene strategies have been trialed. One innovative approach has proposed the use of inducible Fas and Caspase 9 as suicide genes. The activation of both these molecules is dependent on a dimerization process, which can be induced by manufactured chemical inducers of dimerization (CID). T cells transduced with a retroviral vector encoding the dimerizable gene can be selectively eliminated following exposure to CID (92). Another approach has investigated the transgene expression of CD20 as a suicide gene for genetically modified T cells. Human T cells transduced with a retroviral vector encoding the human CD20 molecule have been efficiently lysed following administration of a humanized anti-CD20 Ab (Rituximab) and complement (93). These approaches are yet to be trialed in patients.

The foremost type of potential toxicity associated with redirected T cell therapy is damage to normal tissue expressing the same antigen as that targeted by adoptively transferred cells. Depending on the therapy, autoimmunity may be expected but tolerable, as in the transfer of melanoma-reactive T cells that induced vitiligo (94), or potentially dangerous, as in the administration of T cells expressing anti-CAIX chimeras that led to liver toxicity (45). In addition, autoimmunity against other, nontargeted self-antigens could arise from the use of T cell lines rather than T cell clones, especially when an *in vitro* approach that overcomes tolerance and expands rare endogenous autoreactive T cells is used. In order to prevent or limit possible receptor-mediated damage to normal tissue, it is important to determine the factors that influence target cell susceptibility to lysis by redirected effector cells. In experiments evaluating the functional expression of chimeric anti-CEA or anti-erbB2 scFv- $\zeta$  receptors in MD45 hybridoma cells, it was shown that the degree of target cell lysis mediated by transfected MD45 cells correlated with the level of scFv receptor expression. Furthermore, the cytolytic activity mediated by receptor-modified cells was shown to be greater against tumor targets expressing a higher level of antigen (95).

In another study, the level of cytokine secretion by human PBL engineered with a scFv-anti-gangliosides(GD)2- $\zeta$  receptor was shown to correlate with the level of GD2 expressed on the surface of target cells (41). More recently, it was shown that gene-modified T cells targeting the Le<sup>Y</sup> antigen secreted higher levels of IFN- $\gamma$  in response to target cells expressing high levels of Le<sup>Y</sup> antigen. There was negligible response of modified T cells against tumor targets or normal tissue (i.e. neutrophils) expressing low levels of Le<sup>Y</sup> antigen (47). Taken together, these data suggest that the cytotoxic function of redirected T cells can be influenced by the scFv receptor and TAA density on effector and target cells, respectively. In a further study, the antitumor activity of engineered human T cells, expressing either high or low levels of a scFv-anti-G250- $\gamma$  chimeric receptor, was assessed against renal cell carcinoma cell lines expressing varying amounts of G250 antigen (46). A functional and dynamic balance between scFv receptor densities on engineered T cells and TAA density on target cells was found. T cells expressing high-density levels of scFv receptor were triggered by both high-density and low-density TAA-positive target cells, which led to specific lysis and secretion of cytokines. In contrast, low-density scFv receptor expressing T cells were only triggered for cytolysis and cytokine production by high-density TAA-positive target cells (46). Therefore, even though the redirection of engineered effector cells with high receptor densities may prevent the escape of tumor cells expressing low antigen levels, they may also possess a greater ability to mediate damage of normal tissue expressing the same antigen at physiological levels.

### Future perspectives

The utilization of genetically engineered T cells for disease treatment has developed at a rapid pace with therapeutic efficacy now shown against established cancer in patients that have not responded to conventional therapies. Development of modified TCR transgenes to prevent mispairing with endogenous TCR, generation of second- and third-generation scFv receptors and the ability to engineer multiple immune cell subtypes has broadened the scope of adoptive cellular immunotherapy. These advances have enabled enhanced function of T cells outside the tolerizing environment of the host and overcome limitations presented by poorly immunogenic tumors. Nevertheless, such strategies must be pursued with caution and safeguards put in place to ensure any deleterious side effects are minimized or eliminated.

Recent studies have focused on development of scFv receptor constructs that incorporate novel intracellular signaling domains. T cells gene modified with second-generation scFv receptors incorporating the CD28 costimulatory domain linked in tandem with the TCR $\zeta$  signaling domain have shown increased antitumor function *in vitro* and in experimental murine models and are now being clinically translated. It will be interesting to determine whether development of third-generation scFv receptors may further enhance antitumor function of gene-modified T cells and whether they have

any clinical benefit in patients in future trials. The advance in design of TCR transgenes to reduce potential mispairing with endogenous TCR has been a recent focus from several laboratories. It remains to be determined whether these modifications will translate into better outcomes for patients. Other important developments include the isolation of TCR's recognizing antigen with higher affinity and achieving high level stable expression of these transgenes in effector T cells.

There are a number of new strategies that could be used to increase the efficacy of gene-engineered T cells. To enhance antitumor responses of gene-engineered T cells, it is important that these cells expand, traffic to, persist and function at the tumor site. Studies performed in experimental mouse models and in patients have clearly shown that the environment into which T cells are transferred into can have a significant impact on cell survival and therapeutic efficacy. Lymphodepletion in mouse models and in patients prior to adoptive transfer can improve the antitumor efficacy of transferred cells. It is thought that this may be through the elimination of suppressive cells such as T regulatory cells, the removal of endogenous cells that compete for activating cytokines and/or the increased function and availability of antigen presenting cells (96). Increasing lymphodepletion with total body irradiation, together with the combined transfer of CD34<sup>+</sup> cells and TIL cells, has dramatically improved antitumor responses (97). It will be interesting to see whether this type of regimen can similarly enhance antitumor effects following transfer of gene-engineered T cells into patients.

Current immunotherapy regimens involving TIL cells have used IL-2 to expand and activate cells *in vitro* and *in vivo*. However, IL-2 can induce toxicity in patients. In future studies involving gene-engineered T cells, it will be interesting to determine whether other cytokines such as IL-7, IL-15 and IL-21 may enable greater expansion and functional activity of gene-modified T cells while reducing potential toxicity. For example, priming T cells in the presence of IL-21 has led to increased antitumor effects *in vivo* following adoptive transfer compared with cells primed with IL-2 and IL-15 (98).

A potential problem with transfer of retrovirally transduced effector cells is for these cells to undergo transformation and possibly react against normal tissue expressing target antigen. Encouragingly, there have been no reports of transformation involving transfer of gene-modified T cells both in animal models or patients. Nevertheless, there are now several strategies being developed that may be able to eliminate rogue T cells if required. To reduce potential autoimmunity arising from transfer of gene-engineered T cells, a number of strategies are being pursued. These include the expression of two or more scFv receptors targeting multiple TAAs, and targeting of antigens that are truly tumor specific or expressed only at very low levels on normal host tissue.

In conclusion, genetic modification of T cells with scFv chimeric receptors or TCR transgenes holds great promise for the treatment of cancers of numerous histologies. Ongoing research in optimizing gene-transfer technology and effector cell function will continue to advance this approach. The next era of adoptive cellular immunotherapy is entering an exciting phase with much of the preclinical work performed

on optimizing therapy using gene-engineered T cells to be soon translated into cancer patients.

## Acknowledgments

This work was supported by project grants from the National Health and Medical Research Council (NHMRC), Cancer Council of Victoria and Susan G. Komen Breast Cancer Foundation. Phillip Darcy was supported by an NHMRC Career Development Award.

## References

- Xue SA, Stauss HJ. Enhancing immune responses for cancer therapy. *Cell Mol Immunol* 2007; **4**: 173–84.
- Rosenberg SA. Shedding light on immunotherapy for cancer. *N Engl J Med* 2004; **350**: 1461–3.
- Morgan RA, Dudley ME, Wunderlich JR et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006; **314**: 126–9.
- Gross S, Geldmacher A, Sharav T, Losch F, Walden P. Immunosuppressive mechanisms in cancer: consequences for the development of therapeutic vaccines. *Vaccine* 2009; **27**: 3398–400.
- Zhou J, Dudley ME, Rosenberg SA, Robbins PF. Persistence of multiple tumor-specific T-cell clones is associated with complete tumor regression in a melanoma patient receiving adoptive cell transfer therapy. *J Immunother* 2005; **28**: 53–62.
- Stanislowski T, Voss RH, Lotz C et al. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. *Nat Immunol* 2001; **2**: 962–70.
- Uckert W, Schumacher TN. TCR transgenes and transgene cassettes for TCR gene therapy: status in 2008. *Cancer Immunol Immunother* 2009; **58**: 809–22.
- Eshhar Z, Bach N, Fitzer-Attas CJ et al. The T-body approach: potential for cancer immunotherapy. *Springer Semin Immunopathol* 1996; **18**: 199–209.
- Hakomori S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res* 1996; **56**: 5309–18.
- Westwood JA, Murray WK, Trivett M et al. The Lewis-Y carbohydrate antigen is expressed by many human tumors and can serve as a target for genetically redirected T cells despite the presence of soluble antigen in serum. *J Immunother* 2009; **32**: 292–301.
- Brentjens RJ, Latouche JB, Santos E et al. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat Med* 2003; **9**: 279–86.
- Milone MC, Fish J, Carpenito C et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy *in vivo*. *Mol Ther* 2009; **17**: 1453–64.
- Imai C, Mihara K, Andreansky M et al. Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia* 2004; **18**: 676–84.
- Jensen MC, Cooper LJ, Wu AM, Forman SJ, Raubitschek A. Engineered CD20-specific primary human cytotoxic T lymphocytes for targeting B-cell malignancy. *Cytotherapy* 2003; **5**: 131–8.
- Yu K, Hu Y, Tan Y et al. Immunotherapy of lymphomas with T cells modified by anti-CD20 scFv/CD28/CD3zeta recombinant gene. *Leuk Lymphoma* 2008; **49**: 1368–73.
- Wang J, Jensen M, Lin Y et al. Optimizing adoptive polyclonal T cell immunotherapy of lymphomas, using a chimeric T cell receptor possessing CD28 and CD137 costimulatory domains. *Hum Gene Ther* 2007; **18**: 712–25.
- Gross G, Levy S, Levy R, Waks T, Eshhar Z. Chimaeric T-cell receptors specific to a B-lymphoma idiotype: a model for tumour immunotherapy. *Biochem Soc Trans* 1995; **23**: 1079–82.
- Hombach A, Schneider C, Sent D et al. An entirely humanized CD3 zeta-chain signaling receptor that directs peripheral blood T cells to specific lysis of carcinoembryonic antigen-positive tumor cells. *Int J Cancer* 2000; **88**: 115–20.
- Nolan KF, Yun CO, Akamatsu Y et al. Bypassing immunization: optimized design of “designer T cells” against carcinoembryonic antigen (CEA)-expressing tumors, and lack of suppression by soluble CEA. *Clin Cancer Res* 1999; **5**: 3928–41.
- Haynes NM, Snook MB, Trapani JA et al. Redirecting mouse CTL against colon carcinoma: superior signaling efficacy of single-chain variable domain chimeras containing TCR-zeta vs Fc epsilon RI-gamma. *J Immunol* 2001; **166**: 182–7.
- Arakawa F, Shibaguchi H, Xu Z, Kuroki M. Targeting of T cells to CEA-expressing tumor cells by chimeric immune receptors with a highly specific single-chain anti-CEA activity. *Anticancer Res* 2002; **22**: 4285–9.
- Ma Q, Gonzalo-Daganzo RM, Junghans RP. Genetically engineered T cells as adoptive immunotherapy of cancer. *Cancer Chemother Biol Response Modif* 2002; **20**: 315–41.
- Daly T, Royal RE, Kershaw MH et al. Recognition of human colon cancer by T cells transduced with a chimeric receptor gene. *Cancer Gene Ther* 2000; **7**: 284–91.
- Wang G, Chopra RK, Royal RE, Yang JC, Rosenberg SA, Hwu P. A T cell-independent antitumor response in mice with bone marrow cells retrovirally transduced with an antibody/Fc-gamma chain chimeric receptor gene recognizing a human ovarian cancer antigen. *Nat Med* 1998; **4**: 168–72.
- Hwu P, Shafer GE, Treisman J et al. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain. *J Exp Med* 1993; **178**: 361–6.
- Turatti F, Figini M, Alberti P, Willemsen RA, Canevari S and Mezzanzanica D. Highly efficient redirected anti-tumor activity of human lymphocytes transduced with a completely human chimeric immune receptor. *J Gene Med* 2005; **7**: 158–70.
- Li S, Yang J, Urban FA et al. Genetically engineered T cells expressing a HER2-specific chimeric receptor mediate antigen-specific tumor regression. *Cancer Gene Ther* 2008; **15**: 382–92.
- Moeller M, Haynes NM, Kershaw MH et al. Adoptive transfer of gene-engineered CD4+ helper T cells induces potent primary and secondary tumor rejection. *Blood* 2005; **106**: 2995–3003.

29. Pinthus JH, Waks T, Kaufman-Francis K et al. Immuno-gene therapy of established prostate tumors using chimeric receptor-redirected human lymphocytes. *Cancer Res* 2003; **63**: 2470–6.
30. Altenschmidt U, Kahl R, Moritz D et al. Cytolysis of tumor cells expressing the Neu/erbB-2, erbB-3, and erbB-4 receptors by genetically targeted naive T lymphocytes. *Clin Cancer Res* 1996; **2**: 1001–8.
31. Muniappan A, Banapour B, Lebkowski J and Talib S. Ligand-mediated cytolysis of tumor cells: use of heregulin-zeta chimeras to redirect cytotoxic T lymphocytes. *Cancer Gene Ther* 2000; **7**: 128–34.
32. Ma Q, Safar M, Holmes E, Wang Y, Boynton AL and Junghans RP. Anti-prostate specific membrane antigen designer T cells for prostate cancer therapy. *Prostate* 2004; **61**: 12–25.
33. Maher J, Brentjens RJ, Gunset G, Riviere I and Sadelain M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. *Nat Biotechnol* 2002; **20**: 70–5.
34. McGuinness RP, Ge Y, Patel SD et al. Anti-tumor activity of human T cells expressing the CC49-zeta chimeric immune receptor. *Hum Gene Ther* 1999; **10**: 165–73.
35. Hombach A, Heuser C, Sircar R et al. T cell targeting of TAG72+ tumor cells by a chimeric receptor with antibody-like specificity for a carbohydrate epitope. *Gastroenterology* 1997; **113**: 1163–70.
36. Yun CO, Nolan KF, Beecham EJ, Reisfeld RA, Junghans RP. Targeting of T lymphocytes to melanoma cells through chimeric anti-GD3 immunoglobulin T-cell receptors. *Neoplasia* 2000; **2**: 449–59.
37. Abken H, Hombach A, Heuser C, Reinhold U. A novel strategy in the elimination of disseminated melanoma cells: chimeric receptors endow T cells with tumor specificity. *Recent Results Cancer Res* 2001; **158**: 249–64.
38. Willemsen R, Ronteltap C, Heuveling M, Debets R, Bolhuis R. Redirecting humanCD4+ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR alphabeta gene transfer requires CD8alpha. *Gene Ther* 2005; **12**: 140–6.
39. Kershaw MH, Westwood JA, Zhu ZB, Witte LP, Libutti SK, Hwu P. Generation of gene-modified T cells reactive against the angiogenic kinase insert domain-containing receptor (KDR) found on tumor vasculature. *Hum Gene Ther* 2000; **11**: 2445–52.
40. Niederman TM, Ghogawala Z, Carter BS, Tompkins HS, Russell MM, Mulligan RC. Antitumor activity of cytotoxic T lymphocytes engineered to target vascular endothelial growth factor receptors. *Proc Natl Acad Sci USA* 2002; **99**: 7009–14.
41. Rossig C, Bollard CM, Nuchtern JG, Merchant DA, Brenner MK. Targeting of G(D2)-positive tumor cells by human T lymphocytes engineered to express chimeric T-cell receptor genes. *Int J Cancer* 2001; **94**: 228–36.
42. Ren-Heidenreich L, Hayman GT, Trevor KT. Specific targeting of EGP-2+ tumor cells by primary lymphocytes modified with chimeric T cell receptors. *Hum Gene Ther* 2000; **11**: 9–19.
43. Ren-Heidenreich L, Mordini R, Hayman GT, Siebenlist R, LeFever A. Comparison of the TCR zeta-chain with the FcR gamma-chain in chimeric TCR constructs for T cell activation and apoptosis. *Cancer Immunol Immunother* 2002; **51**: 417–23.
44. Weijtens ME, Willemsen RA, Valerio D, Stam K, Bolhuis RL. Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. *J Immunol* 1996; **157**: 836–43.
45. Lamers CH, Langeveld SC, Groot-van Ruijven CM, Debets R, Sleijfer, S, Gratama JW. Gene-modified T cells for adoptive immunotherapy of renal cell cancer maintain transgene-specific immune functions *in vivo*. *Cancer Immunol Immunother* 2007; **56**: 1875–83.
46. Weijtens ME, Hart EH, Bolhuis RL. Functional balance between T cell chimeric receptor density and tumor associated antigen density: CTL mediated cytolysis and lymphokine production. *Gene Ther* 2000; **7**: 35–42.
47. Westwood JA, Smyth MJ, Teng MW et al. Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice. *Proc Natl Acad Sci USA* 2005; **102**: 19051–6.
48. Mezzananza D, Canevari S, Mazzoni A et al. Transfer of chimeric receptor gene made of variable regions of tumor-specific antibody confers anticarbohydrate specificity on T cells. *Cancer Gene Ther* 1998; **5**: 401–7.
49. Hombach A, Mucic JM, Gerken M et al. T cells engrafted with a recombinant anti-CD30 receptor target autologous CD30(+) cutaneous lymphoma cells. *Gene Ther* 2001; **8**: 891–5.
50. Dall P, Herrmann I, Durst B et al. In vivo cervical cancer growth inhibition by genetically engineered cytotoxic T cells. *Cancer Immunol Immunother* 2005; **54**: 51–60.
51. Finney HM, Akbar AN, Lawson AD. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. *J Immunol* 2004; **172**: 104–13.
52. Kershaw MH, Westwood JA, Parker LL et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 2006; **12**: 6106–15.
53. Cheung NK, Guo HF, Modak S, Cheung IY. Anti-idiotypic antibody facilitates scFv chimeric immune receptor gene transduction and clonal expansion of human lymphocytes for tumor therapy. *Hybrid Hybridomics* 2003; **22**: 209–18.
54. Wilkie S, Picco G, Foster J et al. Retargeting of human T cells to tumor-associated MUC1: the evolution of a chimeric antigen receptor. *J Immunol* 2008; **180**: 4901–9.
55. Carpenito C, Milone MC, Hassan R et al. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci USA* 2009; **106**: 3360–5.
56. Gattenlöhner S, Marx A, Markfort B et al. Rhabdomyosarcoma lysis by T cells expressing a human autoantibody-based chimeric receptor targeting the fetal acetylcholine receptor. *Cancer Res* 2006; **66**: 24–8.
57. Patel SD, Moskalenko M, Tian T et al. T-cell killing of heterogenous tumor or viral targets with bispecific chimeric immune receptors. *Cancer Gene Ther* 2000; **7**: 1127–34.
58. Darcy PK, Haynes NM, Snook MB et al. Redirected perforin-dependent lysis of colon carcinoma by *ex vivo* genetically engineered CTL. *J Immunol* 2000; **164**: 3705–12.
59. Darcy PK, Kershaw MH, Trapani JA, Smyth MJ. Expression in cytotoxic T lymphocytes of a single-chain

- anti-carcinoembryonic antigen antibody. Redirected Fas ligand-mediated lysis of colon carcinoma. *Eur J Immunol* 1998; **28**: 1663–72.
60. Moritz D, Wels W, Mattern J, Groner B. Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proc Natl Acad Sci USA* 1994; **91**: 4318–22.
  61. Altvorschmidt U, Klundt E, Groner B. Adoptive transfer of *in vitro*-targeted, activated T lymphocytes results in total tumor regression. *J Immunol* 1997; **159**: 5509–15.
  62. Hwu P, Yang JC, Cowherd R et al. *In vivo* antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. *Cancer Res* 1995; **55**: 3369–73.
  63. Hekele A, Dall P, Moritz D et al. Growth retardation of tumors by adoptive transfer of cytotoxic T lymphocytes reprogrammed by CD44v6-specific scFv:zeta-chimera. *Int J Cancer* 1996; **68**: 232–8.
  64. Janeway CA, The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu Rev Immunol* 1992; **10**: 645–74.
  65. Riley JL, Carroll RG, Levine BL et al. Intrinsic resistance to T cell infection with HIV type 1 induced by CD28 costimulation. *J Immunol* 1997; **158**: 5545–53.
  66. Krause A, Guo HF, Latouche JB, Tan C, Cheung NK, Sadelain M. Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. *J Exp Med* 1998; **188**: 619–26.
  67. Haynes NM, Trapani JA, Teng MW et al. Rejection of syngeneic colon carcinoma by CTLs expressing single-chain antibody receptors codelivering CD28 costimulation. *J Immunol* 2002; **169**: 5780–6.
  68. Kershaw MH, Teng MW, Smyth MJ, Darcy PK. Supernatural T cells: genetic modification of T cells for cancer therapy. *Nat Rev Immunol* 2005; **5**: 928–40.
  69. Geiger TL, Nguyen P, Leitenberg D, Flavell RA. Integrated src kinase and costimulatory activity enhances signal transduction through single-chain chimeric receptors in T lymphocytes. *Blood* 2001; **98**: 2364–71.
  70. Pule MA, Savoldo B, Myers GD et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 2008; **14**: 1264–70.
  71. Murphy AM, Westwood JA, Brown LE et al. Antitumor activity of dual-specific T cells and influenza virus. *Cancer Gene Ther* 2007; **14**: 499–508.
  72. Kershaw MH, Westwood JA, Hwu P. Dual-specific T cells combine proliferation and antitumor activity. *Nat Biotechnol* 2002; **20**: 1221–7.
  73. Moeller M, Kershaw MH, Cameron R et al. Sustained antigen-specific antitumor recall response mediated by gene-modified CD4+ T helper-1 and CD8+ T cells. *Cancer Res* 2007; **67**: 11428–37.
  74. Kershaw MH, Wang G, Westwood JA et al. Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. *Hum Gene Ther* 2002; **13**: 1971–80.
  75. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* 2005; **106**: 376–83.
  76. Pegram HJ, Jackson JT, Smyth MJ, Kershaw MH, Darcy PK. Adoptive transfer of gene-modified primary NK cells can specifically inhibit tumor progression *in vivo*. *J Immunol* 2008; **181**: 3449–55.
  77. Brocker T, Karjalainen K. Adoptive tumor immunity mediated by lymphocytes bearing modified antigen-specific receptors. *Adv Immunol* 1998; **68**: 257–69.
  78. Roberts MR, Cooke KS, Tran AC et al. Antigen-specific cytotoxicity by neutrophils and NK cells expressing chimeric immune receptors bearing zeta or gamma signaling domains. *J Immunol* 1998; **161**: 375–84.
  79. Paul S, Snary D, Hoebeke J et al. Targeted macrophage cytotoxicity using a nonreplicative live vector expressing a tumor-specific single-chain variable region fragment. *Hum Gene Ther* 2000; **11**: 1417–28.
  80. Hege KM, Cooke KS, Finer MH, Zsebo KM, Roberts MR. Systemic T cell-independent tumor immunity after transplantation of universal receptor-modified bone marrow into SCID mice. *J Exp Med* 1996; **184**: 2261–9.
  81. Pollok KE, van der Loo JC, Cooper RJ, Kennedy L, Williams DA. Costimulation of transduced T lymphocytes via T cell receptor-CD3 complex and CD28 leads to increased transcription of integrated retrovirus. *Hum Gene Ther* 1999; **10**: 2221–36.
  82. Lamers CH, van Elzakker P, van Steenbergen SC, Sleijfer S, Debets R, Gratama JW. Retroviral-assisted retroviral transduction of primary human T lymphocytes under good manufacturing practice conditions: tissue culture bag critically determines cell yield. *Cytotherapy* 2008; **10**: 406–16.
  83. Mitsuyasu RT, Anton PA, Deeks SG et al. Prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous CD4(+) and CD8(+) T cells in human immunodeficiency virus-infected subjects. *Blood* 2000; **96**: 785–93.
  84. Deeks SG, Wagner B, Anton PA et al. A phase II randomized study of HIV-specific T-cell gene therapy in subjects with undetectable plasma viremia on combination antiretroviral therapy. *Mol Ther* 2002; **5**: 788–97.
  85. Lamers CH, Sleijfer S, Vulto AG et al. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 2006; **24**: e20–2.
  86. Marshall E. Gene therapy. Second child in French trial is found to have leukemia. *Science* 2003; **299**: 320.
  87. Li Z, Düllmann J, Schiedmeier B et al. Murine leukemia induced by retroviral gene marking. *Science* 2002; **296**: 497.
  88. Jones S, Peng PD, Yang S et al. Lentiviral vector design for optimal T cell receptor gene expression in the transduction of peripheral blood lymphocytes and tumor-infiltrating lymphocytes. *Hum Gene Ther* 2009; **20**: 630–40.
  89. Huang X, Guo H, Kang J et al. Sleeping Beauty transposon-mediated engineering of human primary T cells for therapy of CD19+ lymphoid malignancies. *Mol Ther* 2008; **16**: 580–9.
  90. Yoon SH, Lee JM, Cho HI et al. Adoptive immunotherapy using human peripheral blood lymphocytes transferred with RNA encoding Her-2/neu-specific chimeric immune receptor in

- ovarian cancer xenograft model. *Cancer Gene Ther* 2009; **16**: 489–97.
91. Verzeletti S, Bonini C, Marktel S *et al.* Herpes simplex virus thymidine kinase gene transfer for controlled graft-versus-host disease and graft-versus-leukemia: clinical follow-up and improved new vectors. *Hum Gene Ther* 1998; **9**: 2243–51.
92. Berger C, Flowers ME, Warren EH, Riddell SR. Analysis of transgene-specific immune responses that limit the *in vivo* persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation. *Blood* 2006; **107**: 2294–302.
93. Introna M, Barbui AM, Bambiacioni F *et al.* Genetic modification of human T cells with CD20: a strategy to purify and lyse transduced cells with anti-CD20 antibodies. *Hum Gene Ther* 2000; **11**: 611–20.
94. Palmer DC, Chan CC, Gattinoni L *et al.* Effective tumor treatment targeting a melanoma/melanocyte-associated antigen triggers severe ocular autoimmunity. *Proc Natl Acad Sci USA* 2008; **105**: 8061–6.
95. Haynes NM, Smyth MJ, Kershaw MH, Trapani JA, Darcy PK. Fas-ligand-mediated lysis of erbB-2-expressing tumour cells by redirected cytotoxic T lymphocytes. *Cancer Immunol Immunother* 1999; **47**: 278–86.
96. Gattinoni L, Powell DJ, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. *Nat Rev Immunol* 2006; **6**: 383–93.
97. Wrzesinski C, Paulos CM, Gattinoni L *et al.* Hematopoietic stem cells promote the expansion and function of adoptively transferred antitumor CD8 T cells. *J Clin Invest* 2007; **117**: 492–501.
98. Hinrichs CS, Spolski R, Paulos CM *et al.* IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood* 2008; **111**: 5326–33.