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Review

Genetic redirection of T cells for cancer therapy

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ABSTRACT

Adoptive immunotherapy can induce dramatic tumor regressions in patients with melanoma or viral-induced malignancies, but extending this approach to many common cancers has been hampered by a lack of naturally occurring tumor-specific T cells. In this review, we describe recent advances in the genetic modification of T cells using genes encoding cell-surface receptors specific for tumor-associated antigen. Using genetic modification, the many functional properties of T cells, including cytokine secretion and cytolytic capacity, are redirected from their endogenous specificity toward the elimination of tumor cells. Advances in gene design, vectors, and cell production are discussed, and details of the progress in clinical application of this approach are provided. *J. Leukoc. Biol.* 87: 791–803; 2010.

The immune system is a crucial ally in protection from infection, and there is growing evidence that it plays an important role in protecting us from neoplasia. Harnessing the immune system by using immunotherapeutic strategies for the treatment of cancer is gaining momentum, and these approaches can already impact a range of malignancies. Adoptive immunotherapy is proving particularly promising at treating malignant disease, even at advanced stages, but significant, durable responses are generally limited to patients with melanoma or viral-induced malignancies such as EBV-associated lymphoproliferative disorders.

A major reason for the failure of adoptive immunotherapy against most common cancers lies in the absence of a source of tumor-specific T cells. To overcome this limitation, genetic modification of T cells is being used to generate T cells with specificity for tumors. Genetic modification redirects the activity of T cells effectively, from its inherent cognate specificity toward reactivity against tumor-associated antigens. Genes used to redirect T cell activity vary in their composition but to date, have been predominantly chimeric in nature, composed of an extracellular domain consisting of a TAA-specific single-chain antibody (scFv) that is linked through hinge and transmembrane regions to cytoplasmic signaling domains. These chimeric molecules are termed CARs. Tremendous advances have been made in vector and gene design to produce enhanced reactivity of T cells against TAA, and application of this approach is in early Phase I trials in the clinic. Several excellent reviews published up to 2006 have described the early development of genetic redirection of T cells [1, 2], and the current review summarizes these early studies briefly but focuses predominantly on advances made in several aspects of this approach since 2006.

ADVANCES IN OPTIMIZING ACTIVITY

Typically, gene-redirected T cells secrete moderate levels of cytokine, rarely reaching 50 ng/ml IFN-γ in response to tumors, and are able to induce 50–80% lysis of tumor cells at effector-to-target ratios of ~20:1. Redirecting T cell activity to this degree is a considerable achievement, but these levels of response do not approach the activity of virus-specific T cells against cognate antigen, where >1000 ng/ml IFN-γ can be secreted, and significant levels of lysis can be achieved with effector-to-target ratios of <1:1 [3]. Clearly, gene-redirected T cells are not performing to their best potential, and there is room for improvement. The reason for the limitations may lie in a number of areas, including deficiencies on the part of the target cell or shortcomings on the part of the T cells. Several approaches aimed at enhancing redirected T cell function are being pursued; principal among these approaches are attempts to combine alternative signaling domains in the cytoplasmic region of chimeric genes.

Abbreviations: ACT=α1 antichymotrypsin, ALL=acute lymphocytic leukemia, CAIX=carboxy-anhydrase-IX, CAR=chimeric antigen receptor, CEA=carcinoembryonic antigen, CR=complete response, erbB2/Her-2=human epidermal growth factor receptor 2, GALV=gibbon ape leukemia virus envelope, GD2=diasialoganglioside, HEL=hen egg lysozyme, IRES=internal ribosomal entry site, LCL=lymphoblastoid cell line(s), MART-1=melanoma antigen recognized by T cells, PR=partial response, scFv=*single-chain* variable fragment, SD=stable disease, TAA=tumor-associated antigens, TAG72=tumor-associated glycoprotein, Treg=regulatory T cell

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The earliest, first-generation CARs were composed of signaling domains derived from single molecules, the CD3-ζ chain or FcRγ chain. These first CARs were followed by second-generation CARs incorporating additional signaling domains derived from costimulatory molecules such as CD28. These second-generation, dual-domain CARs were demonstrated to induce secretion of greater amounts of cytokine than single-domain CARs in response to antigen. These dual-domain CARs were also sometimes observed to be capable of enhancing target cell lysis compared with single-domain receptors [4], but in other cases, this capability was not demonstrated [5, 6]. Second-generation CARs were also demonstrated to possess enhanced proliferative potential and an increased ability to persist and inhibit tumor growth in vivo [7]. CD28 is the costimulating molecule of choice for most chimeric receptors, although there is some evidence to suggest that the cytoplasmic domain of CD137 (4-1BB) can be superior to that of CD28 [6].

More recently, CARs containing tripartite signaling domains have been demonstrated to have a benefit over single- or double-domain CARs with respect to enhancing survival of T cells following antigen engagement (**Table 1**). Domains from various signaling molecules have been incorporated into tripartite receptors, including those from CD137, OX40, and ICOS [8]. Receptors containing elements from CD137, in particular, possess enhanced abilities to induce cytokine release and cytotoxicity from transduced T cells and an ability to inhibit tumor growth in mice [9, 10]. In one study targeting CD19, cytokine secretion was not augmented by a tripartite receptor containing CD137, but cytotoxicity and the ability to inhibit tumor growth in vivo were enhanced [11]. Thus, there is some degree of disagreement with respect to the relative functions imparted on T cells by various costimulating domains, and it is likely that this varies with different receptors and/or targets.

Generally, costimulation is provided within the signaling domains of chimeric receptors or on artificial APCs, but another innovative way of achieving costimulation is through the provision of costimulatory ligands on the T cells themselves. This approach has been demonstrated to costimulate T cells through autoligation of costimulatory molecules as well as through costimulation by neighboring T cells, and T cells modified in this way have been demonstrated to respond to tumor cells and reject systemic human prostate cancer tumors in mice [12].

Although improvements in the magnitude of T cell responses can be achieved using additional or alternative domains, there is evidence to suggest that further improvements are possible. A study targeting the TAA CD19 found that CD28 costimulation was not sufficient to stimulate T cell proliferation, but additional, as yet uncharacterized costimulatory molecules present on allogeneic EBV-transformed LCL could cooperate with chimeric receptor signaling leading to T cell proliferation [13]. Clearly, there is still some way to go before the full activity of T cells can be realized against tumor cells, and testing further signaling domains in single receptors or using combinations of receptors will likely lead to further improvements in T cell function.

Other means of enhancing T cell function include increasing expression levels of CARs or varying their affinity for antigen. As might be expected, higher levels of CAR expression have been found to be associated with greater responses against tumor cells [14, 15]. However, CAR affinity seems to play less of a role in determining T cell responses, and low-affinity ($\sim 1.6\times10^{-6}$ M) receptors are still able to elicit T cell responses comparable with higher-affinity CARs [14, 16]. Other attributes of the ectodomain of CARs can play a role in determining T cell activity; in particular, the nature of the hinge region can affect how well CARs bind to antigen, which can consequently impact T cell function. An elongated hinge region derived from IgD has been shown to improve recognition of the MUC1 antigen [17]. Other Ig hinge regions have

TABLE 1. Composition of CARs Used to Redirect T Cell Function

Antigen	CAR	Details	Ref.
GD2	α-GD2-CD28-OX40-ζ	Significant cytokine release and in vitro and in vivo antimelanoma activity	[8]
Her-2	scFv Herceptin-CD28-ζ+/–CD137	Greater cytokine secretion, lytic activity, and tumor suppression with CD137 in construct	[9]
PSMA	α-PSMA-CD28-4-1BB-ζ	T cells with three domains more effective than those with two domains in eliminating tumor in SCID mice	[10]
CD19	α-CD19-CD28-CD137-ζ	Greater survival of tumor-bearing mice when treated with UCB T cells bearing three domains rather than two domains	[11]
PSMA	α-PSMA-ζ and T cell-expressed CD80 and CD137 ligand	Tumor eradication of tumor-bearing SCID-beige mice when treated with T cells bearing CAR and CD80X CD137L	[12]
CD19	α-CD19-ζ	Transduced EBV-specific CTL suggests multiple costimulatory molecules required	[13]
Her-2	α-Her-2-ζ	Low-affinity receptors still able to elicit T cell responses comparable with higher-affinity CAR	[14]
Lewis-Y	α-LeY-CD28-ζ	Higher levels of CAR expression associated with greater responses against tumor cells	[15]
Lewis-Y	α-LeY-CD28-ζ	Low-affinity receptor on T cells inhibited tumors in NOD-SCID mice	[16]

Abbreviations used for CARs include the specificity of the scFv antibody denoted with the anti (α)-prefix, followed by components of the signaling domains (ζ, CD3-ζ; PSMA, prostate-specific membrane antigen; UCB, umbilical cord blood; CD137L, CD137 ligand).

been used to direct T cells effectively against tumor cells using a tumor-binding peptide fused to the IgG4 hinge region [18].

Of importance in the generation of optimal gene-modified T cells is the vector used. Ideally, stable, high-level expression of CARs is desirable in 100% of T cells. However, the safety of genetic modification also needs to be considered, and vector attributes and titer needed for high expression can also lead to genomic integration of high copy numbers of vectors, which may increase the risk of malignant transformation of T cells similar to that observed with modification of hematopoietic stem cells [19]. See below for more detail of vectors used.

ENHANCING PERSISTENCE OF T CELLS

It seems logical that the longer T cells persist, the better chance they have of impacting tumor cells. Indeed, increased persistence of adoptively transferred T cells has correlated with better tumor responses in the melanoma setting [20, 21]. Several approaches aimed at increasing the survival of T cells are being pursued involving autocrine provision of growth or survival signals or methods for preferential enrichment or selection following transfer.

Enhanced persistence of adoptively transferred tumor-infiltrating lymphocytes has been demonstrated in mouse models and in patients following myelo-depleting regimens prior to cell transfer [22, 23]. The enhanced persistence is thought to be a result of the reduction in homeostatic cytokine consumption by competing endogenous leukocytes and perhaps the removal of regulatory cells. However, myelo-depletion is associated with considerable morbidity, largely as a result of an increased risk of infection, and therefore, other methods of enhancing the persistence of transferred T cells are being developed, including the autocrine production of cytokines.

Cytokines can provide important growth and homeostatic signals to T cells, and the importance of IL-2 and IL-15 is particularly well established. A strategy involving the introduction of the IL-15 gene into T cells has demonstrated the ability of autocrine production of this cytokine to enhance T cell persistence in culture in the absence of exogenous cytokine, and T cell activity against antigen was maintained [24]. Similarly, tumor-reactive T cells maintained their activity and persisted longer in vitro following transduction with a vector encoding an IL-2 gene [25]. However, in a small study involving seven patients, despite durable expression of the transgene, no advantage to T cell persistence or clinical effectiveness compared with administration of exogenous cytokine was demonstrated [26]. Nevertheless, an advantage of the approach lies in not having to administer such high levels of exogenous IL-2, which has been demonstrated to have severe side-effects.

Constitutive expression of cytokine can be concerning from a safety point of view, as T cells constantly producing an autocrine growth factor may have an increased tendency to transform in concert with changes to other proto-oncogenes. Therefore, a conceptually elegant approach would be to link autocrine production of cytokines to antigen engagement. Such an approach has been applied in principal using surrogate antigens, HEL, or fluorescein, in which a chimeric receptor composed of anti-HEL or antifluorescein scFv linked to cytokine receptor intracellular domains was demonstrated to mediate numerical expansion of cytokine-dependent cell lines in the absence of exogenous cytokine [27, 28]. Enhancing responses of T cells to homeostatic cytokines by introduction of genes encoding cytokine receptors is also a promising way of enhancing the persistence of T cells. Expression of the IL-7R in this way has been demonstrated to restore responses to IL-7 [29].

Although a myelo-depleting, preparative regimen can enhance persistence as described above, the rebound in endogenous leukocytes may limit engraftment of transferred cells. An approach aimed at circumventing this and permitting selective depletion of competing leukocytes post-transfer involves endowing T cells with resistance to an immunosuppressive drug. T cells transduced with a gene encoding resistance to mycophenolate were demonstrated to be able to proliferate in the presence of drug [30]. However, this study was restricted to an in vitro characterization, and the feasibility in vivo still needs to be determined.

Perhaps the most "natural" way of enhancing persistence of transferred T cells is to be found in approaches aimed at genetic redirection of T cells already possessing specificity for defined antigens through their endogenous TCR. This would enable reactivation and expansion of redirected T cells by persistent endogenous antigen, such as EBV, or by administration of vaccines. The use of these approaches has been demonstrated in vitro [31, 32] and for allogeneic, influenza, and lymphocytic choriomeningitis virus antigens in mice [3, 33, 34], in addition to the use of EBV-specific T cells in patients [35]. Examples of new functions that can be endowed on T cells through genetic modification, as described in this and the previous section are shown in **Figure 1**.

ADVANCES IN GENE VECTORS

One way to generate T cells with consistent high CAR expression is through cloning cells and expanding them to large numbers. This method has been used in some applications, but it is laborious and results in older cells with short telomeres, which is in conflict with recent findings, showing that younger T cells are better. Therefore, considerable effort is being expended in producing vectors with enhanced abilities to modify genetically large proportions of T cells quickly.

Vectors that have been used most frequently to generate stable CAR-expressing T cells are retroviral, largely as a result of their ability to facilitate integration of constructs and result in more stable expression than plasmid-based methods. Several factors are important in considering the best retroviral vector for transduction of T cells. Gene construct size can impact vector titer, and inserts of >2 kb result in lower titers of $<1 \times 10^6$/ml. Elements such as short-intronic sequences flanked by splice sites can improve titers, likely as a result of stabilization of vector transcripts. The choice of retroviral envelope and producer cell line is also important, and there are reports that the PG13 cell line producing GALV-pseudotyped virus can give optimal transduction rates in human T cells. However, there are also reports that retrovirus produced using Phoenix cells

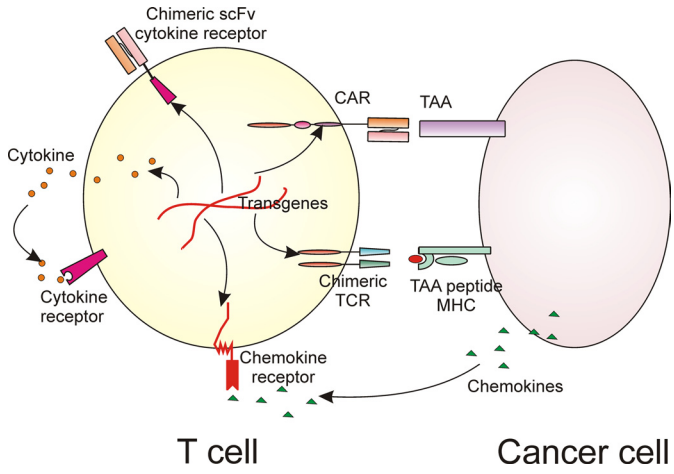

Figure 1. Examples of new functions that can be endowed on T cells through genetic modification. The specificity of T cells can be redirected toward TAA using genes encoding CARs that recognize cell-surface TAA on cancer cells. CARs can consist of extracellular scFv antibody recognizing TAA linked to intracellular domains from several signaling molecules. Specificity can also be mediated by genes encoding tumor-specific TCRs, which can be chimeric (linked directly to signaling molecules) to reduce mispairing with endogenous TCR chains. TCR can respond to intracellular TAA in the context of MHC. Enhanced survival and proliferation of T cells can be achieved through genetic modification for autocrine production of cytokines, and cytokine triggering can also be achieved using genes encoding chimeric cytokine receptors able to signal upon engagement of antigen by the extracellular scFv domain. Localization to tumors can be facilitated by the introduction of genes encoding receptors for chemokines secreted by tumors.

(with an amphotropic envelope) can give superior transduction frequencies than vector produced from PG13 cells [36].

Initially, γ-retroviral vectors were used in preference to lentiviral vectors as a result of concerns over using HIV-based vectors with respect to the potential generation of replication-competent virus, although this was never observed. Nevertheless, more recent lentiviral vectors have incorporated extra safety measures, including the separation of viral elements and the use of self-inactivating 5′ long-terminal repeats. Lentiviral vectors are gaining increasing acceptance for gene-modifying T cells, with claims of improved transduction frequencies over γ-retroviruses [37], and close to 100% transduction efficiency has been demonstrated [6, 38, 39]. Advances in lentiviral vector design continue to result in improved expression of transgenes, and the murine stem cell virus promoter demonstrates optimal expression capabilities and expression of two genes facilitated best by insertion of a 2A-linker peptide rather than using separate promoters or an IRES [40].

The high cost of production associated with viral vectors and safety concerns with respect to potential emergence of replication-competent virus have led investigators to pursue nonviral vectors for the generation of gene-modified T cells. Early studies confirmed the potential of this approach using electroporation of plasmid DNA, but transfection frequencies were low, necessitating cloning and re-expansion of cells. However, more recently, transposon systems have demonstrated promise for genetic modification of T cells. These systems use a transposon-based construct together with a plasmid encoding a transposase that facilitates integration of the construct into thymine/adenine dinucleotide sites within the genome. CAR expression was demonstrated in 5–20% of T cells and functional responses to antigen (CD19) demonstrated [41, 42]. These vectors have also been used to redirect T cell function against MHC-restricted TAA (p53 and MART-1) using TCR gene transfer. Expression frequencies of 15–30% of T cells were observed in this study and function in the forms of cytokine secretion and cytolysis against TAA demonstrated [43]. Interestingly, using RNA to encode the transposase enzyme enhanced expression, perhaps as a result of a relative increase in enzyme levels. The use of RNA in this way in place of plasmid DNA serves also to reduce concerns about genetic instability resulting from integration of the transposase gene, which has been demonstrated previously, albeit infrequently.

Another advantage of transposon systems is the ability to transfer multiple gene constructs, which will likely be necessary to endow T cells with several important functions simultaneously, such as specificity, trafficking, persistence, and safety. The piggyBac transposon system, offering a capacity to carry large DNA constructs, has been used to deliver multiple genes to T cells with stable expression in up to 20% of cells possible, rising to ~40% if T cells were maintained in the presence of IL-15 [44]. Similar levels of expression have been observed with the piggyBac system following expansion of T cells using artificial APCs displaying costimulatory molecules and membrane-bound IL-15 [45].

Retroviral vectors incorporate in a semi-random manner into the genome but appear to have a predilection for transcriptionally active sites. This is a concern from a safety point of view as a result of potential gene dysregulation leading to oncogenic transformation. It is known that γ-retroviral vectors, in particular, integrate into or near active promoter regions and have been demonstrated to contribute to transformation of stem cells [19]. Lentiviral vectors, although integrating into transcriptionally active genes, appear to do so less near promoter regions. Integration of transposon-based vectors is also semi-random into thymine/thymine/adenine/adenine sites but has been reported to have decreased integration frequency within ~50 kb of transcriptional start sites of known proto-oncogenes, which may decrease the risk of transformation using these vectors [46]. However, the tendency for integration of increased copy number by transposon-based vectors compared with retroviral vectors may reduce any relative safety advantages of these vectors, and transfection procedures may need to be adjusted to keep integration levels low.

Another alternative to viral vectors is the use of RNA-encoding chimeric receptors introduced using electroporation. High-level expression of proteins can be achieved using RNA, and the transient nature of expression, typically less than 4 days, can be an advantage if concerns exist about toxicity from long-term persistence of T cells and their reactivity against normal tissues [47]. Several TAA have been targeted using RNA transfection of T cells, including Her-2 and CD19, with function of redirected T cells against tumor cells demonstrated in vitro and in mice [48, 49].

PROGRESS IN MOUSE TUMOR MODELS

Experiments in mouse tumor models can inform us about limitations of various approaches and their potential success in patients. The state of play about 5 years ago was that early stage disseminated disease or micrometastases, and small s.c. disease could be inhibited and eradicated occasionally by adoptive transfer of CAR-redirected T cells, but advanced (>Day 7) and solid tumors were refractory to this treatment [16, 50–52]. Since then, advances in receptor design and adjuvant approaches have allowed more impressive demonstrations of the potential of this approach, as summarized in **Table 2**.

Eradication of longer-term disseminated disease (Day 13) in 100% of mice has been achieved against lymphoma using T cells redirected against CD19 following a lympho-depleting regimen [53]. Attempts to impact Day 21 systemic ALL also produced impressive results with survival of some mice beyond 200 days after tumor injection, although the majority of mice eventually succumbed to disease [6].

In an approach to direct T cell activity against two antigens simultaneously, investigators have gene-modified EBV-specific T cells with a CAR specific for CD30. EBV antigens and CD30 are expressed in many Hodgkin's lymphoma cells. Adoptive transfer of anti-CD30 human T cells was demonstrated to inhibit 7-day established i.p. Hodgkin's lymphoma (L428) for up to 28 days [54]. Simultaneous administration of EBV-infected LCL was shown to enhance the anti-tumor effect, presumably by providing activation and/or proliferative signals to gene-modified T cells.

The range of tumor types was extended to include osteosarcoma by targeting low-level expression of human Her-2. i.p. disease established for 8 days was able to be eradicated in ~40% of mice using three daily injections of T cells modified to express anti-Her-2 linked to CD28 and CD3-ζ. Lung metastases could also be eradicated in ~80% of mice, but this required treatment of earlier (Day 2) disease [55].

Perhaps the most dramatic demonstration of the efficacy of gene-redirected T cells against established, solid tumors was observed in a xenograft model of human mesothelioma. In this model, tumors were injected s.c. and allowed to grow for 6–7 weeks, by which time, they were ~500 mm^3 in size. Treatment with T cells redirected against mesothelin, administered intratumorally or i.v., was able to induce complete regression of tumors in some mice [56].

Tumor models are also becoming more sophisticated. For example, intraventricular injection of medulloblastoma cells was used to establish tumors on leptomeningial surfaces in a xenograft model. These tumors were then treated with T cells redirected against the IL-13R expressed on tumor, leading to substantial regression of established tumors [57]. In a similar model of medulloblastoma, enhanced survival of mice was observed after adoptive transfer of T cells gene-modified to respond against Her-2 [58].

A limitation of these approaches, however, is that they are performed largely in immunodeficient mice, mostly using human T cells. Therefore, we cannot predict the impact of the endogenous immune system, e.g., on persistence through competition for cytokines and Tregs, which might occur in the clinical application of these approaches. Advances in the field will await experiments in transgenic mice expressing human TAA as a self-antigen. Further sophistications in mouse tumor models using various imaging techniques, such as bioluminescence, have contributed already to improving our understanding of adoptive immunotherapy [17, 59–61], and further developments will no doubt lead to more advances.

PROGRESS IN CELL PRODUCTION

The effectiveness of adoptively transferred T cells can be affected by the phenotype and culture conditions. This has been seen most clearly in mouse melanoma models, where

TABLE 2. Effectiveness of Gene-Redirected T Cells in Mouse Models of Cancer

Malignancy	Antigen	Vector(s)	CAR	Details	Ref.
Lymphoma	CD19	rKat.IRES.GFP and pMP71 retroviruses	tCD34-α-CD19-ζ	Eradication of Day 13 tumors in xenograft SCID/beige mice	[53]
ALL	CD19	pRRL-SIN-CMV-eGFP-WPRE lentivirus	α-CD19-CD137-ζ	Survival to >200 days, Day 21 model, NOD-SCID mice	[6]
Hodgkins lymphoma	CD30	SFG retrovirus	α-CD30-ζ	Inhibition for >28 days of Day 7 model, SCID mice	[54]
Osteosarcoma	Her-2	SFG retrovirus	α-Her-2-CD28-ζ	Eradication of Day 8 tumors in Nu/Nu mice	[55]
Mesothelioma	Mesothelin	pELNS	scFv-CD28-CD137-ζ	Eradication of 6 to 7-week tumors in NOD/SCID/IL2rγ−/− (NOG) mice	[56]
Medullo blastoma	IL-13	Electroporation with DNA	IL-13-CD4-ζ	Tumor regression of Day 8 tumors in NOD-SCID	[57]
Medullo blastoma	Her-2	SFG retrovirus	α-Her-2-ζ	Enhanced survival (>55 days), 5 to 7 day model, in NOD-SCID mice	[58]

A range of antigens has been targeted on the malignancies listed in mice. Points of interest include the vector used and the composition of the CAR. A brief summary of the degree of tumor control in each model is also given. t-CD34, Truncated CD34; SFG, retrovirus vector derived from MPG; pRRL-SIN-CMV-eGFP-WPRE, third generation lentiviral expression vector; pELNS, third generation self-inactivating lentivirus based on pRRL-SIN-CMV-eGFP-WPRE.

effector TAA-specific T cells were less effective than T cells of a memory phenotype at eradicating tumors in mice [62]. Subsequently, it was shown that activated T cells derived from T cells with a naïve or stem cell-like phenotype had an enhanced ability to inhibit tumor growth compared with T cells generated from central memory or effector memory subsets [63, 64]. In addition to phenotype considerations, the duration of culture appears to be important, and T cells cultured for shorter periods following stimulation are more effective than those cultured for longer periods when used in adoptive transfer [65, 66]. Although these observations were made in nongene-modified T cells, there is some evidence to suggest that these findings will extend to gene-redirected T cells, as TCR gene-modified T cells derived from naïve cells were demonstrated to be better than those derived from T cells with a memory phenotype at inhibiting melanoma growth in mice [63].

The above observations were restricted to $CD8^+$ T cells, and there is less information about the optimal phenotype of gene-redirected $CD4^+$ T cells for adoptive immunotherapy. Nevertheless, the presence of $CD4^+$ gene-modified T cells has been shown to correlate with enhanced anti-tumor effects [67], and those of the Th1 phenotype were demonstrated to possess better antitumor activity than those of the Th2 phenotype [68].

Traditionally, T cell activation for transduction and expansion of T cell numbers has been accomplished using anti-CD3 and IL-2. However, these conditions are nonspecific, and nontransduced cells are also stimulated. In addition, the resulting phenotype has aspects of effector cells and effector memory cells, which may not be optimal for persistence and function in vivo. Alternate culture conditions being investigated to address these concerns, although still yielding sufficient cell numbers, include the use of artificial APCs. In this approach, a cell line, often derived from the mouse 3T3 fibroblast line or the K562 human erythroblastoid line, is modified genetically to express antigen and ligate adhesion molecules and costimulatory molecules [69]. T cells are then cultured with these APCs (irradiated) and cytokine and with periodic restimulation. There is some evidence that this system can generate populations of T cells enriched for antigen specificity and a modest increase in function when compared with T cells generated using anti-CD3 and IL-2 [70]. However, it is not clear whether this is at the expense of total cell numbers. In addition, other variations in culture conditions, such as different cytokines, make a direct comparison with traditional methods difficult. Indeed, the cytokine composition has been shown to impact production of gene-modified cells, and the inclusion of IL-15 in the culture is demonstrated to increase transgene expression [44].

Currently, production of gene-modified T cells is a cumbersome process with stringent and complex regulatory requirements that restrict its application to relatively few clinical centers with access to clean room facilities. However, progress in T cell production using closed systems is in the development process and may soon be applied to gene-modified cells [71]. Already, the feasibility of generating sufficient numbers of functional gene-redirected T cells using a semiclosed Wave Bioreactor system has been demonstrated [72].

The vast majority of investigations of genetic redirection has involved the modification of T cells. Some earlier studies were performed in other cell types including monocytes [73], neutrophils, NK cells [74], and dendritic cells [75], but these studies were relatively few. Redirection of cellular function was demonstrated against tumor cells that included cytotoxicity and secretion of inflammatory mediators such as IL-12 and MCP-1. Although these studies demonstrated potential for the use of these cell types for therapeutic purposes, follow-up work has been lacking, perhaps as a result of difficulties in generating and gene-modifying these cell types. However, more recently, there has been a resurgence of interest in genetically redirecting cell types other than T cells, in particular, NK cells.

Three studies have introduced chimeric receptors into the human NK cell line, NK-92. Uherek et al. [76] transduced NK-92 cells retrovirally with anti-erbB2-ζ CAR and demonstrated killing of erbB2-expressing tumor cells in vitro. Muller et al. [77] transduced anti-CD20-CD3ζ CAR retrovirally into NK-92 cells and injected them s.c. simultaneously with human Raji Burkitt's lymphoma cells in NOD/SCID$\gamma_c^{-/-}$ mice, demonstrating extended survival of mice and marked suppression of lymphoma. Transfection of NK-92 cells by electroporation with anti-CD19 CAR mRNA was demonstrated to mediate killing of chronic lymphocytic leukemia cells in vitro [78]. Two studies have transduced primary NK cells. Kruschinski et al. [79] transduced anti-Her-2-CD3ζ-CD28 CARs retrovirally into primary human NK cells and when injected simultaneously with SKOV-3 carcinoma cells in $Rag2^{-/-}$ mice, were able to eradicate the tumor. Pegram et al. [80] transfected primary mouse NK cells with anti-erbB2-CD28-ζ CARs and demonstrated enhanced survival of RAG-$1^{-/-}$ mice injected i.p. with erbB2-RMA tumor cells following treatment on Days 3 and 4 with anti-erbB2 NK cells delivered i.p.

In addition to these studies with NK cells, investigators have expressed NK cell receptors in T cells and demonstrated recognition of stress ligands often overexpressed on tumor cells, thereby widening the range of molecular targets of this approach. T cells have been transduced with human NKG2D-CD3ζ and demonstrated significant survival of mice bearing RMA-retinoic acid early incucible-1 tumors [81] and ovarian tumor [82]. In addition, the NKG2D-CD3ζ T cells were able to lyse T cell lymphoma and myeloma cells [81, 83]. Another way in which NK cell receptors have been used is through the incorporation of their signaling domains into chimeric receptors directed against TAA. In this way, the NK receptor 2B4 was demonstrated to play a costimulatory role in human T cells redirected against CD19 or GD2 [84].

REDIRECTING T CELLS USING TCR TRANSGENES

Earlier work in redirecting T cell function focused primarily on the use of chimeric receptors targeting cell surface-expressed TAA in a non-MHC-restricted manner. It was technically easier to introduce one transgene into cells, and much valuable information about T cell redirection was obtained. However, using this method, it was not possible to direct T

cells against intracellular TAA. More recently, with the development of better expression vectors, there has been considerable interest in redirecting T cells using genes encoding α- and β-chains of TCRs. Redirected T cell function has been demonstrated against a variety of antigens including MART-1 [85], gp100 [86], NY-ESO-1 [87], and CEA [88]. Importantly, the ability of redirected T cells to inhibit tumor growth in mice has been demonstrated in melanoma and prostate cancer model systems [89, 90]. As described below, this approach has also been applied in the clinic with some tumor responses observed in melanoma patients [91].

An important factor for consideration in redirecting T cells using TCR genes is the propensity of introduced TCR genes to mispair with endogenous TCR α- and β-chains, thereby decreasing the expression of TAA-specific TCR. A variety of approaches are being tested for their ability to circumvent this problem, including the use of chimeric TCR, where the extracellular domains of α- and β-chains are linked to intracellular CD3-ζ, which results in correct pairing of TCR transgene products [92]. Function of T cells modified in this way was demonstrated, although these receptors do not use the full suite of signaling molecules normally associated with TCR.

Murine TCR do not pair as readily with human TCR chains, and mouse TAA-specific TCR can be generated more easily in transgenic mice expressing the appropriate restriction element. Preferential pairing of mouse TCR transgene products and enhanced expression levels have been observed using this approach [93]. A potential disadvantage of this approach, however, may be the immunogenic nature of murine TCR components when applied in patients. Other approaches to promote correct pairing of introduced TCR α- and β-chains include the introduction of additional disulfide bonds [94] and the use of γ/δ T cells as recipient cells for genes [95]. Both of these latter approaches have achieved improved pairing, expression, and function against TAA.

ADVANCES IN CLINICAL APPLICATION

One of the most important advances in this field in the past 5 years has been the accelerated initiation of clinical trials. Prior to 2005, only three Phase I trials using genetically redirected T cells had been performed [96–98]. Safety of delivering large numbers ($>10^9$) of gene-redirected T cells was demonstrated in these studies. Some potential measure of activity was noted in a minority of colorectal patients, and transient reduction in TAG72 or CEA serum markers was noted. However, no tumor responses were observed in these advanced-stage patient cohorts. These results and others in this section are summarized in **Table 3**.

One hypothesis for this lack of tumor response is that the redirected T cells do not persist as a result of a lack of stimulation. Pule et al. [35] sought to rectify this problem by generating dual-specific T cells expressing a CAR in EBV-specific T cells, which could be able to respond to persistent EBV antigens present in most individuals. A CAR directed to GD2, an antigen expressed on human neuroblastoma cells, was genetically engineered into PBMC activated with diasialoganglioside and IL-2 (CAR-ATC) and EBV-specific CTL (CAR-CTL). Neuroblastoma patients with an evaluable tumor received an equal number of autologous CAR-ATC and CAR-CTL at a dose of 2×10^7–2×10^8 cells in one injection. There was complete remission of disease in one patient by 16 weeks, and three of seven other patients showed necrosis or temporary regression of tumor. The CAR-CTL persisted beyond 6 weeks (compared with CAR-ATC, which persisted for ~2 weeks) and in higher numbers, probably as a result of stimulation through their native receptor by EBV [35].

Another hypothesis for the lack of tumor response in some previous clinical trials is that silencing for the gene may be occurring. Some previous observations of retroviral vectors suggested that methylation of some consensus cytosine-guanine dinucleotides was responsible for gene silencing [99–102]. Other studies suggested that a repressive histone code and deacetylation contributed to transgene silencing. In a study with T cells modified through a retroviral vector with TCR transgenes, transgene expression was shut down even when gene-modified T cells were detected in vivo [103]. However, transgene silencing was not associated with methylation and was reversed following lymphocyte stimulation. It was concluded that transgene silencing reflected global gene downregulation following in vivo administration and was reversible when T cells were reactivated. This study investigated T cells greater than 8 weeks following adoptive transfer. In another study, gene expression and T cell function were found to persist at least for 4 days after transfer [104]. Long-term persistence of gene-modified T cells was also observed in a study using T cells transduced with OVA-specific TCR, and these cells were also able to respond upon re-encounter of antigen [105].

Persistence of genetically engineered cells can be enhanced by host immunodepletion prior to cell transfer [91]. In this study involving 17 patients with metastatic melanoma, patient PBLs were transduced with α- and β-chains of α-MART-1 TCR. Patients received 1×10^9–8.6×10^{10} autologous α-MART-1 T cells. Two patients achieved complete regression of some tumors and coupled with removal of other tumors, achieved CRs that persisted at the time of writing. Fifteen patients demonstrated durable (>2 months) engraftment at levels ranging from 9% to 56% of total PBLs and after 1 year in two patients [91].

More highly reactive TCRs have been identified and used in a clinical trial, which has shown persistence of gene-modified T cells and tumor regression [106]. A high-avidity human TCR recognizing MART-1 or a mouse TCR recognizing gp100 was engineered into patient PBLs and adoptively transferred to lymphodepleted melanoma patients, followed by high-dose IL-2 therapy for 3 days. The 36 patients received 1×10^9–1.1×10^{11} cells. Of the 20 patients who received MART-1-specific T cells, 30% achieved objective cancer regressions, as did 19% of the 16 patients receiving gp100-specific T cells. Tetramer-positive cells persisted in all patients at high levels (≥10% of total PBLs) 1 month following treatment. However, 55% of patients who received MART-1-specific T cells and 25% who re-

TABLE 3. Published Clinical Trials Using Gene-Redirected T Cells in Cancer

Malignancy	Antigen	Vector	Receptor	Details	Ref.
Colorectal carcinoma (liver mets)	TAG-72	Retrovirus	α-TAG-72-ζ	10 patients. No responses. T cells persisted <10 weeks. Toxicity (hyperbilirubinemia) in two patients	[96]
Colorectal and breast cancer	CEA	Retrovirus, MFG-based GALV-pseudotyped	α-CEA-ζ	Seven patients. Decrease in serum CEA and reduced abdominal pain in one patient	[97]
Ovarian cancer	FBP	Retrovirus, MFG with neomycin selection	α-FBP-γ	14 patients. No responses. HAMA in three of six sera. Tumor localization in one patient	[98]
Neuroblastoma	GD2	SFG retrovirus. GALV-pseudotyped	α-GD2-ζ	Eight patients. One CR, three temporary regression or necrosis of tumor. T cells persist >6 weeks	[35]
Metastatic melanoma	MART-1	MFG retrovirus	MART-1 α- and β- chains TCR	17 patients: two objective cancer regression. 15 durable engraftment of T cells >2 months	[91]
Metastatic melanoma	MART-1	pMSGV1 retrovirus	MART-1 α- and β- chains TCR	20 patients: 30% objective cancer regression. 55% developed uveitis, 80% rashes	[106]
Metastatic melanoma	gp100	pMSGV1 retrovirus	gp100 (154)-α/β TCR	16 patients: 19% objective cancer regression. 25% developed uveitis, 80% rashes	[106]
Renal cell carcinoma	CAIX	LXSN retrovirus	G250-γ	Three patients: No responses; Grades 2–4 liver toxicities	[107]
Neuroblastoma	L1-CAM (CD171)	Naked DNA electrotransfer	CE7R-ζ	Six patients: stable (one >4.5 year) and partial responses	[108]
Indolent B cell or mantle cell lymphoma	CD20	Naked DNA electroporation of pcDNAneo plasmid	α-CD20-ζ	Seven patients: two CR, one PR, four SD. T cells persisted 5–9 weeks in four patients	[109]

FBP, Folate-binding protein; HAMA, human anti-mouse antibody; pMSGV1, MSCV-based splice-gag vector 1; LTR-X-SV4D-neo, long terminal repeat-X-simian virus 40 early promoter-neomycin transferase gene; MFG, retroviral vector based on the Moloney murine leukemia virus (MoMLV); L1-CAM, L1-cell adhesion molecule; LXSN, LTR from MoMLV-SV4D fragment containing early promoter-Neo.

ceived gp100 T cells developed uveitis, likely as a result of TAA expression in cells of the retina. In addition, 81% of the 36 patients developed skin rashes, which subsided within days without treatment.

Destruction of normal tissue has also been detected in another study [107], in which CAIX on renal cell carcinoma was targeted by G250-CAR-modified T cells. Three patients received 2×10^7 cells on Day 1, 2×10^8 cells on Day 2, and 2×10^9 cells on Days 3–5. After four to five infusions, Grades 2–4 liver toxicity developed, treatment was stopped, and corticosteroid treatment was given. Biopsy of the liver showed CAIX expression on bile duct epithelial cells, and it was presumed that these had been attacked by the G250-CAR T cells in the three patients. In addition, all three patients developed low levels of antibody to the murine scFv250 receptor (including anti-idiotype antibodies), between 37 and 100 days following ACT, a problem that may have limited the efficacy of ACT in this and other studies.

The problem of antitransgene immune response was also suggested to occur in a study in which six patients received three escalating doses of autologous CE7R-CAR CD8 T cell clones directed against L1-cell adhesion molecule (CD171) on metastatic neuroblastoma [108]. Patients received 10^8 cells/m^2 on Day 0, 10^9 cells/m^2 on Day 14, and 10^{10} cells/m^2 on Day 28, but cells in peripheral blood were detectable only to 1 week after the first and second infusions in a proportion of patients and not after 1 week in the only patient treated with a third infusion, in which cells had been detected previously. Patients exhibited stable (one prolonged survival for 4.5 years) or PRs before all succumbing to disease.

In another trial in patients [109] targeting refractory indolent B cell lymphoma or mantle cell lymphoma with autologous CD20-CAR T cells, plus low dose IL-2 (for 14 days), the engineered T cells persisted 5–9 weeks in vivo in several patients and were able to induce tumor regression. Of seven patients treated, two showed CRs, one partially responded, and four had SD, following three infusions of cells, given in escalating doses (10^8 cells/m^2, 10^9 cells/m^2, and 3.3×10^9 cells/m^2) 2–5 days apart. The authors pro-

posed that the shorter culture time ex vivo was a reason for cells persisting, although lymphodepletion of the patients prior to therapy and IL-2 administration may have also contributed to cell persistence. These were also factors for engineered T cell persistence noted in a study mentioned previously [91]. Reasons given for tumor relapse were hypothesized to be: Numbers of T cells may be insufficient; possible CD20 antigen competition from normal B cells; possible inadequate localization of T cells; and poor killing of T cells as a result of low CAR expression and lack of costimulation (reflected in in vitro killing assays).

Of importance with respect to clinical translation of T cells, redirected in a non-MHC-restricted manner, is the effect of soluble antigen in patient serum or interstitial fluid, in addition to the effect of immune responses against the transgene product, particularly those composed of murine scFv. Surprisingly, the presence of soluble antigen has not been found to inhibit the function of gene-redirected T cells against tumor cells [15, 110–113]. It is not clear why soluble antigen does not inhibit T cell function, although it may be a result of an enhanced avidity of T cell-expressed scFv for surface-displayed antigen compared with the relatively low affinity of the interaction of scFv with soluble antigen. Indeed, this may be an argument for using lower-affinity scFv in chimeric receptor design if levels of soluble antigen are anticipated in patients.

Therefore, many advances have been made in clinical translation of redirected T cells, and continuing developments in the production of T cells with optimal transduction frequencies and high transgene expression levels will no doubt enhance the effectiveness of this approach [114, 115].

CONCLUDING REMARKS

As interest grows in the field of genetic redirection, we are seeing an expansion of the range of antigens targeted in addition to the more common antigens targeted in most studies to date that included Her-2, CEA, and CD19 on breast and colon cancers and lymphoma, respectively. Recent studies have shown an expansion of receptors against other antigen targets on solid tumors, such as epidermal growth factor receptor vIII on glioblastoma [116], prostate stem cell antigen on prostate cancer [117], fetal acetylcholine receptor on rhabdomyosarcoma [118], and MUC1 on breast cancer cells [17]. In addition, novel, antigenic targets on hematological cancers have been addressed, such as CD38 on non-Hodgkin's lymphoma [119].

Although the focus of this review has been on the use of genetically redirected T cells against cancer, other options for the use of genetic redirection are emerging. In particular, novel approaches to target self-reactive T cells are being pursued in which T cells are gene-modified to express a chimeric HLA molecule able to mediate destruction of myelin basic protein-specific T cells that can be responsible for the autoimmune condition, multiple sclerosis [120]. Another novel application of genetic redirection involved the genetic modification of Tregs, which were demonstrated to inhibit autoimmune disease in a mouse model using OVA-specific mouse T cells [121]. Thus, the concept of redirection can be used to accentuate or down-regulate immune responses.

Much progress has been made in the field, but some problems still need to be overcome. Trafficking remains a major hurdle, and pioneering studies addressing this issue through modifying tumor-specific T cells with chemokine receptors specific for chemokines secreted by tumors [122, 123] may one day lead to enhanced localization of transferred T cells to sites of malignant disease.

Toxicity of gene-redirected T cells has been observed in some clinical trials as a result of on-target autoimmunity mediated by transferred cells against normal tissue expressing TAA. This continues to be of concern, and strategies aimed at eliminating gene-modified T cells through the use of suicide genes continue to be pursued [124, 125]. However, the elimination of transferred cells is counterproductive in therapy, and the field would benefit from new approaches to enhance the specificity of T cells for tumors that could reduce autoimmune consequences and retain reactivity against tumor.

Inherent in the genetic redirection approach is the derivation of human TAA-redirecting genes from mice, as humans are frequently deeply tolerant of TAA, and it is difficult to derive tumor-specific receptors from humans. This raises concerns of immunogenicity of transgene products, which has been observed in the clinic [98, 107]. This may not always be the case, but these concerns are being addressed through the use of humanized components in transgenes where possible [14, 16].

The field of genetic redirection has advanced quickly from its beginnings in 1989 [126, 127]. Preliminary results and the obvious potential for clinical application have led to increasing interest, and over 50 research groups are now involved in various aspects of this area. The total monetary investment in this area is difficult to calculate, but it can be estimated to be many millions of dollars annually. Much productive cooperation is evident in this area, and the field will no doubt benefit from collaborative, coordinated approaches similar to the large collection of investigators funded by the European Union under a Framework Program. The generation of tumor-reactive T cells with enhanced functional and survival abilities, together with an ability to localize to tumor sites, holds much promise for the treatment of cancer.

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