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Ex vivo gene transfer for improved adoptive immunotherapy of cancer

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Adoptive immunotherapy is an appealing approach to cancer treatment, with the potential for more precise targeting and reduced toxicity. While early clinical trial data using adoptive T cells against post-transplant virus-associated hematologic malignancies, lymphoma and melanoma have been promising, treating other solid tumors has proven to be more challenging. Adoptive lymphocytes have been genetically modified in many ways to improve activity and circumvent tumor evasion, including transfer of transgenic T-cell receptors and chimeric antigen receptors to redirect T cell and natural killer cell antigen specificity. Gene transfer may also allow expression of homeostatic cytokines or their receptors to overcome the lack of stimulatory signals or expression of dominant-negative receptors for inhibitory cytokines to compensate for an immunosuppressive tumor milieu. In addition, suicide genes can install a 'safety switch' on adoptively transferred cells to allow ablation if necessary. Although further refinement and validation are necessary, these genetic modification strategies offer hope for significant improvements in cancer immunotherapy.

INTRODUCTION

Over the past few decades, advances in cancer immunology have been increasingly translated into clinical testing of immune-based approaches to cancer treatment. By exploiting the exquisite specificity of the immune system, cancer immunotherapy offers the potential for more precise targeting and reduced toxicity compared with traditional chemotherapy. Numerous immune-based therapies have been evaluated, including monoclonal antibody treatment, cell- and DNA-based vaccines and adoptive transfer of natural killer (NK) and T lymphocytes (1).

Adoptive cell therapies have achieved promising results in clinical trials. *Ex vivo*-expanded autologous tumor-infiltrating lymphocytes (TILs) have induced regression in patients with metastatic melanoma (2), and virus-specific T cells have been shown to be effective in treatment of virus-associated hematologic malignancies (3). Unmanipulated donor lymphocyte infusions have been widely used to treat patients with relapsed hematologic malignancies after allogeneic hemopoietic stem cell transplantation (4). Allogeneic NK cell therapies have also shown activity in patients with acute myeloid leukemia with little or no toxicity (5). Although these results are promising, other studies, particularly in solid tumors, have shown less impressive responses, likely due to a combination of immune evasion by tumors and poor function of adoptively transferred cells. In this review, we discuss a variety of *ex vivo* genetic manipulations designed to overcome tumor evasion and improve function of adoptively transferred lymphocytes.

GENETIC AUGMENTATION OF ADOPTIVE T CELLS

The development of T cell immunotherapy for cancer faces significant challenges. Most tumor-associated antigens that serve as targets for T cell therapies are self-proteins which are weakly antigenic, due to the development of tolerance. Therefore, endogenous antigen-specific T cells isolated from patients with cancer may be present in low frequency and will likely have low-affinity T-cell receptors (TCRs) due to negative selection during maturation, (6) limiting their cytotoxic activity against tumor cells. In addition, tumors have evolved to evade the immune system through both passive and active mechanisms (7). Tumors can passively avoid T cell targeting by downregulating major histocompatibility

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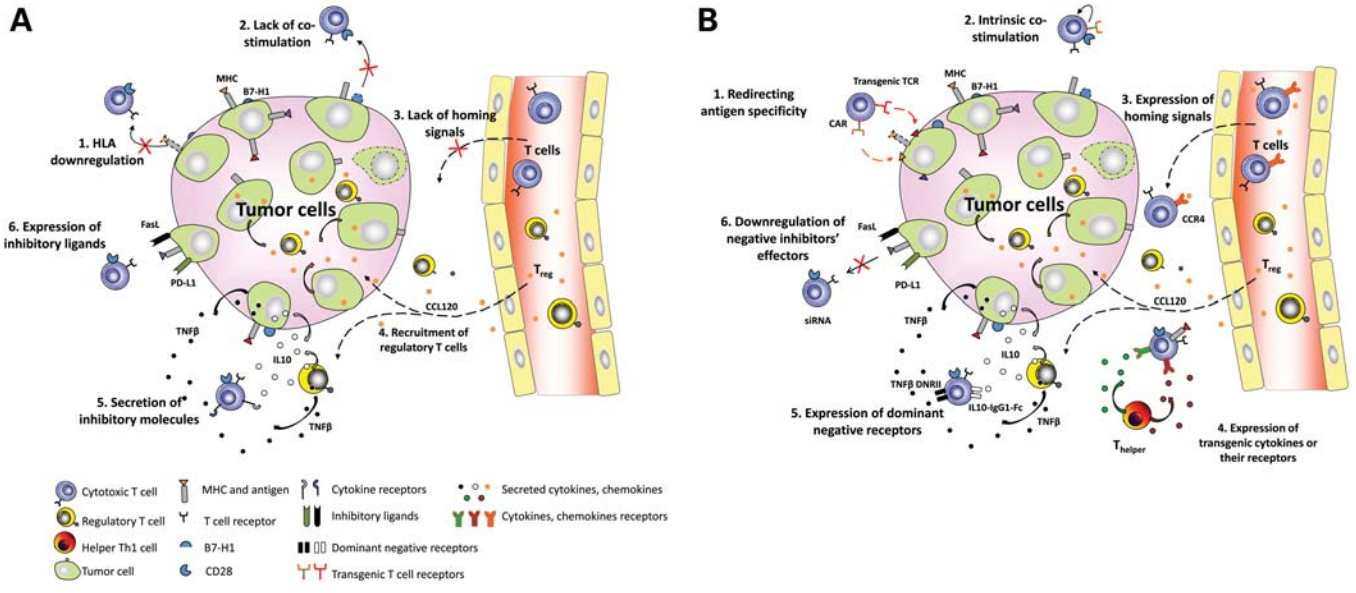

Figure 1. (**A**) Tumor evasion mechanisms. (1) Downregulation of HLA; (2) lack of co-stimulation; (3) lack of homing signals for effector T cells; (4) recruitment of Treg and Th2 subsets; (5) secretion of immunosupressive molecules; (6) expression of inhibitory ligands. (**B**) Genetic modifications of adoptive T cells to overcome tumor evasion. (1) Redirecting T cell antigen specificity with transgenic TCRs or bypassing the need for HLA with CARs; (2) intrinsic co-stimulatory signals; (3) expression of homing signals; (4) expression of transgenic cytokines or their receptors; (5) expression of dominant-negative receptors; (6) down-regulation of negative effectors' signaling cascade.

complex (MHC) or co-stimulatory molecules. Tumors actively antagonize the immune response by expressing inhibitory ligands and secreted factors. All of these evasion mechanisms can potentially be overcome by genetic modification of adoptively transferred T cells as summarized in Figure 1. Redirection of T cells to tumor antigens by expressing transgenic TCRs or chimeric antigen receptors (CARs) can bypass negative selection and yield much higher levels of tumor-specific cells, with reduced dependence on co-stimulation and target cell MHC expression. Lymphocyte activity can be restored by transgenic expression of activating cytokines such as interleukin-2 (IL-2) and IL-15, and T cells can also be made resistant to suppressive factors by overexpression of dominant-negative receptors (8,9). Homing of T cells to the tumor site can be improved by transgenic expression of receptors for tumor-secreted chemokines (10). T cells can also be provided with resistance to immunosuppressive drugs by genetic modification (11).

Redirecting T cell specificity with transgenic TCRs

Tumor-specific T cells are frequently negatively selected during development because tumor antigens are typically recognized as 'self'. Those tumor-specific T cells that survive have low affinity TCRs, are often anergic and consequently have poor tumor-killing activity. However, tumor specificity can be conferred on non-tumor-specific T cells by expression of transgenic TCRs specific for antigens expressed on tumor cells. This approach permits large numbers of highly active, tumor-specific T cells to be generated in a short period of time. Transgenic TCRs are typically generated by cloning the α and β subunits of class I human leukocyte antigen (HLA)-restricted TCRs from tumor-reactive cytotoxic T cell clones. The cloned TCR is then transferred *ex vivo* into patient T cells using integrating retroviral or lentiviral vectors or, in some studies, plasmids.

In one example of this strategy, Morgan *et al.* (12) treated metastatic melanoma patients with autologous T cells genetically modified with a retroviral vector to express a transgenic TCR targeting melanoma antigen recognized by T cells (MART)-1. The infused cells persisted up to a year and objective regression of tumor was seen in 4 of 31 patients. None experienced clinical signs of graft-versus-host disease (GvHD) or melanocyte toxicity (13).

One of the advantages of transgenic TCR expression is the ability to optimize affinity between TCRs and their target antigens to improve activation. In follow-up studies, higher avidity TCRs recognizing MART-1 and the human melanocyte differentiation antigen gp100 were generated. Objective cancer regression was observed in 19–30% of patients who received these higher affinity TCRs (14). However, in the study with the higher affinity TCR targeting MART, some patients also experienced toxicity to normal melanocytes in the skin, eye and ear, requiring administration of steroids to treat hearing loss and uveitis (14). Another example of the effects of highly avid TCRs on normal tissue was observed in a study in which autologous T cells expressing a murine TCR against human carcinoembryonic antigen (CEA) were administered to three patients with metastatic colorectal cancer (15). Although one patient had an objective tumor regression, all three developed an inflammatory colitis, likely due to recognition of low levels of CEA in normal colonic mucosa.

Using a large pool of non-specific T cells as a source for adoptive transfer also has some potential drawbacks. Although these T cells gain new antigen specificity through the presence of a transgenic TCR, their original TCRs are still functional. Studies in murine models with TCR gene transfer have

Table 1. Clinical trials with T cells expressing CARs

Reference	Type of T cell	CAR construct	Cell dose	Targeted cancer/ number of patients	Serious adverse effects	Persistence	Responses
Kershaw *et al.* (57)	OKT3-activated T cells (8 patients) Alloantigen activated T cells (6 patients)	α-Folate receptor CAR retroviral vector with neomycin resistance gene	3×10^9 to 5×10^{10} (OKT3) 4.0×10^9 to 1.69×10^{11} (alloantigen)	Ovarian cancer/14 patients	None (IL-2 effects in cohort receiving high dose IL-2)	Up to 3 weeks in 13 patients 12 months in 1 patient	None
Park *et al.* (58)	OKT3-activated T cells (clones)	CE7R-CAR plasmid with HyTK	10^8 to 10^9 cells/m^2	Neuroblastoma/6 patients	None	1–42 days	1 of 6 with evaluable tumor had a PR
Lamers *et al.* (59)	OKT3-activated T cells	G250-CAR retroviral vector	0.38 to 2.13×10^9	Renal cancer	Grade 2–4 liver toxicity	Up to 53 days	None
Till *et al.* (60)	OKT3-activated T cells (clones in 3 and lines in 4)	CD20-CAR plasmid with neomycin resistance gene	10^8 to 3.3×10^9 cells/m^2	CD20+ low grade B cell lymphoma/7 patients	None	1–3 weeks (clones) 5–9 weeks (T cell lines and low dose IL-2)	4 of 5 with evaluable disease had stable disease and one a PR
Pule *et al.* (22)	OKT3-activated T cells and EBV-specific CTLs	GD2-CAR retroviral vector	2×10^7 to 2×10^8 cells/m^2 of each product	Neuroblastoma/11 patients	None	Up to 3 weeks for the activated T cells and up to 6 months for CTLs	4 of 8 with evaluable tumor had necrosis or responses with 1 CR
Jensen *et al.* (61)	OKT3-activated T cells	CD19 or CD20 CAR plasmid with HyTK	Up to 2×10^9 cells/m^2	Diffuse large cell lymphoma	None	24 h to 7 days	2 of 4 patients with antitransgene immune rejection
Morgan *et al.* (31)	OKT3-activated T cells	Her-2/neu retroviral vector with CD28 and 41BB	10^{10} cells	Colon cancer	Patient died after 5 days due to multiorgan failure		
Brentjens *et al.* (32)	OKT3-activated T cells	CD19-CAR retroviral vector with CD28	$1.2–3 \times 10^8$ cells/kg	CLL	1/6 patient died after 2 days due to sepsis-like syndrome		
Kochenderfer *et al.* (28)	OKT3-activated T cells	CD19-CAR retroviral vector with CD28	10^8 cells day 1, 3×10^8 cells day 2, IL-2 q8hr × 8	Follicular lymphoma	None	27 weeks after infusion	1 out of 1 patient with partial remission at 39 weeks

CR, complete remission; PR, partial remission; HyTK, hygromycin thymidine kinase.

shown that mispairing of endogenous and introduced TCR chains can lead to off-target effects or GvHD (16). However, GvHD has not occurred in the >100 human patients who have received autologous T cells transduced with human or mouse TCRs, illustrating that preclinical models do not always predict clinical effects in humans (17).

While transgenic TCRs overcome the problems of low number and avidity of cancer-specific T cells, they are still HLA restricted, limiting the treatment scope of each transgenic TCR to MHC-matched tumors and tumors whose HLA antigens have not been downregulated. In addition, they can dimerize with native TCR, leading to loss of function. An alternative approach that avoids some of these issues is the use of transgenic CARs instead of TCRs.

Redirecting T cell specificity with CARs

CARs are synthetic constructs that can confer target antigen specificity without HLA restriction and avoid development of hybrid TCRs. A CAR contains an extracellular antigen-binding domain, a transmembrane region and a signaling endodomain. The extracellular domain is typically a single-chain variable fragment (scFv) derived from a tumor-specific monoclonal antibody. The hinge/spacer region between the binding and transmembrane domains permits flexibility and increases the CAR's access to antigens by allowing it to protrude higher than other molecules on the plasma membrane (18). The endodomain consists of one or more intracellular signaling components of the TCR complex.

There are several advantages to using an antibody-derived domain for antigen recognition. Antibodies are not dependent on MHC presentation, so CARs can recognize both protein-derived peptides and surface proteins with varying degrees of post-translational modification (19). In addition, antibodies bind antigens with much greater affinity than TCRs, permitting the formation of a more stable immunological synapse (20).

Current CARs can be grouped into three generations, with progressively increasing co-stimulatory activity. These differ primarily in the structure of the signaling endodomain. First-generation CARs contain a single signaling unit derived from the CD3ζ chain or FcεRIγ IgG receptor (21). Overall, modest clinical responses have been achieved in studies where first-generation CARs have been transferred to adoptively transferred lymphocytes for treatment of lymphoma, neuroblastoma, ovarian and renal cancer as summarized in Table 1. The results from these studies suggest that CAR signaling through the CD3ζ chain alone is not adequate to achieve full T cell activation. Pule *et al*. (22) attempted to overcome this limitation and enhance *in vivo* T cell persistence by expressing a tumor-specific CAR in Epstein–Barr virus (EBV)-specific T cells. Unlike non-specific T cells, EBV-specific T cells experience additional co-stimulation when encountering EBV antigens *in vivo*. These investigators expressed distinguishable CARs targeting the neuroblastoma antigen GD2 in EBV-specific T cells and T cells non-specifically activated with CD3 antibody. They found better persistence of the EBV-specific cytotoxic T lymphocytes (CTLs) and an encouraging response rate, with tumor regression or necrosis in four out of eight patients with active disease.

Because full activation and proliferation of T cells require signaling through the CD28 receptor, in second-generation CARs the CD28 intracellular domain is inserted proximal to the CD3ζ endodomain to enhance the stimulatory effects of the CAR (23). The synergistic effect of combining the two signaling domains results in increased proliferation, decreased activation-induced apoptosis and increased cytokine secretion in response to antigen (19).

The improvement in T cell function with addition of the CD28 endodomain to the CAR encouraged further addition of other signaling sequences such as CD137 (4-1BB) and CD134 (OX40) to third-generation CARs (24,25). In preclinical studies, these second- and third-generation CARs showed superior ability to eliminate tumor xenograft models (26) and are currently under evaluation in the clinic (27). In one recent report, a complete response was seen in a patient with high-grade progressive follicular lymphoma who received T cells transduced with an anti-CD19 CAR (FMC63 antibody-CD28-CD3ζ) (28). A summary of other active clinical trials involving all three generations of CARs as of 2010 is listed by Cooper and colleagues (29) in a recent review.

While second- and third-generation CARs showed superior ability to target tumor xenografts (26), their potentially supra-physiological signal is also a source of concern. By lowering the activation threshold, later generation CARs have a higher risk for low-avidity off-target binding (30) and may also produce an overly vigorous activation signal, with on-target binding resulting in adverse effects from cytokine release. Morgan *et al*. (31) recently reported an adverse event when a patient with metastatic colon cancer receiving autologous T cells transduced with an ERBB2-specific CAR (herceptin-CD28-CD137-CD3ζ) rapidly developed acute respiratory distress syndrome within 15 min after infusion, and died 5 days after treatment despite intensive medical intervention. This event was associated with very high cytokine levels attributed to cross-reactivity with normal tissues expressing HER2. In a second report, a patient with bulky chronic lymphocytic leukemia developed fever and hypotension with elevated cytokine levels within 24 h of receiving autologous T cells transduced with a CD19-28ζ CAR (32). In this case, low-grade sepsis was considered the most likely cause, but elevated cytokine levels may also have enhanced the *in vivo* activation of modified T cells. It is worth noting that similar 'on-target, off-organ' adverse events have been observed in other studies using native T cells (33), suggesting that this is not solely due to genetic modifications. Modification to reduce T cell dose, as well as splitting infusions across multiple days, has been suggested to reduce the risk of such serious adverse events (31,32).

Supplying homeostatic cytokines

Administration of cytokines such as IL-2 and IL-15 can overcome the lack of stimulatory signals within the tumor microenvironment and enhance antitumor effects of adoptively transferred T cells (9). However, systemic toxicity and expansion of regulatory T cells limit the use of these cytokines when administered systemically. An alternative approach is to modify T cells to express cytokine or cytokine receptor genes that recapitulate the milieu found during lymphoid

regeneration and restoration of homeostasis. Transgenic expression of IL-2 and IL-15 has been shown to increase antigen-specific T cell expansion *in vivo* and enhance antitumor activity without systemic toxicity in preclinical models (34). For cytokines like IL-7, whose receptors are downregulated upon exposure, T cells can be modified to stably express IL-7Rα (35). Antigen-specific T cells transduced with transgenic cytokines or cytokine receptors showed improved antitumor effects in animal models (36,37). However, in a clinical trial of adoptive transfer of TILs to treat metastatic melanoma, overexpression of IL-2 did not increase *in vivo* persistence or overall clinical effectiveness compared with unmodified T cells (38).

Enhancing homing signals

It is well established that tumors secrete chemokines that selectively recruit only Th2 and regulatory subsets of T cells. In murine studies, transgenic expression of CCR4 on CD8(+) T cells that are specific for the Hodgkin's lymphoma marker CD30 helped to direct them to the tumor site while retaining their cytotoxic function and cytokine secretion *in vivo* (10). Similar enhancements in antitumor effects were obtained when T cells were co-transduced with anti-GD2 CAR and the receptor for CCL2, a chemokine which is highly secreted by neuroblastoma cells (39). At present, strategies involving genetic modification of T cells to enhance homing signals are still in the preclinical phase and need to consider chemokines produced by tumor stromal cells as well as tumor cells.

Resisting hostile tumor environment

Even after migration to tumor tissues, T cells continue to encounter challenges such as suppressive cytokines and inhibitory ligands produced by tumors and their stroma. One of the most potent inhibitory cytokines is transforming growth factor (TGF)β, and expression of a dominant-negative TGFβ type II receptor in T cells rendered them resistant to TGFβ-secreting EBV-positive lymphoma in *in vitro* studies and murine models (40). This approach is currently being evaluated in a phase I clinical trial. Similar strategies to confer resistance to other negative signals such as IL-10 are also under evaluation (41). T cells can also be protected from apoptosis by knockdown of inhibitory ligands such as Fas using retrovirally encoded small interfering RNAs (siRNAs) (42).

Resisting concomitant immunosuppressive therapy

Adoptive transfer of EBV-specific T cells has been shown to be safe and efficacious in preventing post-transplantation lymphoproliferative diseases (43,44). However, most solid organ transplant recipients require continuous administration of immunosuppressive drugs to prevent graft rejection, which can limit the efficacy and long-term persistence of adoptively transferred T cells (45). By knocking down FK506-binding protein (FKBP12) with a stably expressed siRNA, De Angelis *et al*. (11) enabled EBV-specific T cells to continue to expand and maintain their cytotoxicity in the presence of FK506 in a xenogenic mouse model.

Installing a safety switch on adoptively transferred T cells

Along with enhancing potency, sustainability and resistance to suppressive signals, genetic modification can also install a 'safety switch' so that genetically modified T cells can be ablated if adverse effects occur. One of the most well-studied suicide strategies is the herpes simplex viral thymidine kinase (*TK*) gene. In the presence of ganciclovir or acyclovir prodrugs, TK will phosphorylate the substrate to produce a toxic product that can interfere with the infused T cell's DNA synthesis. Thus, prodrugs can be administered when transferred cells have deleterious effects. T cells genetically modified with *TK* have been infused in clinical trials to >120 patients after allogeneic stem cell transplants (13) and have controlled GvHD in every occurrence, confirming the ability of this approach to control alloreactivity. The strategy is now being evaluated in phase III clinical trials in preventing GvHD when donor lymphocytes are infused following stem cell transplantation (46).

There are, however, several shortcomings to using *TK* as a suicide gene. One is its immunogenicity, which might lead to premature clearance of infused cells. Second is the removal of a therapeutically valuable drug as an option in treating viral infection post-transplant. Another concern is the time required to ablate infused cells, usually days to weeks. This is acceptable in treatment of GvHD, but would be inadequate in cases where infused cells cause acute on- or off-target toxicity. An attractive alternative suicide strategy is the inducible *Caspase9* transgene (*iCaspase9*), which is non-immunogenic and rapidly produces apoptosis even in non-dividing cells (47). iCaspase9 is trigged upon administration of a small molecule dimerizer, AP20187, yields >90% apoptosis and is currently being tested in a phase I study.

GENETIC MODIFICATIONS OF NK CELLS

In recent years, interest in using NK cells for cancer immunotherapy has been increasing. In a study in which acute myeloid leukemia patients were infused with freshly isolated and expanded haploidentical, related-donor NK cells, complete hematologic remissions were seen in 5 out of 19 patients (5). Unlike T cells, NK cells are not antigen-specific, and their cytotoxicity is directed at a number of targets on cells expressing low levels of MHC class I (48). Genetic modification with CARs can retarget NK cells specifically to tumor antigens, as demonstrated by reports of improved killing of a Her-2/neu breast cancer cell line and CD20-positive lymphoma (49,50). A recent comparison between the classical CAR endodomain of CD28-CD3ζ versus 2B4 (CD244), an important regulator of NK cell activation, showed that 2B4 signaling on its own can only trigger a degranulation response but not full NK cell activation (51). However, addition of the 2B4 endodomain proximally to CD3ζ significantly enhances NK cell activation as well as cytokine secretion in a tumor-specific manner (51). As with T cells, genetic modifications to produce cytokines (IL-2, IL-12 and IL-15) can increase NK cell *in vivo* survival and antitumor activities (52–54). NK cells may be an attractive platform for redirecting antigen specificity modification after allogeneic transplant due to their lower potential to induce GvHD compared with T cells. However, NK cell

trials are at an earlier stage compared with T cells, mostly due to technical difficulties in NK cell *in vitro* expansion (29). This limitation may be overcome by development of an efficient *ex vivo* culturing system using gas-permeable cultureware (55) and artificial antigen-presenting cells (56).

CONCLUSION

Potentially immunogenic tumors have evolved many different immune evasion strategies, including reduced antigen presentation and inhibition of effector lymphocyte function. Genetic modifications of adoptively transferred cells may be able to overcome these challenges and improve clinical outcomes. Early clinical reports have been promising; however, there have also been increased rates of both on- and off-target toxicities, some of which may be amenable to dose optimization. Inclusion of a safety switch with other genetic modifications can further ensure long-term safety of adoptively transferred lymphocytes. With these novel approaches, the results of on-going and future clinical trials with genetically modified NK and T cells promise to be exciting.

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