GENE-MODIFIED T CELLS: CHALLENGES IN CLINICAL TRIAL DESIGN WITH NOVEL RECEPTORS

Hilton Rockville Hotel & Executive Center

Meeting Materials

June 15, 2010

NATIONAL INSTITUTES OF HEALTH (NIH)
SAFETY SYMPOSIUM

GENE-MODIFIED T CELLS: CHALLENGES IN CLINICAL TRIAL DESIGN

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8:00 AM **Welcome and Opening Remarks**

Scott E. Strome, M.D., Member, Recombinant DNA Advisory Committee
John A. Zaia, M.D., Member, Recombinant DNA Advisory Committee
Daniel Takefman, Ph.D., Federal Food and Drug Administration, Rockville, MD

8:15 AM **Evolution of Trial Design using Gene Modified T Cells**

Presenter: Richard P. Junghans, M.D., Ph.D., Roger Williams Hospital, Providence, RI

8:35 AM **Serious Adverse Events in Trials Utilizing Chimeric Antigen Receptors: Lessons for Trial Design**

Presenters: Steven A. Rosenberg, M.D., Ph.D., National Cancer Institute, NIH, Bethesda, MD
Renier J. Brentjens, M.D., Ph.D., Memorial Sloan-Kettering Cancer Center, New York, NY

9:15 AM **Questions**

9:25 AM **Designing Novel T Cells: Balancing Efficacy and Toxicity**

Choosing an Appropriate Target and Targeting Strategy

- What criteria were used in choosing the target?
- What approach was employed in evaluating potential off target toxicities?
- How did that data inform the starting dose?
- Were there unexpected toxicities?

***Engineered T Cell Receptors*:**

Solid Tumor Antigens (MART-1, gp-100, p53, ESO-1)

Presenter: Steven A. Rosenberg, M.D., Ph.D.

9:45 AM **Break**

9:55 AM ***Chimeric Antigen Receptors (CARs)*:**

Solid Tumor Antigens (CEA, PSMA, carbonic-anhydrase IX, Anti-Lewis Y, Tag-72, Her2/neu)

Presenters: Richard P. Junghans, M.D., Ph.D.
Mitchell H. Finer, Ph.D., Chief Scientific Officer, Genetix Pharmaceuticals, Cambridge, MA
Stephen M. Gottschalk, M.D., Texas Children's Cancer Center, Houston, TX

Hematologic Antigens (CD19, CD20)

Presenters: Michael C.V. Jensen, M.D., City of Hope, Duarte, CA
Dario Campana, M.D., St. Jude Children's Research Hospital, Memphis, TN

11:10 AM **Discussion**

Moderator: Laurence J. Cooper, M.D., Ph.D., M.D. Anderson Cancer Center, Houston, TX

- In evaluating the potential for off-target expression, how do the experiences with anti-Her2/neu, anti-CEA, anti-CD+ 19, and anti-carbonic-anhydrase IX CARs inform the selection of future targets?
- For CARs that target antigens such as CD20, what is the affect on long term immunity if such cells persist?
- Are there additional features that can be added to enhance safety?
    - Selective Promoters
    - Suicide gene

11:55 AM **Public Comment**

12:00 PM **Lunch**

12:45 PM ***Co-signaling Moieties***

Presenter: Carl H. June, M.D., University of Pennsylvania, Philadelphia, PA
Panelists: George Coukos, M.D., Ph.D., University of Pennsylvania, Philadelphia, PA
Michel Sadelain, M.D., Ph.D., Memorial Sloan-Kettering Cancer Center, New York, NY
Gianpietro Dotti, M.D., Baylor College of Medicine, Houston, TX

- How do co-signaling moieties change the cytokines that are released?
- What is the potential for reverse signaling through 4-1BB or B7-1 (CD80)?
- Can the strength of T cell activation and co-stimulation be titrated?
- What are the considerations in using naturally inducible versus constitutively expressed molecules?

1:30 PM **Preconditioning Regimens: Improved Engraftment vs. Systemic Toxicity**

Presenter: Steven A. Rosenberg, M.D., Ph.D.

Panelists: Laurence J. Cooper, M.D., Ph.D.
Richard E. Champlin, M.D., M.D. Anderson Cancer Center, Houston, TX

- What is the evidence for efficacy and improved engraftment with lymphodepletion?
- What is the anti-tumor effect of lymphodepletion alone?
- How does the preconditioning used for T cell protocols compare to that used for bone marrow transplantation and in particular regarding potential toxicity?
- Are there modifications to preconditioning that should be considered in initial trials with new receptors?

2:30 PM **Break**

2:45 PM **Cytokine Support for Gene Engineered T Cells**

Presenter: Cassian Yee, M.D., Fred Hutchinson Cancer Research Center, Seattle, WA *(via teleconference)*

Panelists: Michael C.V. Jensen, M.D.
Carl H. June, M.D.

- What is the optimum dose to balance T cell support and toxicity?
- How does one discern adverse events related to the cytokine support from toxicity related to the T cells?

3:15 PM **Designing Clinical Trials with New Receptors and Endodomains**

Moderators: Scott E. Strome, M.D.
John A. Zaia, M.D.

Dose Escalation

- What are alternative methods of dose escalation and how should initial dose escalation be based on target antigen?
- What do we understand about persistence of cells and how should this guide dosing intervals?

Panelists: Antoni Ribas, M.D., University of California School of Medicine, Los Angeles, CA
Stephen M. Gottschalk, M.D.
Richard P. Junghans, M.D., Ph.D.

4:00 PM ***Subject Selection: Optimizing Safety***

- Should certain subjects who are at higher risk be excluded from initial trials (e.g., elderly, pulmonary disease, high disease burden)?

Panelists: Renier J. Brentjens, M.D., Ph.D.
Oliver W. Press, M.D., Ph.D., Seattle Cancer Care Alliance, Seattle, WA

4:20 PM **Public Comment**

4:30 PM ***Points to Consider for Trial Design Involving New Receptors: Initial Dose, Co-signaling Moieties, and Subject Selection***

- Choosing an Appropriate Target
- Initial Dose Selection
- Monitoring Strategies
- Preconditioning Options
- Subject Selection

All Participants

5:30 PM **Adjourn**

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REVIEW ARTICLE

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

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

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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 idiotype 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). 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.

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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-α and -β chains from a donor T cell to a recipient T cell of any specificity. Retroviral vectors containing TCR-α and -β 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-α/β 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-α/β transgene transduction to oligoclonal or γδ 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-α and -β 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 α- and β-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-ζ or FcεRI-γ. 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-ζ 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-ζ	Human	*In vivo*	(11)
		scFv-CD28-CD137-ζ	Human	*In vivo*	(12)
	CD20	scFv-4-1BB-ζ	Human	*In vitro*	(13)
		scFv-ζ	Human	*In vitro*	(14)
		scFv-CD28-ζ	Human	*In vitro*	(15)
		scFv-SP163-CD28-CD137-ζ	Human	*In vivo*	(16)
	B lymphoma idiotype	scFv-γ	Mouse	*In vitro*	(17)
Colon	CEA	scFv-ζ	Mouse, human	*In vivo*	(18)
		scFv-ζ	Human	*In vitro*	(19)
		scFv-CD28-ζ	Mouse, human	*In vivo*	(20)
		scFv-CD28-ζ	Human	*In vitro*	(21)
		scFv-ζ	Human	Clinical	(22)
	EGP40	scFv-γ	Human	*In vitro*	(23)
Ovarian	FBP	scFv-γ	Mouse	*In vivo*	(24)
		scFv-γ	Human	*In vitro*	(25)
Breast and associated	erbB2,3,4	scFv-ζ	Mouse, human	*In vivo*	(26)
		scFv-γ	Mouse	*In vivo*	(27)
		scFv-CD28-ζ	Mouse, human	*In vivo*	(28)
		scFv-CD28-ζ	Human	*In vivo*	(29)
		heregulin-ζ	Rat, mouse	*In vitro*	(30,31)
Prostate	PSMA	scFv-ζ	Human	*In vivo*	(32)
		scFv-CD28-ζ	Human	*In vivo*	(33)
Adeno-	TAG-72	scFv-ζ	Human	*In vivo*	(34)
Carcinoma		scFv-ζ	Human	*In vitro*	(35)
Melanoma	GD3	scFv-ζ	Human	*In vitro*	(36)
	HMW-MAA	scFv-γ	MD45	*In vitro*	(37)
	HLA-MAGE-A1	scFv-γ	Hybridoma	*In vitro*	(38)
Many	KDR	scFv-γ	Human	*In vitro*	(39)
(neovas-culature)	VEGFR2	VEGF-ζ	Mouse	*In vivo*	(40)
Neuro-blastoma	GD2	scFv-ζ	Human	*In vitro*	(41)
Many	EGP2	scFv-ζ	Human	*In vitro*	(42)
		scFv-γ	Human	*In vitro*	(43)
Renal cell carcinoma	CA9	scFv-γ	Human	*In vitro*	(44)
	CAIX	scFv-CD4-γ	Human	*In vitro*	(45)
		scFv-CD4-γ	Human	Clinical	(45)
		scFv-γ	Human	*In vitro*	(46)
Epithelia	Lewis-Y	scFv-CD28-ζ	Human	*In vivo*	(47)
		scFv-γ	MD45 hybridoma	*In vitro*	(48)
Lymphoma	CD30	scFv-γ	Human	*In vitro*	(49)
Cervical	CD44 v7/8	scFv-ζ	Mouse	*In vivo*	(50)
Leukemia	CD33	scFv-ζ	Human	*In vitro*	(51)
		scFv-CD28-ζ	Human	*In vitro*	(52)
		scFv-ICOS-ζ	Human	*In vitro*	(52)
		scFv-4-1BB-ζ	Human	*In vitro*	(52)
Multiple cancers	8H9	scFv-CD28-ζ	Human	*In vivo*	(53)
	MUC1	scFv-CD28-OX40-ζ	Human	*In vivo*	(54)
	Mesothelin	scFv-CD28-CD137-ζ	Human	*In vivo*	(55)
Rhabdomyosarcomas	(fAChR)	scFv-ζ	Human	*In vitro*	(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 $CD8^+$ 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)-γ, granulocyte-macrophage colony-stimulating factor and TNF-α, 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, $erbB2^+$ NIH-3T3 tumor cells were combined with either nontransduced or scFv-anti-erbB2(FRP5)-ζ transduced mouse T cells (C196), prior to subcutaneous injection into athymic nude mice. Mice injected with gene-modified C196 cells showed inhibition of $erbB2^+$ 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)-ζ 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 $erbB2^+$ 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)-ζ 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 $CD8^+$ lymphocytes expressing a scFv-anti-CD44v6-ζ 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)-ζ 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 $CD8^+$ T cells engineered with a scFv-anti-VEGFR-ζ 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-ζ or FcεRI-γ 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-ζ receptor showed greater reduction in tumor burden, including established metastatic disease, compared with mice that received T cells expressing the first-generation scFv-ζ 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-ζ 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-TCRζ receptor improved the antigen-specific activation, proliferation and cytolytic activity compared with T cells engineered with an anti-CD19-CD28-TCRζ receptor (12). Another study by the same group reported enhanced persistence of T cells gene engineered with an anti-mesothelin scFv-CD28-4-1BB-TCRζ 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

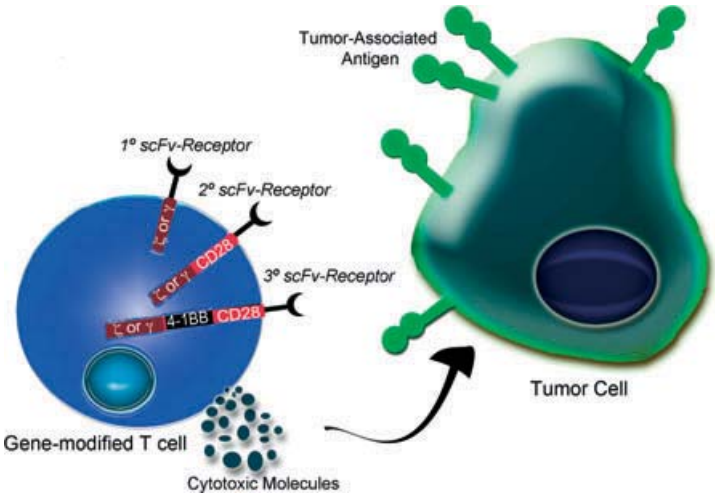

Figure 1 Genetically modified T cells expressing first-, second- and third-generation scFv receptors. The single-chain variable fragment (scFv) of an antibody that specifically binds tumor-associated antigen (TAA) is linked via a hinge region to a transmembrane and cytoplasmic signaling tail. First-generation receptors comprise one signaling domain, such as T cell receptor (TCR)-ζ or FcεRI-γ. Second-generation receptors contain two signaling domains such as the costimulatory CD28 domain linked in tandem with the TCR-ζ domain. Third-generation receptors comprise three stimulatory domains, such as CD28, 4-1BB and TCR-ζ 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-TCRζ-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 downregulated 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^+ 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_L, 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 $CD4^+$ and $CD8^+$ 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 $CD4^+$ and $CD8^+$ T cells at the tumor site (73). Critical for this effect was the requirement that the transferred $CD4^+$ 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 $CD4^+$ subtypes (Th_1 *vs* Th_2) showed that help provided by engineered Th_1 $CD4^+$ 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-γ 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, $CD56^+CD3^-$ NK cells retrovirally transduced with a scFv-ζ 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-ζ) enhanced the activation, cytotoxicity and cytokine secretion of transduced NK cells *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-ζ 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-ζ 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 $CD34^+$ 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-γ or scFv-ζ chimeric receptor, reactive against gp120, could mediate target-specific cytolysis *in vitro*. Interestingly, neutrophils transduced with the scFv-ζ construct showed greater cytolytic function than the scFv-γ 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-1β and TNF-α 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 preclinical 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 (retronectin)that enhance virus and T cell interactions (82).

Initial clinical trials evaluated the function of engineered T cells using scFv-CD4-ζ 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 $CD4^+$ and $CD8^+$ T cells. The transferred T cells were well tolerated in patients and circulating anti-gp120 $CD4^+$ and $CD8^+$ 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-γ 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-ζ (scFv-CD28-ζ) 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^Y-CD28-ζ 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-ζ 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-ζ 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^Y antigen secreted higher levels of IFN-γ in response to target cells expressing high levels of Le^Y antigen. There was negligible response of modified T cells against tumor targets or normal tissue (i.e. neutrophils) expressing low levels of Le^Y 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-γ 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 TCRζ 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 $CD34^+$ 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.

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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 (1.6×10^{-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

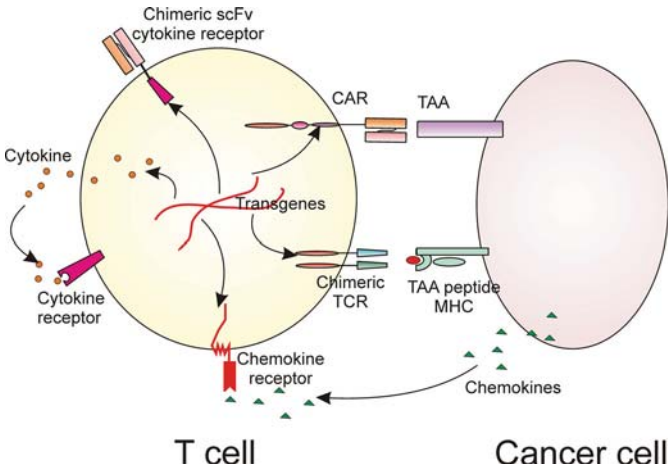

Figure 1. Examples of new functions that can be endowed on T cells through genetic modification. The specificity of T cells can be redirected toward TAA using genes encoding CARs that recognize cell-surface TAA on cancer cells. CARs can consist of extracellular scFv antibody recognizing TAA linked to 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- . 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-	Eradication of Day 13 tumors in xenograft SCID/beige mice	[53]
ALL	CD19	pRRL-SIN-CMV-eGFP-WPRE lentivirus	α-CD19-CD137-	Survival to >200 days, Day 21 model, NOD-SCID mice	[6]
Hodgkins lymphoma	CD30	SFG retrovirus	α-CD30-	Inhibition for >28 days of Day 7 model, SCID mice	[54]
Osteosarcoma	Her-2	SFG retrovirus	α-Her-2-CD28-	Eradication of Day 8 tumors in Nu/Nu mice	[55]
Mesothelioma	Mesothelin	pELNS	scFv-CD28-CD137-	Eradication of 6 to 7-week tumors in NOD/SCID/IL2rγ−/− (NOG) mice	[56]
Medullo blastoma	IL-13	Electroporation with DNA	IL-13-CD4-	Tumor regression of Day 8 tumors in NOD-SCID	[57]
Medullo blastoma	Her-2	SFG retrovirus	α-Her-2-	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- CAR and demonstrated killing of erbB2-expressing tumor cells in vitro. Muller et al. [77] transduced anti-CD20-CD3 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-CD3 -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- 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-CD3 and demonstrated significant survival of mice bearing RMA-retinoic acid early incucible-1 tumors [81] and ovarian tumor [82]. In addition, the NKG2D-CD3 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- , 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 ($\geq$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-	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-	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-	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-	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-	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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KEY WORDS:
adoptive immunotherapy · chimeric receptor · tumor · retroviral vector · lentiviral vector · TCR

See pages 661 and 843

Treatment of Chronic Lymphocytic Leukemia With Genetically Targeted Autologous T Cells: Case Report of an Unforeseen Adverse Event in a Phase I Clinical Trial

To the editor:

Most patients with B-cell malignancies will die from their disease or are incurable. For this reason, innovative therapeutic approaches are direly needed. Patient T cells may be genetically modified to target antigens expressed on tumor cells through the expression of chimeric antigen receptors (CARs), which are antigen receptors designed to recognize cell surface antigens in a human leukocyte antigen–independent manner.[1] CD19, which is expressed on most B-cell malignancies—including most non-Hodgkin's lymphomas, acute lymphoblastic leukemias, and chronic lymphocytic leukemias (CLLs)—is an attractive antigen for this approach.[2] It is present on normal B-lineage cells from the early pre–B-cell stage until plasma cell differentiation. In a model of CD19$^+$ acute lymphoblastic leukemia, we found that that a CAR termed 19-28z, which comprises the CD28 cytoplasmic domain in addition to that of the CD3 ζ-chain,[3] induced better responses than a ζ-chain-based receptor.[4] In preclinical *in vitro* studies, we demonstrated that human T cells that express CD19-specific CARs efficiently lyse human CD19$^+$ tumor cell lines and that CLL patient–derived T cells effectively lyse autologous tumor cells.[2,4] These results, and others,[5] supported a phase I clinical trial treating refractory CLL patients with autologous T cells modified by retroviral gene transfer of the 19-28z CAR.

We have thus far enrolled six patients in this clinical trial. The cohort of subjects treated with modified T cells alone at the first dose level of T cells tolerated therapy well without dose-limiting toxicities. However, the first subject (subject 4) enrolled in the second cohort of patients, in whom cyclophosphamide lymphodepleting chemotherapy was administered before infusion of the same T-cell dose, developed a syndrome of hypotension, dyspnea, and renal failure following T-cell infusion. Subject 4 died 4 days after administration of cyclophosphamide and modified T cells. Herein we describe the chronology of his treatment and report the findings of an extensive postmortem analysis.

Clinical trial design

Subject 4 was treated in a phase I clinical trial (IRB no. 06-138, NIH-RAC no. 0507-721, NCT00466531) designed to assess the safety of infusing autologous T cells modified to express the CD19-targeted CAR 19-28z in subjects with relapsed or purine analog–refractory CLL. For 2–3 days following T-cell infusion, the subjects are closely monitored for tumor lysis and unforeseen adverse events. If stable, subjects are discharged and subsequently closely followed in the outpatient clinic setting.

This phase I clinical trial has a three-step design (**Table 1**). In the first step, subjects are treated with dose level 1 of modified T cells ($1.2–3.0 \times 10^7$ CAR$^+$ T cells/kg) without prior lymphodepleting chemotherapy. The subject of the current report was enrolled in cohort 1 of step 2 and treated with 1.5 g/m^2 of cyclophosphamide followed 2 days later by infusion of modified T cells at dose level 1. The enrollment thus far is summarized in **Table 1**.

Case report

Subject 4 was a 69-year-old man with refractory CLL who was enrolled in clinical trial IRB no. 06-138. At the time of enrollment, three previous subjects had been treated on this protocol without significant adverse events in the first planned cohort, receiving the lowest planned dose of modified T cells alone. Subject 4 was the first to receive lymphodepleting chemotherapy with cyclophosphamide (1.5 g/m^2) followed 2 days later by infusion of modified T cells at the same dose tolerated earlier by the first three subjects enrolled in cohort 1 of this trial.

Subject 4's CLL treatment history. Subject 4 was initially diagnosed with CLL 8 years before treatment on this protocol, when he was noted to have an elevated lymphocyte count on a routine complete blood count in the context of lymphadenopathy. The subject had a significant past medical history of myocardial infarction, coronary artery disease, hypertension, and chronic renal insufficiency. Two years after diagnosis, because of progressive symptomatic abdominal lymphadenopathy and a rapidly doubling peripheral blood lymphocyte count, he was treated per Memorial Sloan–Kettering Cancer Center (MSKCC) IRB protocol no. 98-080 with sequential fludarabine (25 mg/m^2) daily for 5 days every 4 weeks for six cycles, followed by high-dose cyclophosphamide (3 g/m^2) given once every 3 weeks for three cycles, followed by rituximab (375 mg/m^2) given weekly for 8 weeks. He achieved a durable partial response. Five years later, he developed evidence of progressive disease as shown by increasing lymphadenopathy, increasing peripheral blood lymphocyte counts, and cytopenias. The subject was enrolled in MSKCC IRB protocol no. 05-077 and treated with six monthly cycles of combination therapy with pentostatin (4 mg/m^2), cyclophosphamide (600 mg/m^2), rituximab (375 mg/m^2 given only on cycles 2–6), and mitoxantrone (10 mg/m^2). The subject once more achieved a partial response. Two years later, the subject presented with a rapidly increasing peripheral blood lymphocyte count, worsening cytopenias, and

Table 1 Cyclophosphamide and T-cell doses in IRB protocol no. 06-138

Step	Cyclophosphamide	CAR + T-cell dose	No. of enrolled subjects
1	0	$1.2–3.0 \times 10^7$/kg	3
2	1.5 g/m^2	$1.2–3.0 \times 10^7$/kg	1
	3.0 g/m^2	$1.2–3.0 \times 10^7$/kg	0
3	MTD	$0.4–1.0 \times 10^8$/kg	0
–1	1.5 g/m^2	$4.0–10 \times 10^6$/kg	2

CAR, chimeric antigen receptor; MTD, maximum-tolerated dose.

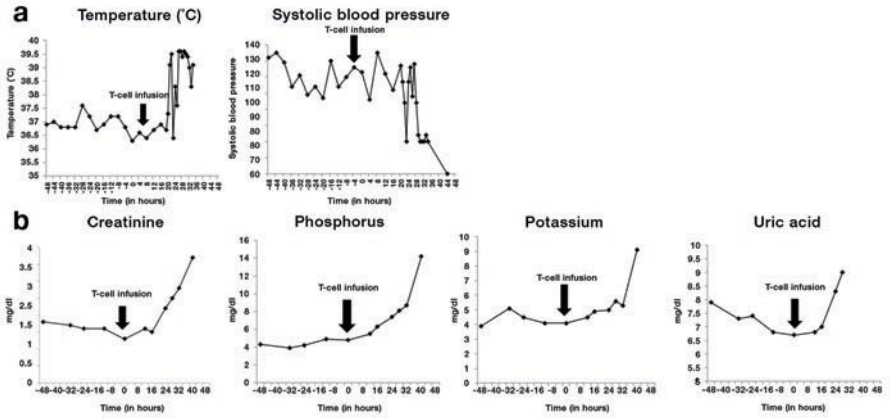

Figure 1 Clinical assessment of subject 4 on IRB no. 06-138, NIH-RAC no. 0507-721, NCT00466531. Assessment of patient from the time of admission to the hospital before cyclophosphamide chemotherapy (–48 hours), through time of modified T-cell infusion (0 hours), to time of death (44 hours). Clinical status was assessed by routine vital sign parameters, including (**a**) temperature and systolic blood pressure, as well as by laboratory chemistry measurements, including (**b**) renal function as measured by creatinine, potassium, phosphorus, and uric acid concentrations. Vital signs over time are consistent with a sepsis syndrome (fever with hypotension), whereas laboratory chemistry studies demonstrate an initial rise in creatinine coinciding with the patient's anuric state, followed by rising potassium, phosphorus, and uric acid at concentrations that are consistent with tumor lysis but confounded by the antecedent acute renal failure. The vertical arrow indicates the time of T-cell infusion.

increasing lymphadenopathy and he was enrolled in IRB protocol no. 06-138 (NIH-RAC no. 0507-721, NCT00466531). The subject was assessed and met all criteria for enrollment in this trial.

Treatment course. For IRB protocol no. 06-138, the subject underwent a leukapheresis procedure, and the product was processed and frozen. Subsequently, T cells were activated and retrovirally transduced with the 19-28z retroviral vector as described.[6] The subject was then admitted and, per protocol, received tumor lysis prophylaxis with hydration and allopurinol followed by cyclophosphamide (1.5 g/m^2) infusion. He tolerated therapy well, and twice-daily serum electrolyte studies revealed no evidence of tumor lysis. On the day of T-cell infusion, the subject's tumor lysis laboratory results were unremarkable, with the exception of a mildly elevated phosphorus level (4.8 mg/dl). His creatinine was 1.3 mg/dl. The T-cell infusion was completed over 3 hours without complication. Twenty hours after modified T-cell infusion, the subject developed a fever, a transient finding observed in all three subjects treated on step 1 of this protocol; however, in contrast to previously treated subjects, subject 4's fever persisted and was associated with concomitant hypotension (**Figure 1a**). At the same time, the subject developed respiratory distress despite a negative chest X-ray. After bacterial blood cultures had been obtained, the patient was started on broad-spectrum antibiotics (piperacillin/tazobactam and ciprofloxacin) with pressor support. He was subsequently transferred to the intensive care unit. Laboratory studies obtained at 24 hours after T-cell infusion demonstrated an elevated creatinine concentration (2.2 mg/dl) and rising phosphorus (7.4 mg/dl), potassium (5.0 mEq/L), and uric acid (8.3 mg/dl) concentrations.

The subject became anuric, consistent with acute renal failure. In the intensive care unit, his blood pressure responded to inotropic support, from which he was successfully and fully weaned over the course of the day. However, he remained anuric with increasing serum potassium and phosphorus concentrations (**Figure 1b**). By the early evening, he once more became hypotensive, and inotropic support was restarted. A worsening respiratory status led to intubation and mechanical ventilation. Supportive care was withdrawn shortly thereafter at the request of the subject's health-care proxy. The subject expired 44 hours after infusion of modified T cells. Laboratory studies just before his death demonstrated an increasing serum creatinine concentration at 3.7 mg/dl, as well as markedly elevated potassium and phosphorus concentrations at 9.1 mEq/L and 14.2 mg/dl, respectively (**Figure 1b**). The subject's peripheral blood lymphocytosis remained generally stable over time, beginning with initial chemotherapy and following modified T-cell infusion (data not shown).

Postmortem pathology report. Both gross and histologic postmortem analyses of tissues from subject 4 revealed extensive CLL with diffuse bulky adenopathy, including a large abdominal tumor (2.5 kg) and associated increased mesentery lymphadenopathy. Microscopic evaluation revealed diffuse CLL involvement in multiple organs, including the liver, pancreas, adrenal glands, and bone marrow, as well as the lymph nodes. Renal tissues were generally normal, other than scattered calcium crystals. Overall, these data fail to support a diagnosis of tumor lysis syndrome as the primary source of renal failure. Histology of the lung and cardiac tissues showed no significant pathology. Furthermore, initial blood cultures, as well as all subsequent cultures obtained after antibiotic administration, were negative. The infused cell product was sterile at the time of infusion, a result we reconfirmed after the occurrence of the serious adverse event (SAE).

Analysis of serum cytokines. As stipulated in the protocol, serial serum samples were routinely obtained from all subjects before and after each stage of the treatment regimen. Analyses of these serum samples revealed a significant increase in the concentrations of interleukin (IL)-2, IL-7, IL-15, and IL-12 cytokines following cyclophosphamide chemotherapy as compared with the pretreatment serum sample obtained 30 days earlier (**Figure 2**). An interpretation implicating the cyclophosphamide chemotherapy in this rise in serum cytokines is hampered by the 30-day time lag between obtaining the pre- and post-chemotherapy serum samples. Alternatively, the elevation of these cytokines may have been secondary to a prior subacute infectious process that was subsequently exacerbated by cyclophosphamide-mediated immune suppression, resulting in the sepsis syndrome seen in this subject. Significantly, serum tumor necrosis factor-α and interferon-γ were unchanged immediately before and after T-cell infusion (**Figure 2**).

Conclusion

Etiology of SAE. Subject 4, a 69-year-old patient with bulky CLL, was the first to receive T cells following prior lymphodepleting chemotherapy (IRB protocol no. 06-138, step 2, cohort 1). In the first cohort (step 1), in which three subjects were treated with the lowest planned modified T-cell dose alone, all experienced transient fevers following T-cell infusion but otherwise tolerated therapy well. These subjects had no evidence of hypotension, tumor lysis, or acute renal failure. In contrast to subjects treated in the first cohort, subject 4 developed persistent fevers following T-cell infusion, became hypotensive, and developed acute renal failure, all consistent with a clinical picture of sepsis. Significantly, acute renal failure developed in the absence of any clinical evidence suggestive of tumor lysis syndrome. A later rise in serum potassium, phosphorus, and uric acid concentrations may be indicative of a subsequent incipient tumor lysis. This sequence of events thus suggests that the patient developed renal failure due primarily to hypotension as a consequence of sepsis-like syndrome, a conclusion supported by the postmortem examination of renal tissues.

Although consistent with an infectious etiology, the subject's blood cultures as well as postmortem cultures failed to detect any bacterial growth, noting that the latter may have been compromised by broad-spectrum antibiotic therapy.

Serum cytokine analysis revealed markedly elevated levels of the proinflammatory and homeostatic cytokines IL-2, IL-7, IL-15, and IL-12 following cyclophosphamide chemotherapy and preceding the T-cell infusion. The etiology of this elevated cytokine profile remains unclear, because the pretreatment serum sample was obtained 30 days before chemotherapy. Nevertheless, regardless of the etiology, this cytokine milieu, highly favorable to T-cell persistence, activation, and proliferation, may account for the possible incipient tumor lysis seen in subject 4 but not in subjects 1–3.

Combining this biological evidence with the unremarkable lung, heart, and kidney pathology, this SAE, despite negative blood cultures, is consistent with sepsis due to infection, leading to hypotension, leading to acute renal failure and, ultimately, death. This scenario is also consistent with infection as a prevalent and leading cause of morbidity and mortality in patients with advanced CLL.

Consequent modification of clinical trial protocol. Our findings fail to directly attribute the SAE in subject 4 (IRB protocol no. 06-138) to the modified T cells. Nevertheless, because of the temporal relationship of the autologous T-cell infusion to this SAE, we conservatively attributed infusion of modified T cells as a "possible" source contributing to this SAE. As stipulated in the protocol, we reduced the CAR^+ T-cell dose in the next cohort of patients to the –1 dose ($0.4–1 \times 10^7$ modified T cells/kg, **Table 1**). As a further precaution to enhance patient safety, we modified the protocol by administering T cells as a split infusion, infusing one-third of the dose on day 2 following cyclophosphamide therapy and, in the absence of evidence of tumor lysis, hypotension, or renal failure, administering the remaining two-thirds of the planned T-cell dose on day 3 following cyclophosphamide therapy. Significantly, the subsequent subject treated on this trial under these modified conditions did not exhibit evidence of a "cytokine storm" following cyclophosphamide chemotherapy and further tolerated infusion of modified T cells without any notable toxicities. We will continue to focus on analyses of serum cytokine studies before and following both cyclophosphamide chemotherapy and modified T-cell infusion, with special attention to the conditioning-induced cytokine response and its relationship to post-T-cell infusion cytokine concentrations.

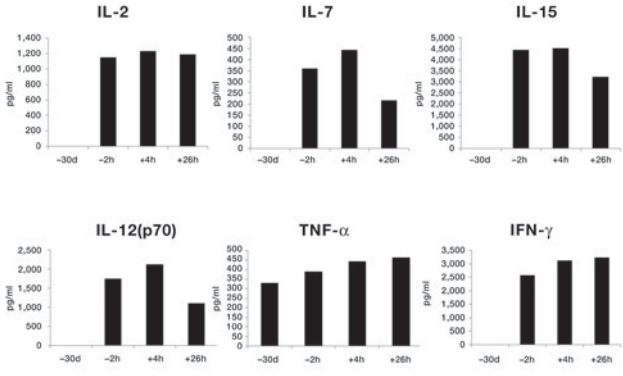

Figure 2 Serum cytokine concentrations measured in subject 4. Serum samples were obtained 30 days before cyclophosphamide (–30 d), 2 hours before T-cell infusion (–2 h), and 4 and 26 hours after T-cell infusion (+4 h, +26 h, respectively). The –2-h sample is therefore post-cyclophosphamide but pre-T-cell infusion. Pretreatment tumor necrosis factor-α (TNF-α) serum values were 200, 50, and 59 ng/ml in subjects 1, 2, and 3, respectively. IFN-γ, interferon-γ; IL, interleukin.

Note

Since the submission of this letter, we have treated a sixth subject at the –1 treatment dose (see **Table 1**). This subject exhibited a transient hypotensive episode 24 hours after T-cell infusion that responded to increased intravenous hydration, accompanied by a mild, transient fever. This episode rapidly resolved with no evidence of infectious etiology or renal compromise. Cytokine serum analyses were unremarkable.

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Case Report of a Serious Adverse Event Following the Administration of T Cells Transduced With a Chimeric Antigen Receptor Recognizing *ERBB2*

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In an attempt to treat cancer patients with *ERBB2* overexpressing tumors, we developed a chimeric antigen receptor (CAR) based on the widely used humanized monoclonal antibody (mAb) Trastuzumab (Herceptin). An optimized CAR vector containing CD28, 4-1BB, and CD3ζ signaling moieties was assembled in a γ-retroviral vector and used to transduce autologous peripheral blood lymphocytes (PBLs) from a patient with colon cancer metastatic to the lungs and liver, refractory to multiple standard treatments. The gene transfer efficiency into autologous T cells was 79% CAR^+ in $CD3^+$ cells and these cells demonstrated high-specific reactivity in *in vitro* coculture assays. Following completion of nonmyeloablative conditioning, the patient received 10^{10} cells intravenously. Within 15 minutes after cell infusion the patient experienced respiratory distress, and displayed a dramatic pulmonary infiltrate on chest X-ray. She was intubated and despite intensive medical intervention the patient died 5 days after treatment. Serum samples after cell infusion showed marked increases in interferon-γ (IFN-γ), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-10, consistent with a cytokine storm. We speculate that the large number of administered cells localized to the lung immediately following infusion and were triggered to release cytokine by the recognition of low levels of ERBB2 on lung epithelial cells.

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INTRODUCTION

ERBB2 (HER-2/neu) is a member of the epidermal growth factor receptor family. Epidermal growth factor receptor–ligand interaction induces the heterodimerization of receptors, which in turn results in the activation of intracellular tyrosine kinase domain signaling cascades that mediate cell growth, differentiation, and survival.[1–3] Overexpression of *ERBB2* can induce dimerization of ERBB2 and initiates signal transduction activities without ligand binding. *ERBB2* overexpression/amplification occurs in ~15–25% of human breast cancer patients, and is associated with more aggressive disease.[4] A proportion of other human cancers are also associated with *ERBB2* gene amplification and protein overexpression; including cancers of the colon, ovary, stomach, kidney, melanoma, and others.[5–7] Investigation of agents that target the *ERBB2* protein led to the development of Trastuzumab (Herceptin), a humanized monoclonal antibody (mAb) that binds to the extracellular domain of the receptor.[8] Trastuzumab has been shown to be of clinical benefit for metastatic breast cancer patients with *ERBB2* overexpression/amplification, either alone or in combination with chemotherapy regimens.[9,10] *ERBB2* has also been the target of several cancer vaccine trials,[11–13] as well as, adoptive cell therapy using anti-ERBB2 cytotoxic T lymphocyte lines.[14]

Adoptive cell therapy has emerged as the most effective treatment for patients with metastatic melanoma. Adoptive cell therapy using tumor-reactive autologous tumor infiltrating lymphocytes (TIL) in combination with nonmyeloablative but lymphodepleting conditioning resulted in 50% objective clinical regression in melanoma patients.[15] Intensifying the lymphodepletion by adding total-body irradiation to the chemotherapy conditioning regimen improved the objective response rate to 72%.[16] This potent therapy, however, has been limited by the requisite surgery to procure tumor-reactive TIL, by *ex vivo* identification and expansion of these cells, and by the failure to reproducibly isolate similar cells from common epithelial tumors.

The transfer of genes into primary human lymphocytes permits the introduction of tumor antigen receptor molecules that can endow the engineered cell with antitumor specificity.[17–19] We reported the first clinical trials using autologous peripheral blood lymphocytes (PBLs) modified to express a tumor antigen-reactive T-cell receptor in the treatment of patients with metastatic melanoma that resulted in objective tumor regressions.[20,21] These strategies, however, have a lower response rate than TIL, and only a minority of patients are eligible for current protocols, as they must express human leukocyte antigen-A*0201 in order to be recognized by the T-cell receptor-engineered cells.

An alternative to T-cell receptor gene therapy is the use of a chimeric antigen receptor (CAR) that is capable of relaying excitatory signals to T cells in a non-Major histocompatibility

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complex-restricted manner. These hybrid proteins, composed of an extracellular antigen recognition domain fused to an intracellular T-cell activation domain,[22,23] may therefore be used in patients regardless of their human leukocyte antigen genotype. The absence of human leukocyte antigen-restricted antigen recognition is achieved by harnessing the antigen-binding properties of mAb; this recognition is also independent of antigen processing, thus bypassing a potential mechanism by which tumor cells can evade the immune system *in vivo*. Several clinical trials using CAR-transduced T cells have been reported.[24–27] ERBB2-based CARs reported thus far are composed of single-chain Fv fragment from murine mAb, which have been shown to induce anti-CAR immune responses in humans.[25,26] The anti-ERBB2 CAR used in this case report was a next generation CAR containing both the humanized Herceptin single-chain Fv fragment and optimized costimulatory signaling domains designed for increased cytokine secretion, lytic activity, and shown to display robust *in vivo* antitumor activity in a human breast cancer xenograft model.[28]

RESULTS

In vitro characteristics of the ErbB2-CAR transduced T cells for patient treatment

Leukophoresis was performed to obtain patient peripheral blood mononuclear cells (PBMCs), which were stimulated with an anti-CD3 mAb and interleukin-2 (IL-2) to initiate T-cell expansion followed by transduction with the 4D5-CD8-28BBZ ERBB2-CAR vector as described in Materials and Methods section. At 4 days before infusion, cells were analyzed for the expression of the ERBB2 CAR using an ERBB2-Fc fusion protein as previously described.[28] As shown in **Figure 1**, 79% of $CD3^+$ T cells expressed the CAR with gene transfer into both $CD4^+$ (17%) and $CD8^+$ (63%) T-cell subsets. To determine functional activity, transduced T cells were cocultured with $ERBB2^+$ melanoma, breast cancer, and ovarian cancer cell lines, or $ERBB2^-$ breast cancer and T lymphoblastoid cell lines. ERBB2-specific reactivity was demonstrated by production of effector cytokine interferon-γ (IFN-γ) only in cell lines expressing ERBB2 (**Table 1**). Background cytokine production of ERBB2-CAR transduced cells, when cocultured with ERBB2-targets, was similar to untransduced control cells. To complete the certificate of analysis for patient treatment, transduced cells were also tested to be negative for the presence of replication competent retrovirus by PCR and passed sterility testing (data not shown). Retrospective testing using an amplification-based S^+/L^- assay was also negative for replication competent retrovirus.

Clinical course

The patient was a 39-year-old female who 3 years earlier had undergone a sigmoid resection for colon cancer that on pathologic analysis exhibited lymphatic invasion and vascular involvement, with spread to 6 of 21 lymph nodes and the presence of synchronous liver metastases. She was treated with a

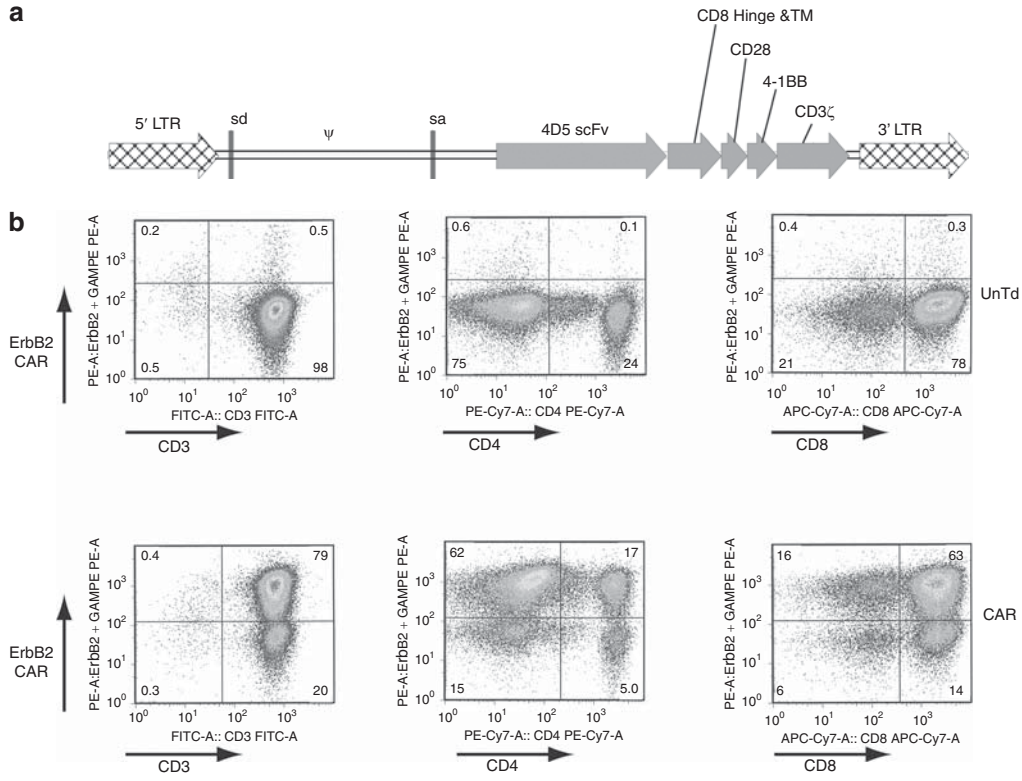

Figure 1 Expression of the ERBB2 CAR. Diagram of the ERBB2-CAR vector (MSGV1-4D5-CD8-28BBZ) used in this trial is as shown on the top of the figure. As described in Materials and Methods section, patient PBMC were stimulated to induced T-cell division and then transduced with the CAR vector. Four days before cell infusion samples were removed for analysis of *ERBB2-CAR* gene expression by FACS (along with the CD3, CD4, or CD8 T-cell markers). CAR, chimeric antigen receptor; LTR, long terminal repeats; PBMC, peripheral blood mononuclear cell; scFv, single-chain Fv fragment.

Table 1 ErbB2-CAR certificate of analysis

	ErbB2$^+$ target								ErbB2$^-$ target	
Effector	**Media**	**Mel888**	**Mel938**	**Mel526**	**Mel624**	**MDA361**	**SK-BR3**	**SK-OV3**	**CEM**	**MDA468**
None	24	49	68	44	66	57	143	82	37	99
MART-1 TCR	26	37	35	**3,700**	**4,900**	37	68	47	29	55
Patient CAR	46	**12,745**	**13,210**	**11,430**	**>21,065**	**>26,815**	**>33,385**	**>58,480**	114	134
Mock Td PBL	46	69	81	93	127	82	144	146	117	103

Abbreviations: CAR, chimeric antigen receptor; IFN-γ, interferon-γ; PBL, peripheral blood lymphocyte.
Cell reactivity following coculture of 1e5 effectors with 1e5 targets. Data are IFN-γ (pg/ml) following overnight incubation. Mock Td PBL, untransduced PBL maintained under identical culture conditions; MART-1 TCR, MART-1 TCR-transduced PBL; patient CAR, ErbB2 CAR-transduced patient PBL. ErbB2 antigen expression was as indicated. Only melanoma lines 526 and 624 expressed both HLA-A2 and MART-1, required for TCR recognition.

chemotherapy regimen consisting of 5-fluorourocil, leucovorin and oxaliplatin plus the antivascular endothelial growth factor mAb, bevacizumab. The tumor progressed and the patient was then treated with an alternate chemotherapy regimen in which irinotecan was substituted for oxaliplatin (FOLFIRI). The patient again progressed and after desensitization to oxaliplatin for an allergic reaction, she received a third chemotherapy regimen consisting of capecitabine, oxaliplatin, and bevacizumab. The tumors in the lung and liver continued to progress and the patient was referred to the Surgery Branch, National Cancer Institute (NCI; Bethesda, MD) and signed an informed consent for our protocol. The protocol was reviewed and approved by the National Institutes of Health Institutional Biosafety Committee, the NCI Institutional Review Board, the National Institutes of Health Office of Biotechnology Activities, and the Food and Drug Administration (all Bethesda, MD). Patient inclusion criteria included metastatic cancer that expressed ERBB2 (Her-2/neu) at ≥2$^+$ as assessed by immunohistochemistry.

To facilitate homeostatic expansion of the transduced cells, the patient received a lymphodepleting regimen (60 mg/kg cyclophosphamide daily for 2 days followed by flurodarabine 25 mg/m^2 for the next 5 days). On the day following the last chemotherapy dose the patient received an intravenous infusion of 10^{10} cells transduced with the ERBB2 CAR in 125 ml over 30 minutes. This was the largest number of cells permitted in the first dose-escalation cohort. Within 15 minutes after completing the infusion, the patient developed respiratory distress with decreased blood oxygen saturation that worsened over the next hour. Chest X-ray obtained 40 minutes after completion of the infusion showed pulmonary edema, which appeared worse on chest X-rays repeated at 2 and 4 hours after the infusion. Because of decreasing respiratory function the patient was transferred to the intensive care unit and was intubated about 1 hour after the cell infusion. The patient then developed severe hypotension requiring vasopressors. Dexamethasone, 8 mg every 6 hours, was administered starting at about 5 hours after the cell infusion (it was continued for 2 days, after which the dose was tapered). The patient experienced two cardiac arrests in the next 12 hours after cell infusion both requiring cardiopulmonary resuscitation. She was maximally supported with vasopressors and ventilatory support. She remained severely ill with maximum intensive care unit support for the next 5 days at which time progressive hypotension and bradycardia as well as gastrointestinal bleeding resulted in cardiac arrest from which she could not be resuscitated.

Postmortem analysis

At autopsy, multiple organs exhibited signs of systemic ischemia and hemorrhagic microangiopathic injury. The lungs also showed diffuse alveolar damage consistent with the clinical findings of acute respiratory distress syndrome. Autopsy also revealed a generalized rhabdomyolysis. Copious blood in the small intestine indicated that the patient succumbed to hemorrhage in the setting of multiple organ failure secondary to systematic microangiopathic injury. The autopsy findings appeared to be a combination of the initial pulmonary injury followed by the sequela of several days of hypotension and organ ischemia.

Beginning at about 4 hours after cell infusion serum samples were obtained and stored for analysis. Compared to pretreatment samples, the patient's serum displayed a rapid and marked increase in the levels of five cytokines; IFN-γ, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), IL-6, and IL-10 (**Figure 2**). At 4 hours after infusion, four of the five cytokines displayed peak serum levels: IFN-γ,11, 456 pg/ml; TNF-α, 380 pg/ml; GM-CSF, 10,191 pg/ml; and IL-6, 34,467 pg/ml. Preinfusion levels for IFN-γ, GM-CSF, and IL-6 were undetectable, whereas TNF-α values varied from 0 to 51 pg/ml. The levels of these cytokines decreased over the next 3 days but remained above baseline values. The levels of seven additional cytokines (IL-1β, IL-2, IL-4, IL-7, IL-10, IL-12, and TRAIL) were determined by cytokine array (SearchLight assay) with only IL-10 showing increased levels after infusion, which unlike the other cytokines, was sustained through the study period (8 pg/ml preinfusion, 219 pg/ml at about 100 hours after infusion, **Figure 2**). An increased amount of IL-2 was observed at 4 hours only and was likely associated with the administration of the cell product, which was given in saline with 300 IU/ml IL-2 (the patient received no other IL-2). The possible involvement of an anaphylactic response to the infused cells was deemed unlikely because we measured only a modest twofold increase in serum tryptase levels at the 4 hours time point (from 7–9 pg/ml preinfusion to 15 pg/ml at 4 hours).

To determine the relative tissue distribution of vector-containing cells, DNA was isolated from samples obtained at autopsy and subjected to quantitative-PCR using vector-specific primers and probe. As a reference for this analysis, DNA was extracted from the infusion sample (79% ERBB2-CAR$^+$) and arbitrarily assigned a value of 100 for comparison to tissue samples. There was a wide variation in the presence of vector-containing cells found in multiple tissues, though the highest levels were seen in the lung and

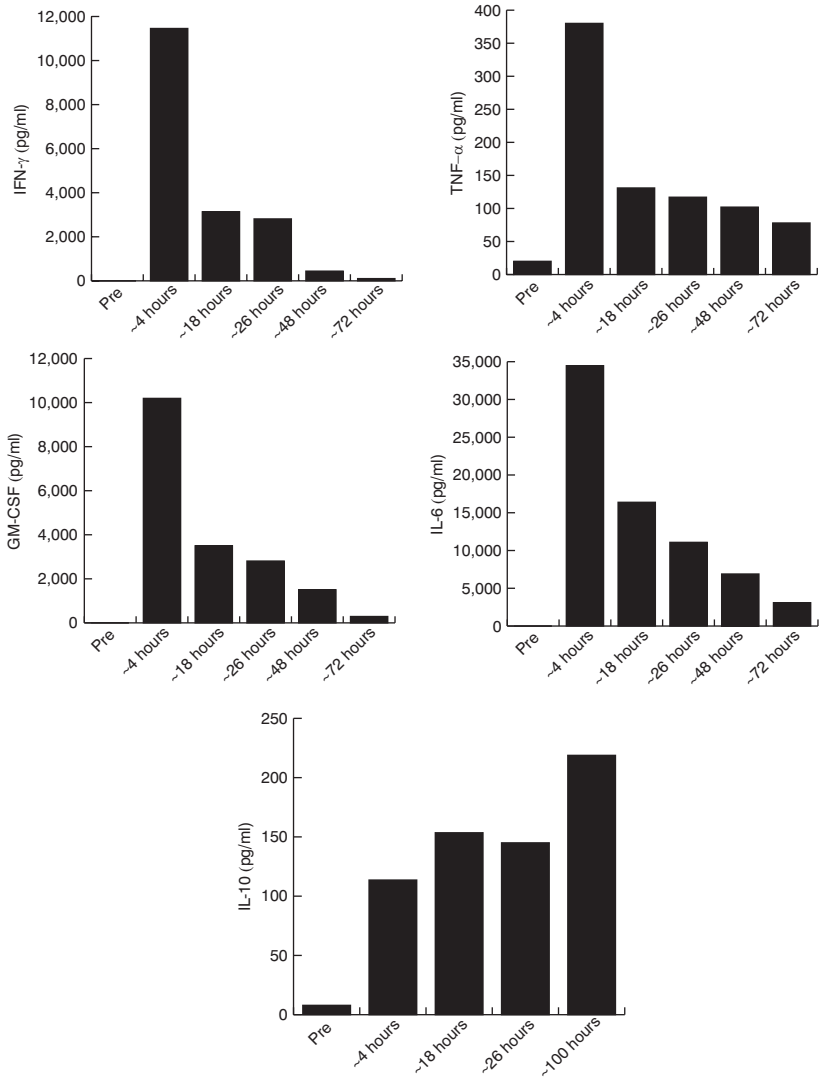

Figure 2 Serum cytokine levels. Serum samples obtained at the approximate times indicated after cell infusion were assayed for cytokine expression using commercial ELISA kits for cytokines IL-6, TNF-α, GM-CSF, and IFN-γ. The levels of cytokine IL-10 were independently determined by cytokine array (SearchLight) assay. All samples were diluted as necessary as to be in the linear range of the assay. ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α.

abdominal/mediastinal lymph nodes (**Table 2**). There did not appear to be a preferential accumulation of vector-containing cells in metastatic deposits in the liver or lungs. DNA from the pretreatment PBMC was also subjected to analysis of single-nucleotide polymorphisms associated with the activity/function of five cytokine genes (*IL-6*, *IL-10*, *IFN-γ*, *TGF-β1*, and *TNF-α*). PCR using sequence-specific primers (**Figure 3**) indicated that the patients' genotype was: IL-6, heterozygous −741G/C; IL-10, homozygous −1082G, −819C, −592C; IFN-γ, homozygous 874A; transforming growth factor-β1, homozygous 10T, 25G; and TNF-α homozygous −308G. This genotype is consistent with a phenotype of increased synthesis of transforming growth factor-β, IL-10, and IL-6, but lower production of TNF-α and IFN-γ.

Analysis of the ERBB2-CAR transduced T cells before infusion demonstrated specific recognition of ERBB2-expressing tumor cells (**Table 1**). To confirm and extend this analysis, an aliquot of the cryopreserved infusion sample was thawed and retested. In addition to cell lines used for the certificate of analysis, the patient's PBMC were used to derive autologous dendritic cell and macrophage cultures and several cultures of allogeneic primary cells adapted for growth in culture by a commercial supplier were obtained. Coculture results presented in **Figure 4** confirm the data obtained in the certificate of analysis. There was no reactivity seen to the dendritic cell and macrophage autologous cell cultures. Cytokine release was observed in several cocultures using allogeneic primary cells adapted for growth in culture.

DISCUSSION

The *ERBB2* gene has been extensively studied as a target for both chemotherapy and immunotherapy. In breast cancer, overexpression of *ERBB2* is correlated with a poor clinical outcome and at the same time, is a positive predictive factor for those women who are most likely to respond to therapy with the anti-ERBB2 antibody, trastuzumab.[10] In randomized clinical

Table 2 Vector tissue distribution

Normal tissue	Signal
Brain (left frontal lobe)	0.00
Pectoralis muscle (left)	0.00
Aorta	0.01
Atrium (right)	0.04
Neck LN (left)	0.05
Ventricle (left)	0.08
Small bowel	0.09
Liver (left lobe)	0.11
Liver (right lobe)	0.13
Abdominal para-aortic LN	0.14
Liver	0.15
Auxiliary LN	0.15
Kidney (left)	0.16
Adrenal gland (left)	0.17
Kidney (right)	0.25
Spleen	0.30
Lung left—A	0.44
Adrenal gland (right)	0.49
Lung left—B	0.56
Mediastinal	0.70
Lung right—A	0.78
Lung right—B	1.59
Para-aortic/mediastinal LN	1.99
Abdominal para-aortic LN	2.24
Abdominal LN	2.58
Mediastinal LN	2.64
Metastasis	**Signal**
Liver metastasis	0.03
Lung metastasis (left)	0.08
Liver metastasis	0.09
Liver metastasis (left lateral)	0.11
Lung metastasis (left)	0.29
Liver metastasis (left anterior)	0.34

Abbreviations: CAR, chimeric antigen receptor; FACS, fluorescence-activated cell sorting; LN, lymph node; PBL, peripheral blood lymphocyte; Q-PCR, quantitative-PCR.
DNA extracted from tissues and metastasizes at autopsy were subjected to Q-PCR using ErbB2 CAR-vector specific primers/probe. Values were normalized to the infusion PBL (arbitrarily assigned a value of 100). Gene transfer efficiency of the infusion PBL was 79% as measured by FACS.

trials, trastuzumab in combination with chemotherapy lead to increased disease-free and overall survival in breast cancer patients.[9,29,30] The mechanism of action of trastuzumab appears to be multifactorial, but there are several studies that indicated the involvement of cell-mediated immunity (natural killer cell–based antibody-dependent cellular cytotoxicity) in patient responses.[31–33] Furthermore, patient immune responses (antibody-dependent cellular cytotoxicity) can be increased when trastuzumab is combined with concomitant cytokine (IL-2, IL-12) administration,[34,35] or in vaccine trials where anti-ERBB2 T cells responses have been reported.[11,13]

Safety considerations that preceded our clinical trial included the use of trastuzumab in thousands of cancer patients, the lack of toxicity seen in multiple studies immunizing against epitopes of ERBB2, and the lack of toxicity seen in a report of the adoptive transfer of autologous anti-ERBB2 cytotoxic T lymphocyte clones in the setting of breast cancer.[14] In this report, three different cytotoxic T lymphocyte clones were administered in five transfers given 2 weeks apart. A total of 2.65×10^9 total cells were administered along with low-dose IL-2. With the exception of low-grade fever and chills following the third and fourth infusions, no side effects were noted. The therapy was associated with a decrease in tumor cells within the patient's bone marrow, but larger metastatic sites (such as liver) were not impacted. Radioimaging was performed using ^{111}In-labeled cytotoxic T lymphocyte that demonstrated an immediate accumulation of cells in the lung that decreased over 72 hours, whereas uptake to the liver and spleen increased over the first 24 hours then remained stable for the 72-hour study period. We have demonstrated similar uptake of ^{111}In-labeled TIL[36] and have observed that lung uptake of TIL happens at the first pass (at the 90% retention level) after which the TIL then leak out of the lungs to the liver and other organs (ref. 36 and J.C. Yang, unpublished results).

The γ-retroviral vector construct used in this cancer gene therapy trial was designed for optimal *ERBB2-CAR* gene expression and anti-ERBB2 reactivity. It was demonstrated to be highly specific, was able to recognize a wide range of tumor histologies, and was able to significantly prevent the growth of human breast cancer cells orthotopically implanted into the mammary fat pad of severe combined immunodeficiency mice.[28] Part of the process of optimizing this anti-ERBB2-CAR vector was the inclusion of two T-cell costimulatory domains from CD28 and 4-1BB (CD137) that were linked to the CD3z signaling element. 4-1BB is essential for the optimal activity of $CD8^+$ T cells[37,38] and inclusion of 4-1BB signaling domains was shown to enhance the *in vivo* antitumor activity of CARs in tumor xenograft models.[28,39–41] To date, the reported clinical application of CAR-engineered T cells has been limited to constructs containing CD3z alone.[24–27,42] In one report targeting carbonic anhydrase IX, on-target toxicity was observed most likely due to recognition of antigen expression on biliary epithelium. The results with the carbonic anhydrase IX directed CAR suggest that on-target toxicity may be antigen dependant and does not require the presence of costimulatory signals (such as CD28 and 4-1BB) in the CAR construct.

In our initial report we observed, in some transductions, T-cell recognition of the $ERBB2^-$-tumor line MDA468 (this line is negative for ERBB2 expression by fluorescence-activated cell sorting, but ERBB2 mRNA can be detected by PCR). Transduction of the current patient's T cells with the identical ERBB2-CAR vector did not result in recognition of the MDA468 cell line (**Table 1**, **Figure 4**). The significance of the recognition of allogenic primary cells adapted for growth in culture (**Figure 4**) is not clear, as it is not known how the adaptation of these cells for growth in *ex vivo* culture influences the expression of ERBB2. For example, it was reported that while normal rat liver hepatocytes do not express

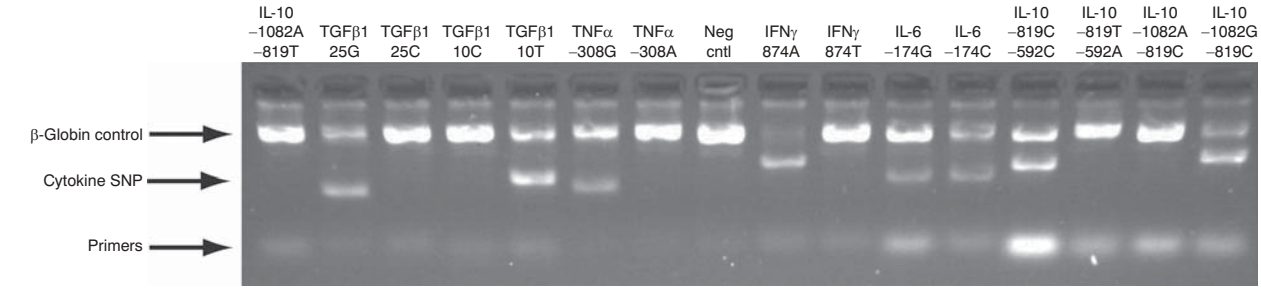

Figure 3 Cytokine genotype. DNA extracted for patient PBMC was subject to PCR with sequence-specific primers (PCR-SSP) as described in Materials and Methods section. Primer pairs are designed to have perfect matches only with a single allele or group of alleles. Matched primer pairs result in the amplification of target sequences (i.e., a positive amplification band), whereas mismatched primer pairs do not result in amplification (i.e., a negative result). The specific genotypes detected by the SSP are shown above each lane (Neg cntl, reaction without cytokine primers). Shown is one of duplicate determinations. IFN-γ, interferon-γ; IL, interleukin; SNP, single-nucleotide polymorphism; TNF-α, tumor necrosis factor-α.

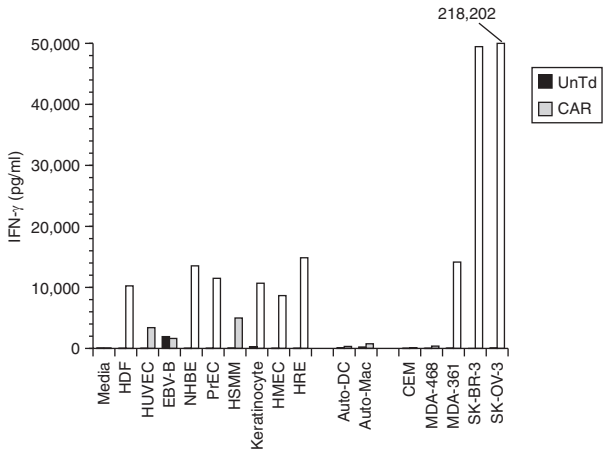

Figure 4 *In vitro* cytokine production. An aliquot of the ErbB2-CAR transduced (CAR) T lymphocytes infused into the patient or untransduced control T cells (UnTd) where assayed for IFN-γ cytokine production following overnight coculture with the indicated cell lines. ErbB2$^+$ target cells were SK-OV3, SK-BR3, and MDA361. ErbB2$^-$ tumor lines were MDA468 and CCRF-CEM (CEM). Primary cells adapted for growth in culture were HDF, human diploid fibroblast, HUVEC, human umbilical vein endothelial cells; EBV-B-EBV transformed B cell line, NHBE, normal human bronchial/tracheal epithelial cells; PrEC, human prostate epithelial cells, HSMM, human skeletal muscle myoblasts, keratinocytes-human keratinocytes, HMEC, human mammary epithelial cells, and HRE, human renal epithelial cells. Autologous patient cells were auto-DC-patient 6-day dendritic cell culture, and auto-mac-patient 6-day macrophage culture. All samples were diluted as necessary as to be in the linear range of the assay (sample SK-OV3 was off-scale in this assay and the value from a repeat determination was as indicated). CAR, chimeric antigen receptor; IFN-γ, interferon-γ.

ERBB2, that upon *ex vivo* culture, ERBB2 expression was rapidly induced.[43]

The most compelling finding in this case was the rapid rise in serum cytokine levels that has been associated with a multiple organ dysfunction syndrome.[44–46] The administration of biologics resulting in cytokine release syndrome was first reported to be associated with the administration of anti-CD3 mAb OTK3, which was administered as a systemic immunosuppressive agent during organ transplantation.[47,48] Within 1–4 hours after OKT3 injection, serum levels of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-6 were markedly elevated. Most recently a cytokine storm was reported in six of six patients that were treated with anti-CD28 mAb TGN1412.[48–50] In that report,[50] TNF-α levels peaked within 1 hour after infusion and IL-2, IL-6, IL-10, and IFN-γ reached maximum levels at the next time point, 4 hours after infusion (elevation in other cytokines included IL-4, IL-8, IL-12, and IL-1β). All six patients in that study required supportive care in an intensive care unit and two of the six required extensive intensive care unit stays of 11 and 21 days. Similar to patients suffering serious adverse events from other mAb infusions, these patients showed signs of cardiovascular instability and disseminated intravascular coagulation, but unlike other treatments, these patients manifested additional sequelae including early and acute lung injury. Of interest was the lack of toxicity seen with this agent in preclinical tests of the anti-CD28 mAb TGN1412 in nonhuman primate studies.[48,49]

Perhaps the most relevant to our patient, were results observed in a phase I trial of a bispecific antibody that targeted both ERBB2 and FcγRIII.[51] In this study, attempting to target FcγRIII-expressing cells (e.g., natural killer cells) to ERBB2-expressing tumors, some patients experienced side effects including dyspnea with arterial oxygen desaturation and hypotension. Analysis of serum cytokine levels demonstrated an increase in TNF-α within 30 minutes that peaked at 2.3 hours before declining over the next 24 hours with slightly delayed increases in IL-6, IL-8, IL-2, IL-1β, GM-CSF, and IFN-γ. A major difference between therapeutic antibody administration and CAR-engineered T cells, is that while antibodies are subject to clearance by the body (e.g., the bispecific mAb used in the cited trial had a $t_{1/2}$ of 20 hours), T cells can continuously produce effector cytokines and can expand in cell numbers following antigen stimulation. The severity of this patients' response lead us to investigate her cytokine genotype (**Figure 3**), as polymorphisms in cytokine genes are associated with both levels of cytokine production and immunological responses, such as organ graft rejection.[52–54] Although her genotype for IFN-γ (−874A) and TNF-α (−308G) are associated with lower levels of cytokine production, her IL-6, IL-10, and transforming growth factor-β1 genotypes are associated with higher levels of cytokine production, and the specific IL-6 (−174G/C) and the IL-10 (−1082G) genotypes are associated with shock in patients with sepsis[55] and increased mortality in severe sepsis,[56] respectively.

Since 2004, the Surgery Branch, NCI has conducted clinical trials involving the transfer of T-cell receptor genes into autologous lymphocytes (either PBL or TIL) using the identical

nonmyeloablative conditioning regimen used in the present study (in some cases total body irradiation was added to further reduce endogenous lymphocytes). These treatments were undertaken in a variety of cancers (melanoma, synovial cell sarcoma, and cancers of the breast, colon, and kidney) and there was no indication that this conditioning regime was associated with the tumor lysis syndrome most frequently observed in cancers of hematopoietic origin.[57] Similar numbers of cells were administered in these trials as well as the present trial, and review of the certificates of analysis of these products demonstrated similar levels of background cytokine production. A total of 143 patients were treated during this period without any treatment related mortality. These trials, included 11 patients treated with PBL engineered to target the wild-type p53 tumor suppressor protein,[58,59] and no significant treatment related toxicity was seen (data not shown). In our recent report describing the use of high-avidity T-cell receptors targeting melanocyte differentiation antigens, on-target toxicity was observed in the skin, ears, and eyes, with serum IFN-γ levels peaking at days 3–6 after infusion at cytokine levels over tenfold less than observed in this patient.[21] ERBB2 is known to be expressed at low levels in a variety of normal tissues including the lung.[60] Histological examination of tissues obtained at autopsy confirmed ERBB2 overexpression in both liver metastases (3^+ positivity, >50%), and lung metastases (2^+ positivity, >50%), but also noted much lower levels of ERBB2 expression in normal lung parenchyma, normal liver tissue, and normal breast lobules consistent with the known tissue distribution of ERBB2 expression.

We postulate that the death of this patient was the result of the transfer highly active anti-ERBB2 directed T cells that upon first-pass clearance in the lung, recognized ERBB2 expressed by normal lung cells and released inflammatory cytokines (including TNF-α and IFN-γ) that caused pulmonary toxicity and edema followed by a cascading cytokine storm resulting in multiorgan failure similar to multiple organ dysfunction syndrome caused by a variety of acute physiological insults (e.g., trauma or severe infections). It is likely that the lack of toxicity seen in multiple vaccine trials that induced the generation of anti-ERBB2 lymphocytes was due to the very low levels of reactivity of these cells following immunization with self-antigens.

In initiating any new effort in patients with terminal cancer, there is a tension between administering a sufficient dose to provide clinical benefit while paying strict attention to patient safety. Our recommendation for future first-in-man trials of similar regents would be to conduct a more restricted dose-escalation trial starting at low doses that are unlikely to cause serious toxicity.

MATERIALS AND METHODS

Clinical protocol. The clinical trial under which this patient was treated was: NCI-09-C-0041 entitled Phase I/II Study of Metastatic Cancer that Expresses Her-2 Using Lymphodepleting Conditioning Followed by Infusion of Anti-Her-2 Gene Engineered Lymphocytes. Patient inclusion criteria included metastatic cancer that expressed ErbB2 (Her-2/neu) at $\geq 2^+$ as assessed by immunohistochemistry in a Clinical Laboratory Improvement Amendments approved laboratory. Patients must have previously received systemic standard care (or effective salvage chemotherapy regimens) for metastatic disease, if known to be effective for that disease, and have been either nonresponders (progressive disease) or have recurred. Patients must be ≥18 years of age. Declaration of Helsinki protocols were followed and patients gave their written informed consent. Before receiving treatment with transduced PBLs, patients were transiently lymphoablated using a nonmyeloablative lymphodepleting regimen as previously described, by intravenous administration of cyclophosphamide 60 mg/kg for 2 days followed by fludarabine 25 mg/m^2 for 5 days. One day after completion of their lymphodepleting regimen, patients were to receive transduced lymphocytes infused intravenously followed by high-dose (720,000 U/kg) IL-2 (Aldesleukin; Chiron, Emeryville, CA) every 8 hours to tolerance, although the patient reported here did not receive additional IL-2. The protocol was designed as a cell dose escalation in cohorts of three patients each. The lowest cell dose cohort was $\leq 10^{10}$ cells/infusion.

Gene transfer procedure. A detailed description of the generation of γ-retroviral vector construct designed to express the ERBB2-specific single-chain Fv fragment (4D5-CD8-28BBZ) from Herceptin mAb was recently published.[28] In brief, the single-chain Fv fragment from mAb 4D5 was linked the CD8α-chain hinge and transmembrane region with CD28, 4-1BB, and CD3z intracellular signaling domains and this cassette inserted into the MSGV-1 γ-retroviral vector. The complete sequence of the vector is presented in **Supplementary Figure S1**. A high-titer PG13 cell-based producer cell line was selected and current good manufacturing practice grade retroviral vector supernatant produced by the Indiana University Vector Production Facility (Indianapolis, IN). The vector supernatant was tested and passed all currently required US Food and Drug Administration guidelines for the production of recombinant γ-retroviral vectors for clinical application.

The transduction procedure was initiated by stimulating PBMCs with anti-CD3 mAb OKT3 (Ortho Diagnostic Systems, Raritan, NJ) at a final concentration of 50 ng/ml with recombinant human IL-2 at a final concentration of 300 IU/ml in AIM-V medium (Invitrogen, Carlsbad, CA) containing 5% human serum (Surgery Branch, NCI). Cells were harvested for retroviral transduction on day 2 and resuspended in the same medium without OKT3. Retroviral vector supernatant was thawed and diluted with two parts of medium before being loaded onto RetroNectin (CH-296; Takara Bio, Ohtsu, Japan) coated (coated using 10 mg/ml of CH-296) non-tissue culture treated six-well plates. Vector supernatant was "spun loaded" onto coated plates by centrifugation at 2,000 *g* for 2 hours at <32 °C. Retroviral vector was aspirated from the wells and 1–2 × 10^6 activated PBMC were added pre-well followed by centrifugation at 1,000*g* for 10 minutes. Plates are incubated at 37 °C overnight and the next day all wells are harvested, pooled, and the transduction procedure repeated. Following the second transduction, cells are collected and maintained in medium at 0.5–2.0 × 10^6 cells/ml for a total of 10 days after stimulation. At day 10 after stimulation, cells were subject to a rapid expansion procedure for an additional 14 days using 6,000 IU/ml IL-2 with 50 ng/ml anti-CD3 mAb OKT3 and 100-fold excess 5 Gy irradiated allogeneic PBL feeder cells. Treatment cells were washed in saline before infusion and resuspended in 125 ml containing 300 IU/ml IL-2 then administered to the patient intravenously.

In vitro *assays.* Two to four days before infusion, CAR-transduced PBLs were evaluated for ERBB2-specific CAR expression using an ERBB2-Fc fusion protein, or as control, VEGFR2-Fc (R&D Systems, Minneapolis, MN) followed by phycoerythrin-conjugated antihuman IgG Fc antibody (eBioscience, San Diego, CA). Immunofluorescence was analyzed as the relative log fluorescence of live cells, was measured using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cell function was evaluated by overnight coculture with ERBB2-expressing and nonexpressing target cells (1 × 10^5 target plus 1 × 10^5 effector T cells) followed by enzyme-linked immunosorbent assay measurement (Pierce Endogen, Rockford, IL) of IFN-γ. ERBB2$^+$ target cell were melanoma cell lines 526, 624, 888, 938 (generated at the Surgery Branch, NCI) and tumor lines, SK-OV3, SK-BR3, MDA361 (American Type Culture Collection, Rockville, MD), and ERBB2$^-$ tumor lines MDA468 and CCRF-CEM

(CEM) obtained from ATCC. All tumor cell lines were cultured in media consisting of RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (Biofluids, Rockville, MD), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen). Nontransformed human cell cultures were purchased from Lonza (Walkersville, MD), and maintained in media recommended by the supplier. Autologous dendritic cells and macrophage cultures were obtained from patient PBMC as previously described.[61]

Genomic DNA was isolated from flash-frozen tissue samples using Maxwell 16 Tissue DNA Purification kit (Promega, Madison, WI) according to the manufacture's instruction. One hundred nanogram of each DNA was used for the Real-time quantitative-PCR assay (TaqMan; Applied Biosystems, Foster City, CA). All PCR were performed using an ABI 7500 Fast Real-time PCR System instrument (Applied Biosystems). The TaqMan gene-specific assay was designed by ABI Assays-by-Designs software (Applied Biosystems). Primers and probe used for detection of the ERBB2-CAR vector were: 4D5BBCD3Z-F TGCCGATTTCCAGAAGAAGAAGAAG, 4D5BBCD3Z-R TGCGCTC CTGCTGAACT, 4D5BBCD3Z-M FAM probe CACTCTCAGTTCAC ATCCT. The reference standard curve was established using the DNA extracted from the cells infused into the patient, with undiluted infusion DNA being given a value of 100 as reference. TaqMan β-actin control reagents kit (Applied Biosystems) was used to normalize reactions to input DNA amounts. Cytokine genotype was determined using a commercially available PCR-sequence specific primer kit (Cytokine Genotype Tray; One Lambda, Canoga Park, CA) as directed by the supplier.

Serum cytokine levels were assayed for using commercially available enzyme-linked immunosorbent assay kits [IFN-γ, TNF-α, GM-CSF, and IL-6 (Endogen, Cambridge, MA)] or SearchLight cytokine array (Aushon Biosystems, Billerica, MA). Cytokine secretion was measured in samples diluted to be in the linear range of the assay.

SUPPLEMENTARY MATERIAL

Figure S1. Nucleotide sequence of ERBB2-CAR vector.

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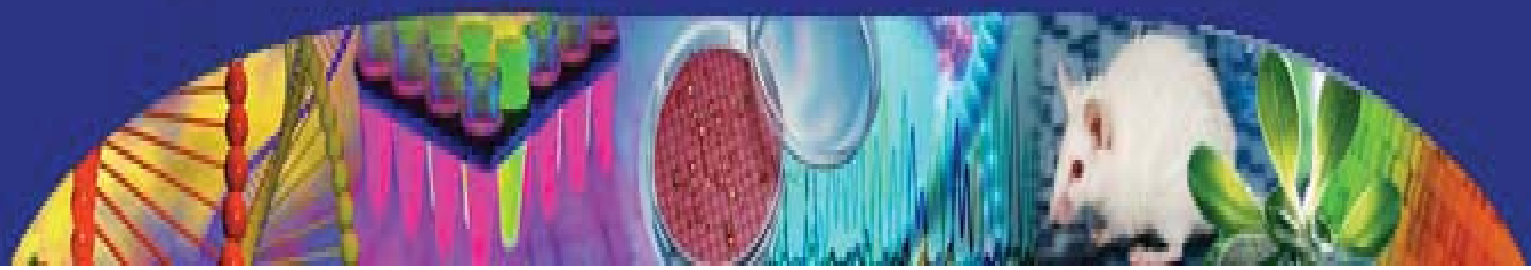

MART-1 and gp100 Protocols

Antigen Target	**MART-1**
Antigen Expression	**Melanoma tumor antigen expressed on normal melanocytes in the skin, eye and ear.**

	Engineered T Cell Trials Registered with OBA
Title	**Protocol # 599: A Study in Metastatic Melanoma Using a Lymphodepleting Conditioning followed by infusion of anti-Mart-1 TCR Gene Engineered Lymphocytes**
PI	**Steve Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/alpha and beta chains for anti-Mart-1 T cell receptor**
Status	**Closed**
Doses Proposed	**$1x10^9$ to $8.6x10^{10}$ cells**
Lymphodepletion	**Cyclophosphamide 60mg/kg, Fludarabine 25mg/m^2**
Subjects Enrolled	**17**
Summary of Unexpected and Related Events	**None**
Summary of Results	**13% response rate overall with 2 sustained objective regressions** **Morgan, R. A., *et. al*. (2006). Cancer Regression in Patients after Transfer of Genetically Engineered Lymphocytes. *Science* 314:126-129.**

Title	**Protocol # 504: A Phase I Study of Genetically Modified Autologous Peripheral Blood T-Cells Expressing a Retrovirally Encoded, MART-1 Specific αβ T-Cell Receptor, With and Without Recombinant Human Interleukin-2, in HLA-A2+ Adult Patients with Advanced Malignant Melanoma**
PI	**Kevin McDonagh, M.D.**
Vector and Signaling Moieties	**Retrovirus/alpha and beta chains for anti-Mart-1 T cell receptor**
Status	**No evidence initiated**
Doses Proposed	**$1x10^8$ – $1x10^{10}$ cells**
Lymphodepletion	**No**
Subjects Enrolled	**Unknown**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

Title	**Protocol # 791: Treatment and Biological Imaging of Patients with Locally Advanced or Metastatic Melanoma with Lentiviral Vector MART-1 TCR/HSV1-sr39k (FUW-M1-TCR/sr39k) Engineered Lymphocytes, MART-126.35-Pulsed Dendritic Cells, and Interleukin-2 after a Nonmyeloablative Conditioning Regimen**
PI	**Antoni Ribas, M.D.**
Vector and Signaling Moieties	**Lentiviral vector co-expressing the two chains of the MART-I TCR and the PET reporter gene sr39tk. These transgenes are linked by 2A self cleaving picornavirus sequences, which are co-translationally cleaved to result in the three independent protein products in the target cells: the alpha and beta MART-I TCR chains and the sr39tk enzyme.**
Status	**No information received**
Doses Proposed	**10^7 – 10^9 $CD8^+$ cells**
Lymphodepletion	**Cyclophosphamide 60mg/kg, Fludarabine 25mg/m^2**
Subjects Enrolled	**No information received**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

Title	**Protocol # 840: Phase II Study of Metastatic Melanoma Using Lymphodepleting Conditioning Followed by Infusion of Anti-MART-1 F5 TCR-Gene Engineered Lymphocyte**
PI	**Steve Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/alpha and beta chains for anti-MART-1 F5 TCR**
Status	**Active**
Doses Proposed	**Up to $3x10^{11}$ cells**
Lymphodepletion	**Cyclophosphamide 60mg/kg, Fludarabine 25mg/m^2**
Subjects Enrolled	**24**
Summary of Unexpected and Related Events	**There have been several incidences of hearing loss, tinnitus, uveitis, rash and symptoms of dizziness observed in patients on this trial and the adverse events have been attributable to the anti-Mart-1 F5 TCR transduced cells.**
Summary of Results	**Twenty-one subjects have been dosed with the anti-Mart-1 F5 TCR peripheral blood lymphocytes (cohort 1). In this cohort, there have been 6 subjects who have experienced partial responses to the intervention, 2 of which are currently ongoing. Three subjects have been treated with the anti-Mart-1 F5 TCR tumor infiltrating lymphocytes (cohort 2), and all three have been removed for progressive disease (2009 data).** **Johnson, L. A., *et. al.* (2009). Gene Therapy with Human and Mouse T-cell Receptors Mediates Cancer Regression and Targets Normal Tissues Expressing Cognate Antigen. *Blood* 114: 535-546.**

Title	**Protocol # 882: Phase II Study of Metastatic Melanoma Using Lymphodepleting Conditioning Followed by Infusion of Anti-MART-1 F5 TCR-Gene Engineered Lymphocytes and ALVAC Virus Immunization**
PI	**Steve Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/alpha and beta chains for anti-MART-1 TCR**
Status	**Active**
Doses Proposed	**$5x10^8$ to $3x10^{11}$ cells**
Lymphodepletion	**Cyclophosphamide 60mg/kg, Fludarabine 25mg/m^2**
Subjects Enrolled	**2**
Summary of Unexpected and Related Events	**One subject presented with Grade 3 hearing loss, Grade 2 dizziness and Grade 2 uveitis.**
Summary of Results	**None**

Title	**Protocol # 885: Transfer of Autologous T Cells Transduced with the Anti-MART-1 F5 T Cell Receptor in High Risk Melanoma (Phase II study)**
PI	**Steve Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/alpha and beta chains for anti-MART-1 TCR**
Status	**Active**
Doses Proposed	**$1x10^9$ – $7x10^{10}$ cells**
Lymphodepletion	**No**
Subjects Enrolled	**25 subjects who per protocol must be clinically disease free at the time of protocol entry as documented by radiologic studies within 4 weeks of patient entry.**
Summary of Unexpected and Related Events	**None**
Summary of Results	**Of the 25 subjects enrolled, 2 were taken off study due to recurrences of tumor 6 months after administration of cells and the remainder are either disease free or it is too early to evaluate (2009 data).**

Title	**Protocol # 901: Adoptive Transfer of MART-1 F5 TCR Engineered Peripheral Blood Mononuclear Cells (PBMC) after a Nonmyeloablative Conditioning Regimen, with Administration of MART-1:26•35-Pulsed Dendritic Cells and Interleukin-2, in Patients with Advanced Melanoma**
PI	**Antoni Ribas, M.D.**
Vector and Signaling Moieties	**Retrovirus/alpha and beta chains for anti-MART-1 TCR**
Status	**Active**
Doses Proposed	**$1x10^7 – 10^9$ cells**
Lymphodepletion	**Cyclophosphamide 60mg/kg, Fludarabine 25mg/m^2**
Subjects Enrolled	**At least 1**
Summary of Unexpected and Related Events	**None**

Antigen Target	**gp-100**
Antigen Expression	**Melanoma antigen also expressed in normal melanocytes, including in the eye and striae vascularis of the inner ear.**

	Engineered T Cell Trials Registered with OBA
Title	**Protocol # 599: Treatment of Patients with Metastatic Melanoma by Lymphodepleting Conditioning Followed by Infusion of TCR-Gene Engineered Lymphocytes and Subsequent Fowlpox gp100 Vaccination**
PI	**Steven Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/TCR alpha beta chains against gp100**
Status	**Closed**
Doses Proposed	**$1x10^9$ – $3x10^{10}$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m^2**
Subjects Enrolled	**14**
Summary of Unexpected and Related Events	**None**
	One partial clinical response (of at least 8 months) has been observed in the tumor infiltrating lymphocyte (TIL) arm but no responses have occurred in the peripheral blood lymphocyte (PBL) arms.

Title	**Protocol # 830: Phase II Study of Metastatic Melanoma Using Lymphodepleting Conditioning Followed by Infusion of Anti-gp100:154-162 TCR-Gene Engineered Lymphocytes**
PI	**Steven Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/TCR alpha and beta chains (human gp100) fused to a high avidity xeno TCR (of murine origin)**
Status	**Active**
Doses Proposed	**$5x10^8$ – $3x10^{11}$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m2**
Subjects Enrolled	**21**
Summary of Unexpected and Related Events	**Two grade 3 hearing loss; one has resolved completely, and the other has decreased to a grade 1 adverse event.**
Summary of Results	**Nineteen subjects were treated in Cohort 1 which uses peripheral blood lymphocytes (PBL) and 2 subjects were treated on Cohort 2 that uses tumor infiltrating lymphocytes (TIL). There have been 3 partial responses (PR) of 3, 6 and 4 months and 1 ongoing complete remission (CR). Two of the PRs and the CR were in the PBL arm and 1 PR was in the TIL arm. Twenty subjects have been removed from the protocol due to progressive disease.** **Johnson, L. A., *et. al.* (2009). Gene Therapy with Human and Mouse T-cell Receptors Mediates Cancer Regression and Targets Normal Tissues Expressing Cognate Antigen. *Blood* 114: 535-546.**

Title	**Protocol # 883: Phase II Study of Metastatic Melanoma Using Lymphodepleting Conditioning Followed by Infusion of Anti-gp100:154-162 TCR-Gene Engineered Lymphocytes AND ALVAC Virus Immunization**
PI	**Steven Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/TCR alpha and beta chains (human gp100) fused to a high avidity xeno TCR (of murine origin)**
Status	**Active**
Doses Proposed	**$5x10^8$ - $3x10^{11}$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m^2**
Subjects Enrolled	**2**
Summary of Unexpected and Related Events	**None**
Summary of Results	**Too early to evaluate**
Title	**Protocol # 939: Phase II Study of Metastatic Melanoma Using a Chemoradiation Lymphodepleting Conditioning Regimen Followed by Infusion of Anti-Mart-1 and Anti-gp100 TCR-Gene Engineered Lymphocytes and Peptide Vaccines**
PI	**Steven Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/TCR alpha and beta chains (human gp100) fused to a high avidity xeno TCR (of murine origin); alpha and beta chains for anti-Mart-1 T cell receptor**

Status	**Active**
Doses Proposed	**$1x10^9$ – $1x10^{11}$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg and Fludarabine 25 mg/m^2**
Subjects Enrolled	**3**
Summary of Unexpected and Related Events	**Hearing loss, uveitis, rash, and symptoms of dizziness observed in subjects on this trial and these adverse events have been attributed to either the anti-gp100:154 TCR transduced cells or the anti-MART-1 F5 TCR-transduced cells. There have been two Grade 3 hearing losses, one which resolved to Grade 1 and the other remains at Grade 3.**
Summary of Results	**Of the three subjects, one died of viral infection 40 days after the treatment began and the other 2 are two early for evaluation (2009 data).**

Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen

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Gene therapy of human cancer using genetically engineered lymphocytes is dependent on the identification of highly reactive T-cell receptors (TCRs) with antitumor activity. We immunized transgenic mice and also conducted high-throughput screening of human lymphocytes to generate TCRs highly reactive to melanoma/melanocyte antigens. Genes encoding these TCRs were engineered into retroviral vectors and used to transduce autologous peripheral lymphocytes administered to 36 patients with metastatic melanoma. Transduced patient lymphocytes were CD45RA$^-$ and CD45RO$^+$ after ex vivo expansion. After infusion, the persisting cells displayed a CD45RA$^+$ and CD45RO$^-$ phenotype. Gene-engineered cells persisted at high levels in the blood of all patients 1 month after treatment, responding patients with higher ex vivo antitumor reactivity than nonresponders. Objective cancer regressions were seen in 30% and 19% of patients who received the human or mouse TCR, respectively. However, patients exhibited destruction of normal melanocytes in the skin, eye, and ear, and sometimes required local steroid administration to treat uveitis and hearing loss. Thus, T cells expressing highly reactive TCRs mediate cancer regression in humans and target rare cognate–antigen-containing cells throughout the body, a finding with important implications for the gene therapy of cancer. This trial was registered at www.ClinicalTrials.gov as NCI-07-C-0174 and NCI-07-C-0175. (Blood. 2009;114:535-546)

Introduction

Tumor-associated antigens have been identified on a wide variety of human cancers. Many of these antigens are normal, nonmutated self-proteins selectively expressed or overexpressed on cancers.[1] Antigens such as MART-1 and gp100 are expressed on melanomas and normal melanocytes in the skin, eye, and ear.[2,3] Other cancer-associated antigens such as carcinoembryonic antigen (CEA), Her2/neu, and Muc-1 are expressed at low levels on some normal tissues, whereas antigens such as NY-ESO-1 and the MAGE family of proteins are expressed on fetal tissue and the adult testes but not on other normal adult tissues.[4] The presence of these normal proteins during fetal development leads to central tolerance based on negative selection in the thymus of lymphocyte clones bearing high-affinity self-reactive T-cell receptors (TCRs). Occasionally, lymphocytes bearing high-affinity TCR escape thymic deletion; and in these instances, mechanisms of peripheral tolerance can suppress their activity.[5]

Cell transfer therapies have emerged as a tool to overcome the limitations imposed by both central and peripheral tolerance.[6-9] Transfer of antitumor T cells to lymphodepleted mice can mediate the rejection of large, vascularized tumors,[10] and the administration of naturally occurring antimelanoma tumor-infiltrating lymphocytes (TILs) can mediate objective cancer regressions in 51% to 72% of lymphodepleted patients with metastatic melanoma.[7,8,11]

A major obstacle to the widespread application of cell transfer therapies is the difficulty in identifying human T cells with antitumor recognition. Only approximately half of melanomas reproducibly give rise to antitumor TILs,[12] and other cancer types only rarely contain identifiable tumor-reactive lymphocytes. An alternative to finding these natural tumor-reactive cells for every patient is the transfer to normal lymphocytes of tumor-reactive TCR genes recognizing shared tumor antigens.

In a prior study, we identified a TCR (MSGV1AIB, here referred to as DMF4) that recognized the MART-1 melanoma/melanocyte antigen cloned from the TILs of a resected melanoma lesion.[13] We treated 31 patients with autologous peripheral blood lymphocytes (PBLs) transduced with genes encoding this receptor (17 were previously reported),[14] and 4 patients (13%) experienced an objective regression of metastatic melanoma. None of the 31 patients exhibited skin rash or normal melanocyte toxicity in the eye or ear. The DMF4 receptor had only moderate ability to recognize limiting amounts of antigen, and we hypothesized that a more highly reactive TCR might be clinically more effective in recognizing malignant cells that expressed the target antigen.

We thus generated a high-avidity TCR from a human T cell that recognized the MART-1:27-35 epitope (here referred to as DMF5).[15] The gp100:154-162 epitope from the gp100 melanoma-melanocyte antigen is the most highly expressed peptide from this protein, displayed

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on the cell surface. Attempts to generate a high-avidity human TCR against this epitope were unsuccessful. We were able, however, to generate a highly avid TCR against this epitope in human leukocyte antigen-A2 (HLA-A2) transgenic mice. These anti–MART-1 and anti-gp100 TCRs were used to treat patients with metastatic melanoma.

Methods

Patients

Thirty-six patients with metastatic melanoma were treated at the Surgery Branch, National Cancer Institute, between July 2007 and March 2008, in protocols approved by the Institutional Review Board and Food and Drug Administration, 20 with TCR recognizing the HLA-A*02–restricted melanoma antigen MART-1 (DMF5)[15] and 16 with TCR recognizing the HLA-A*02–restricted melanoma antigen gp100(154).[16] All patients gave informed consent for treatment in accordance with the Declaration of Helsinki. Patients were HLA-A*02$^+$, 18 years of age or older, had measurable metastatic melanoma, and Eastern Cooperative Oncology Group status 0 or 1. All patients had progressed after prior treatment with interleukin-2 (IL-2)/Aldesleukin and had tumors that expressed the appropriate antigen (MART-1/Melan-A or gp100/HMB45). Contraindications were as follows: concurrent major medical illnesses, any form of primary or secondary immunodeficiency, severe hypersensitivity to any of the agents used in this study, contraindications for high-dose IL-2 administration, systemic steroid treatment within 30 days before treatment, and untreated intracranial metastases more than 1.0 cm in diameter.

TCR recognizing shared melanoma antigens

The generation of the DMF5 TCR has been previously described.[15] The patient from whom the MART-1–reactive TCR was derived experienced vitiligo but no eye or ear toxicity. To generate a high-avidity TCR against the gp100:154-162 epitope, HLA-A*0201 mice were immunized twice with the human gp100:154-162 peptide and the I-A^b-binding synthetic T helper peptide representing residues 128 to 140 of the hepatitis B virus core protein emulsified in incomplete Freund adjuvant. Seven days after the second immunization, splenocytes were stimulated in vitro with equal numbers of irradiated, lipopolysaccharide-activated, A2.1 transgenic mouse splenocytes pulsed with 0.01 μg/mL human gp100:154-162 peptide and 10 μg/mL human β2-microglobulin in media containing 5 IU IL-2. Eight days after a third stimulation, T cells were cloned by limiting dilution in presence of irradiated T2 cells pulsed with 0.01 μg/mL human gp100:154-162 peptide and irradiated C57Bl/6 splenocytes. 5′ RACE TCR isolation and RNA electroporation into donor PBLs were as previously described.[15] The gp100(154) TCR conferring the highest antitumor avidity to donor PBLs functioned independently of CD4 or CD8 coreceptor and was selected for clinical use.

Retroviral gene therapy vectors

pMSGV1 is derived from pMSGV murine stem cell virus long terminal repeat containing an extended gag region and Kozak sequence.[13] Vector pMSGV1 gp100(154)-AIB was produced by linking the *TCR-α* via an internal ribosome entry site (IRES) element followed by insertion of the *TCR-β* chain. Vector pMSGV DMF5 furin 2-A was generated by introducing DMF5 *TCR-α* cDNA[15] followed by a furin T2A cleavage sequence and DMF5 *TCR-β*. Clinical grade cGMP-quality retroviral supernatants were produced by the National Gene Vector Laboratories at Indiana University. Patient PBLs were stimulated with anti-CD3 monoclonal antibody (mAb) OKT-3 2 days before transduction using retronectin (Takara Bio Inc)–coated plates, per the manufacturer's recommendations.

Preparation of PBLs transduced with TCR

A total of 5 to 10 × 10^8 patient PBLs were obtained by leukapheresis and stimulated in vitro at 10^6/mL with 50 ng/mL anti-CD3 mAb OKT-3, in complete AIMV media (Invitrogen) supplemented with 5% human serum (Surgery Branch, National Cancer Institute), and 300 IU IL-2 (TCR media). Two days later, TCR-encoding retroviral supernatant was rapid-thawed, diluted 1:1 in TCR media, and added to plates that had been coated overnight with 10 μg/mL retronectin (Takara Bio Inc). Supernatant was spin-loaded onto plates by centrifuging 2 hours at 2000*g* at 32°C. The stimulated PBLs were washed and resuspended at 0.25 to 0.5 × 10^6/mL in TCR media, and 1 to 2 × 10^6 PBLs were added per well to the retrovirus-loaded plates. Plates were spun at 1000*g* at 32°C for 10 minutes and incubated overnight at 37°C, 5% CO_2. The next day (day 3), PBLs were transferred to newly prepared retroviral-coated 6-well plates as on day 2. The following day, transduced PBLs were washed, resuspended in fresh TCR media, and transferred to flasks at 37°C, 5% CO_2. On days 9 to 12, cells were expanded or not an additional 9 to 14 days in 6000 IU IL-2 with 50 ng/mL anti-CD3 mAb OKT-3 and 100-fold excess 5 Gy irradiated allogeneic PBL feeder cells. Before treatment, TCR-transduced PBLs from all patients were evaluated for expression of the appropriate TCR by tetramer staining and flow cytometric analysis, and cell function was evaluated by overnight coculture with cognate antigen-bearing target cells (1 × 10^5:1 × 10^5) and enzyme-linked immunosorbent assay (ELISA) measurement (Pierce Endogen) of interferon-γ (IFN-γ) produced in the culture supernatant. Treatment cells were washed in saline before infusion into patients intravenously.

Clinical protocol

The clinical trial registration numbers and approved registry names are as follows: NCI-07-C-0175, Phase II Study of Metastatic Melanoma Using Lymphodepleting Conditioning Followed by Infusion of Anti-MART-1 F5 TCR-Gene Engineered Lymphocytes; NCI-07-C-0174, Phase II Study of Metastatic Melanoma Using Lymphodepleting Conditioning Followed by Infusion of Anti-gp100:154-162 TCR-Gene Engineered Lymphocytes.

Before receiving treatment with transduced PBLs, patients were transiently lymphoablated using a nonmyeloablative lymphodepleting regimen as previously described,[8] by intravenous administration of cyclophosphamide 60 mg/kg for 2 days followed by fludarabine 25 mg/m^2 for 5 days. One day after completion of their lymphodepleting regimen, patients received transduced lymphocytes infused intravenously followed by high-dose (720 000 U/kg) IL-2 (Aldesleukin; Chiron Corp) every 8 hours to tolerance. Five DMF5 patients (patients 7-11), and 4 gp100(154) patients (patients 7-10) received TCR-transduced cells on days 10 to 12 after stimulation. The remaining 15 DMF5 patients and 12 gp100(154) patients received a larger number of TCR-transduced cells, which were grown for an additional 9 to 14 days after a second OKT-3 stimulation.

Patients received baseline computed tomography (CT) and/or magnetic resonance imaging before treatment and underwent pretreatment and posttreatment ocular and audiology examinations. Tumor size was evaluated monthly by CT, magnetic resonance imaging, or documented with photography for cutaneous/subcutaneous lesions. Tumor measurements and patient responses were determined according to Response Evaluation Criteria in Solid Tumors (RECIST).[17] Samples of patient PBLs and serum were taken after transduced PBL infusion. Serum cytokine levels were measured by ELISA assay (Pierce Endogen).

Evaluation of cell activity and persistence

Skin and tumor biopsies were obtained and immunohistochemically stained for the presence of CD4 and CD8 T cells. In one patient with uveitis, ocular fluid was evaluated for the presence of transduced antitumor T cells by flow cytometry. Cell activity was evaluated by coculturing patient PBLs with cognate antigen on T2 target cells, or HLA-matched and mismatched melanomas mel526, mel624 (HLA-A*02$^+$), or mel888 and mel938 (non-HLA-A*02). IL-2 and IFN-γ were measured by ELISA (Pierce Endogen), ELISPOT (reagents from Mabtech Inc, Millipore Corp, Invitrogen, BD Biosciences PharMingen, and Kirkegaard & Perry), or intracellular staining (mAbs from eBiosciences) and flow cytometry. Lysis was evaluated by target cell ^{51}chromium-release assay. Transferred cell persistence in blood was followed by tetramer staining with HLA-A2/MART-1:27-35 or HLA-A2/gp100:154-162 tetramer (Beckman Coulter Immunotech).

Statistical significance was evaluated using the paired *t* test.

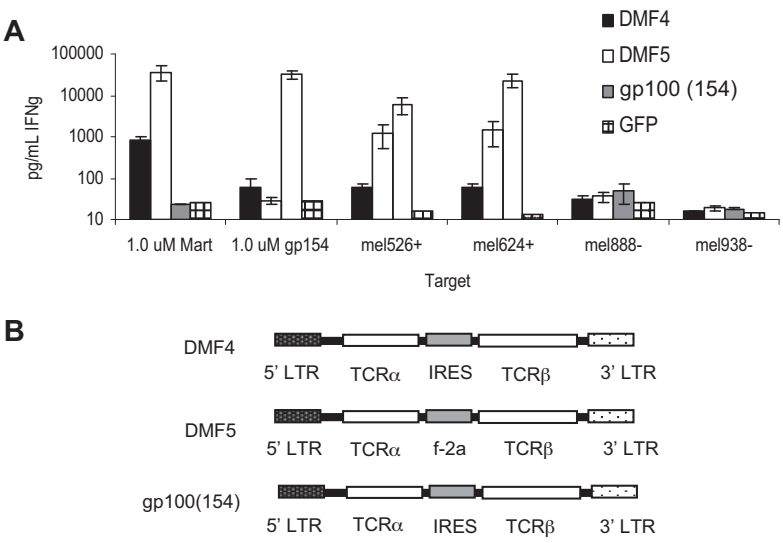

Figure 1. Tumor-reactive DMF5 or gp100(154) alpha and beta TCR chain RNA electroporated into PBLs confer high reactivity to melanoma tumor antigens. (A) Ten-day anti-CD3–stimulated donor PBLs were electroporated with in vitro–transcribed RNA encoding paired DMF4, DMF5, or gp100(154) TCR alpha and beta chains, or GFP control. Cells were cocultured for 18 hours with T2 cells pulsed with peptide, HLA-A*02$^+$ melanomas mel624$^+$ or mel526$^+$, or HLA-A*02$^-$ melanomas mel888$^-$ or mel938$^-$. IFN-γ in the supernatant was detected by ELISA. (B) Structure of the MSGV-based γ-retroviral vectors DMF4 and gp100(154), incorporating an IRES and DMF5 with a furin 2A ribosomal skip sequence, allowing for dual gene expression.

Results

DMF5 and gp100(154) are highly reactive TCRs that confer melanoma tumor reactivity to donor PBLs

We have used 2 highly reactive TCRs capable of recognizing the MART-1 or gp100 melanocyte antigens overexpressed on melanomas. To overcome the problem of central deletional tolerance of lymphocytes expressing high-affinity antitumor TCRs, we raised a highly reactive TCR called gp100(154) against the human melanocyte gp100:154-162 epitope by immunizing HLA-A*0201 transgenic mice with this peptide that differs from the mouse sequence by a single amino acid. In addition, extensive screening of more than 600 clones in TILs from multiple patients revealed a lymphocyte clone called DMF5 with far greater reactivity than the previously identified DMF4 against the MART-1:27-35 peptide epitope.[15] The TCR genes from the mouse cells and from the human DMF5 clone were isolated, and equivalent amounts of RNA were generated in vitro and transferred into both Jurkat and donor PBLs. After transferring the same amount of exogenous TCR (evaluated by CD3 surface expression in Jurkat cells),[15] the higher-avidity DMF5 and gp100(154) TCR conferred higher reactivity to donor PBLs than the previously identified DMF4 in recognizing tumor antigen (Figure 1A). The genes encoding the alpha and beta chains from these 2 receptors, gp100(154) and DMF5, were each cloned into bicistronic gamma-retroviral vectors using an IRES or a furin 2-A picornavirus-like cleavage sequence, respectively, to drive expression of the second gene (Figure 1B). These retroviral vectors were used to transduce normal human peripheral lymphocytes (Figure 2). It has been demonstrated that DMF4 binds tetramer weakly, underrepresenting the amount of surface TCR,[14,15] a result also observed here (Figure 2A). Using similarly prepared retroviral supernatants in the same donor cell transduction procedure, DMF4 on the surface of transduced CD8$^+$ PBLs only bound 2% of MART-1 tetramer and transduced CD4$^+$ cells bound no tetramer. In contrast, 30% to 60% of both CD4$^+$ and CD8$^+$ lymphocytes transduced with the improved gene constructs encoding the highly reactive DMF5 or gp100(154) TCR bound tetramer efficiently (Figure 2A). These new TCR constructs conferred high expression of TCR, which was coreceptor independent on donor PBLs. A comparison of the functional reactivity of donor lymphocytes transduced with the respective gamma-retroviruses encoding these DMF4, DMF5, or gp100(154) TCR are shown in Figure 2B and C. Cells expressing either the highly reactive DMF5 or gp100(154) TCR recognized 100-fold lower peptide concentrations, produced more IFN-γ, and lysed melanoma targets more effectively than those expressing DMF4.

Administration of TCR-transduced autologous PBLs to patients with metastatic melanoma

To investigate the in vivo activity of autologous cells transduced with these highly reactive TCRs, 36 patients with heavily pretreated, progressive metastatic melanoma received transduced cells (20 patients with DMF5 and 16 with gp100(154)), after a lymphodepleting preparative regimen to deplete endogenous circulating lymphocytes.[8] Patient demographics and treatment details are presented in Tables 1 and 2. Intravenous IL-2 was administered to patients starting 8 hours after adoptive cell transfer and continued every 8 hours for up to 3 days. All of the patients were refractory to prior treatment with IL-2, and 42% and 33% had progressed through prior chemotherapy and radiotherapy, respectively.

Based on tetramer staining, the mean transduction efficiencies for cells administered to these 36 patients were 71% and 82% for DMF5 and gp100(154) TCR, respectively (Tables 1-2). All treatment cells showed high levels of specific reactivity against cognate antigen-bearing tumor targets as assessed by intracellular cytokine staining and ELISPOT analysis (both IFN-γ and IL-2; Tables 1-2) and for IFN-γ release (Table 3) and target cell lysis (data not shown).

Adoptively transferred highly reactive antitumor cells persisted at high levels in patients

In prior trials of patients receiving unmodified TILs, in vivo cell persistence of the transferred cells highly correlated with antitumor response.[18] We thus evaluated the persistence of the human DMF5 and the murine gp100(154) TCR-transduced cells using tetramer binding and ELISPOT assays. All patients had measurable levels ($\geq$ 1%) of tetramer-positive T cells in their circulation at 1 month after treatment (Figure 3A-B). There was no difference in the persistence of cells bearing the human DMF5 TCR (22% $\pm$ 6%) or the murine gp100(154) TCR (22% $\pm$ 5%; $P = .4$; Figure 3B). To measure functional recognition of tumor cells, posttreatment PBLs were cocultured with major histocompatibility complex–matched or mismatched melanomas, and cell reactivity was assessed using

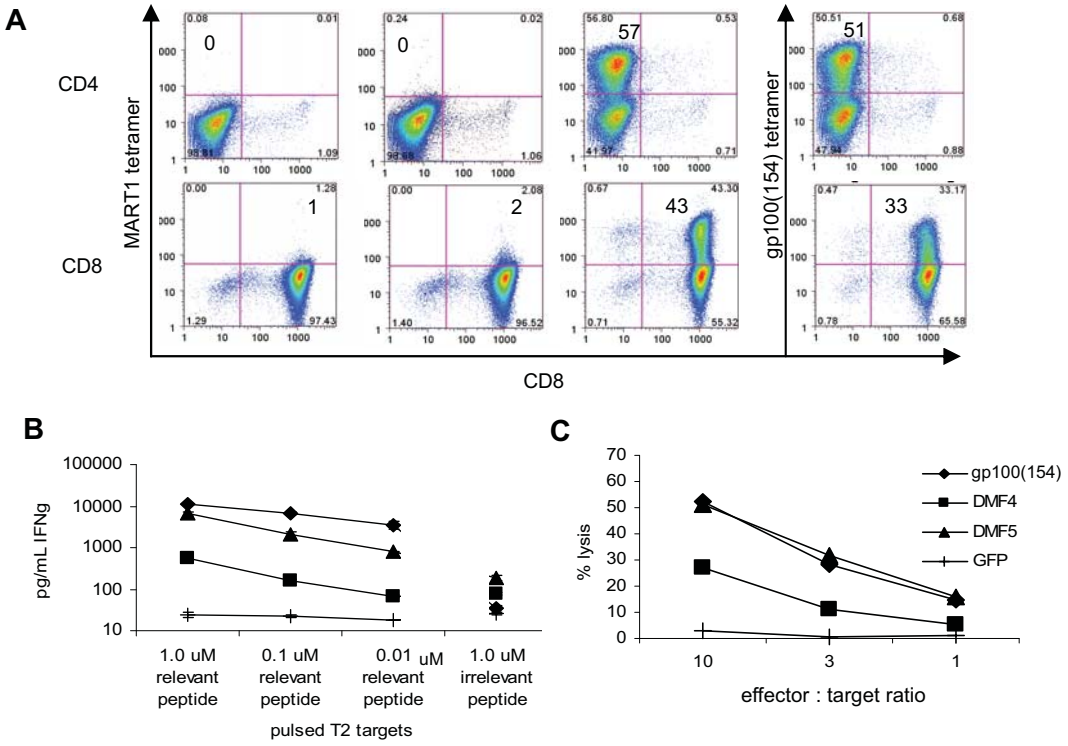

Figure 2. DMF5 and gp100(154) TCR retroviral constructs conferred greater antitumor reactivity to donor PBLs than the original DMF4 receptor. (A) Donor PBLs were stimulated with anti-CD3 mAb OKT-3 and separated into CD4 and CD8 populations before retroviral transduction with DMF4, DMF5, or gp100(154) TCR constructs. TCR expression was analyzed 7 days later by tetramer staining and flow cytometry. (B) Donor PBLs transduced with retroviral TCR constructs were cocultured with T2 cells pulsed with MART-1:27-35 or gp100:154-162 peptide, and IFN-γ secretion was measured by ELISA. (C) Transduced PBLs were cocultured with mel624$^+$ melanomas, and tumor target lysis was evaluated by ^{51}Cr-release assay. Cells did not lyse HLA-mismatched tumors (data not shown).

ELISPOT and intracellular fluorescence-activated cell sorter (FACS) assays (Figure 3). IFN-γ ELISPOT assay showed persistence (> 20 specific spots/100 000 cells) of tumor-reactive transduced cells in 11 of 20 DMF5 and 7 of 16 gp100(154) patients (Figure 3B). Similarly, 11 and 7 of the DMF5 and gp100(154) patients, respectively, exhibited persistence of active cells via IL-2 ELISPOT assays of PBLs at one month after treatment (Figure 3B). At this same 1-month time point, specific intracellular IFN-γ staining was seen in 12 of the 20 DMF5 patients (Figure 3A). Thus, as determined in multiple assays, both murine- and human-derived TCR gene–modified cells persisted in the circulation at 1 month in the majority of patients. Comparing between responding and nonresponding patients, responding patients all had highly persistent tumor-reactive cells at more than or equal to 10% tetramer-positive T cells in the blood 1 month after treatment. However, some nonresponding patients also had high levels of active persistent cells, suggesting that persistence may be necessary, but not sufficient, to cause tumor regression in patients.

Adoptively transferred T-cell phenotype reverts from CD45RA$^-$, CD45RO$^+$ to CD45RA$^+$, CD45RO$^-$ in vivo

We evaluated the phenotype of the DMF5 MART-1–reactive TCR-treated patients' cells before, during, and after treatment by flow cytometry for the costimulatory molecules CD27 and CD28 and the cell differentiation markers CD45RA and CD45RO. Before TCR gene modification, on average (± SEM), patients' peripheral T lymphocytes consisted of 53% plus or minus 5% CD27$^+$, 62% plus or minus 4% CD28$^+$, 42% plus or minus 3% CD45RA$^+$, and 53% plus or minus 3% CD45RO$^+$ cells (Figure 4). After expansion ex vivo, the tetramer-positive infusion cells displayed less CD27 (36% ± 6%), similar CD28 (59% ± 6%), almost complete loss of CD45RA (3.5% ± 1.2%), and a gain of CD45RO to 94% plus or minus 2% (Figure 4). Evaluating tetramer-positive cells in the blood 1 month after treatment showed levels of CD27 similar to infusion (30% ± 5%), reduced levels of CD28 (36% ± 5%), and intriguingly, levels of CD45RA increased and CD45RO decreased, reverting back to levels similar to those seen in the pretreatment PBL samples (27% ± 4% CD45RA$^+$, 66% ± 4% CD45RO$^+$; Figure 4). This suggests that either the few tetramer-positive CD45RA$^+$ cells present at infusion persisted and expanded, or, in agreement with a previous published clinical report of a patient receiving a gene-marked allogeneic cell transfer to treat leukemia,[19] cells that had been CD45RA$^-$ and CD45RO$^+$ reverted to CD45RA$^+$ and CD45RO$^-$ in vivo. There were no substantial differences in the cell phenotype between responding and nonresponding patients.

Clinical course of patients receiving TCR-transduced cells: reactivity against normal tissues

The clinical course of patients receiving the highly tumor-reactive DMF5 and gp100(154) TCR-transduced cells were quite different from that of our prior study with the DMF4 receptor.[14] In the current trial, increased levels of IFN-γ were detected in the serum of patients, peaking around day 3 to 5 after treatment (Figure 5A). The mean peak IFN-γ serum values for patients receiving the improved DMF5 and gp100(154) gene constructs were 128 pg/mL and 210 pg/mL, respectively, compared with 14 pg/mL for the previous DMF4 patients ($P = .03$; Figure 5B). As IFN-γ is an effector cytokine produced by activated T lymphocytes and patients remained depleted of their endogenous lymphocytes during the first week after the preparative lymphodepletion, it is presumed that the transferred cells were the source of this cytokine production.

Table 1. DMF5 patient cell treatment characteristics

Patient no.	Age, y/ sex	Prior treatment*	Sites of disease†	No. of cells $\times 10^9$	Percentage CD4/8	Percentage Tet	Percentage IC IFN-γ‡	ELISPOT		Doses IL-2	Toxicity¶ (skin/eye/ear)	Tumor response, mo**
								IL-2§	IFN-γ‖			
1	36/F	R,S,I	bo, li, lu	22.2	17/83	73	52	95	173	10	1/2/3	NR
2	43/F	S,I	lu	10.5	15/85	46	37	37	166	5	2/2/3	PR (17+)
3	60/F	S,I	bo, ln, sc	6.5	7/93	56	40	1	56	3	0/0/0	NR
4	38/M	S,C,I	eye, sc	12.0	22/78	65	38	24	> 112	8	2/2/1	NR
5	47/F	R,S,I	sc, cu	23.3	37/63	61	21	91	53	5	1,1††/2,2††/0,3††	PR (17+)
6	57/M	R,S,C,I	ln, bo, sc, lu	17.6	27/73	69	29	38	52	7	1/0/0	NR
7	33/M	S,I	ln, li, lu	1.5	11/89	33	19	68	132	10	1/0/0	NR
8	46/M	S,I	ln, br, lu	5.7	32/68	49	17	26	65	7	0/2/0	PR (16+)
9	54/M	S,I	ln, sp, sc	3.8	28/72	69	28	860	164	5	1/0/0	PR (9)
10	35/M	R,S,I	ln, lu, sc	2.0	61/39	48	7	1200	32	11	1/2/0	NR
11	60/F	R,S,I	br, sc, lu	3.0	27/73	60	19	310	152	15	1/0/0	NR
12	49/F	S,I	ln, ki, li, lu, sp	4.8	2/98	85	61	66	34	4	0/0/0	NR
13	54/M	R,S,I	ln, sc	38.0	6/94	91	70	2250	> 292	9	0/2/3	PR (4)
14	24/F	R,S,I	ln, sc	80.0	10/90	75	64	4767	> 349	6	1/1/3	NR
15	56/M	R,S,I	ip, li, lu	30.5	13/87	95	72	990	> 191	9	1/1/1	NR
16	54/M	S,C,I	ln, sc	29.1	4/96	89	54	> 270	> 253	7	0/0/0	NR
17	37/M	S,I	adr, ln, lar, sc	9.9	2/98	77	50	83	27	10	0/0/0	NR
18	56/F	S,I	ln, sc	48.2	21/79	92	68	1410	> 157	3	1/2/3	PR (3)
19	31/F	R,S,C,I	br, sc	73.7	13/60	92	ND	166	> 332	8	2/2/3	NR
20	56/M	S,C,I	adr, ln, lu, im, panc	107.0	33/66	91	ND	1500	> 376	11	1/0/3	NR

ND indicates not done.

*R indicates radiation therapy; S, surgery; C, chemotherapy; and I, immunotherapy (high-dose IL-2).

†In indicates lymph node; li, liver; lu, lung; sp, spleen; sc, subcutaneous; br, brain; bo, bone; ki, kidney; ip, intraperitoneal; adr, adrenal; lar, larynx; im, intramuscular; and panc, pancreas.

‡Treatment cell intracellular IFN-γ detected by flow cytometry after 18-hour coculture with mel526$^+$ tumor cells (gated on CD3$^+$).

§Number of positive IL-2 ELISPOTs per 100 000 infusion PBMCs after 4-hour coculture with mel624$^+$ tumor cells.

‖Number of positive IFN-γ ELISPOTs per 10 000 infusion PBMCs after 4-hour coculture with mel624$^+$ tumor cells.

¶Skin toxicity: grade 1, erythema; grade 2, desquamation < 50%; grade 3, desquamation > 50%. Eye toxicity (uveitis): grade 1, asymptomatic; grade 2, anterior, steroid eye drops; grade 3, pan uveitis, surgery. Ear (hearing): grade 1, hearing loss 15 to 25 dB at 2 frequencies; grade 2, > 25 dB at 2 frequencies; grade 3, > 25 dB at 3 frequencies (per CTCAE, Version 3.0).

**PR indicates partial responder and NR, nonresponder (all by RECIST criteria); and +, ongoing. Values in parentheses are response duration (in months) after treatment.

††Second treatment.

Table 2. gp100(154) patient cell treatment characteristics

Patient no.	Age, y/ sex	Prior treatment*	Sites of disease†	No. of cells $\times 10^9$	Percentage CD4/8	Percentage Tet	ELISPOT		Doses IL-2	Toxicity‖ (skin/eye/ear)	Tumor response, mo¶
							IL-2‡	IFN-γ§			
1	31/F	S,C,I	ln, ip, li	3.7	35/65	60	7100	> 137	5	1/0/0	NR
2	32/M	S,C,I	ln, im, sc	10.0	36/54	78	5300	> 362	5	2/0/0	NR
3	54/M	S,I	sc	9.8	13/82	87	2590	> 302	4	1/0/0	NR
4	50/M	S,I	ln, gb	4.6	11/78	97	12 050	> 409	2	1/0/0	NR
5	49/F	S,I	ln, lu, sc	9.9	21/80	91	11 683	> 451	2	2/0/0	NR
6*	36/F	R,S,C,I	ln, bo, li, sp	5.8	6/89	77	2370	> 336	5	1/0/0	NR
7	60/M	S,I	ln, bo, li, lu, sc	1.8	40/56	83	6117	> 213	4	1/0/0	NR
8	50/F	S,C,I	st, li, lu, int, sc	19.4	44/53	55	2900	294	5	2/2/0	NR
9	25/M	S,I	ln, bo, ip	2.3	44/56	84	ND	46	9	0/0/0	NR
10	40/F	S,C,I	br, ln, im	2.7	24/69	59	6700	> 445	10	1/0/0	NR
11**	50/M	R,S,C,I	br, ln	68.8	5/96	97	8167	> 495	8	1/2/3	CR (14+)
12	62/M	S,C,I	lu, ln	46.5	20/82	85	14 583	> 526	7	1/2/2	NR
13**	44/F	R,S,C,I	li, ln	54.0	2/97	90	8700	> 339	13	1/2/1	NR
14	51/F	S,I	ln, li, lu, sp	110.0	11/88	92	11 817	> 421	6	1/0/2	PR (4)
15	33/M	S,I	ln	94.1	16/78	89	6833	> 389	6	2/0/0	NR
16	41/M	S,I	ln	39.1	18/79	90	10 967	> 485	7	1/0/1	PR (3)

ND indicates not done.

*R indicates radiation therapy; S, surgery; C, chemotherapy; and I, immunotherapy (high-dose IL-2).

†In indicates lymph node; li, liver; lu, lung; br, brain; sp, spleen; sc, subcutaneous; bo, bone; ki, kidney; ip, intraperitoneal; adr, adrenal; lar, larynx; im, intramuscular; panc, pancreas; st, stomach; int, intestine; gb, gallbladder.

‡Number of positive IL-2 ELISPOTs per 100 000 infusion PBMCs after 4-hour coculture with mel624$^+$ tumor cells.

§Number of positive IFN-γ ELISPOTs per 10 000 infusion PBMCs after 4-hour coculture with mel624$^+$ tumor cells.

‖Skin toxicity: grade 1, erythema; grade 2, desquamation < 50%; grade 3, desquamation > 50%. Eye toxicity (uveitis): grade 1, asymptomatic; grade 2, anterior, steroid eye drops; grade 3, pan uveitis, surgery. Ear (hearing): grade 1, hearing loss 15 to 25 dB at 2 frequencies; grade 2, > 25 dB at 2 frequencies; grade 3, > 25 dB at 3 frequencies (per CTCAE, Version 3.0).

¶CR indicates complete responder; PR, partial responder; NR, nonresponder (all by RECIST criteria); and +, ongoing. Values in parentheses are response duration in months after treatment.

**Prior treatment with DMF4 TCR.

Table 3. Interferon-γ production by TCR-transduced infusion cells

	Melanoma cell line					T2 cells + μM peptide*					
		A2−		A2+		Negative		Positive			
Patient no.	None	888	938	526	624	None	1.0	1.0	0.1	0.01	0.001
DMF5											
1	0	6	49	62 300	111 200	102	236	132 400	56 700	12 330	1139
2†	7	133	175	16 800	99 400	159	141	117 400	53 700	6910	1182
3	0	125	163	3830	27 700	37	64	50 500	15 000	984	241
4	0	1	0	983	148 400	7	4	45 000	11 760	798	139
5†	0	2	3	7760	180 800	20	21	34 000	17 000	1076	298
6	1	3	4	3020	2980	13	17	9580	8880	4810	960
7	13	16	41	8420	39 700	102	134	50 400	32 200	11 950	1556
8†	744	663	1320	37 500	88 000	1299	1254	113 100	14 510	> 16 460	> 3278
9†	0	1	6	7440	13 400	176	270	68 000	54 800	14 290	> 1649
10	51	80	155	102 300	119 200	527	857	93 400	104 800	> 5226	2005
11	17	8	77	6460	26 700	221	321	42 300	36 200	12 860	1576
12	0	0	8	1300	19 700	24	190	16 150	4720	338	124
13†	0	6	0	3120	117 900	78	156	137 900	41 900	1750	345
14	0	0	31	> 131 600	426 600	84	352	> 283 400	38 800	7950	956
15	5	91	152	9400	47 200	54	295	69 400	71 400	> 1575	271
16	NA	40	15	5410	17 525	NA	247	52 650	20 225	6370	1830
17	0	11	8	7100	63 200	46	394	70 200	3260	118	73
18†	42	56	226	71 800	328 900	651	1400	> 283 400	62 800	11 450	47 510
19	2	34	82	> 142 700	275 590	829	1155	329 600	> 184 200	7100	> 2470
20	NA	17	9	9995	24 900	NA	81	22 750	6710	1560	590
gp100(154)											
1	46	25	32	14 460	74 900	282	275	7540	9100	4210	3900
2	0	26	42	88 300	199 400	46	37	2080	40 900	> 19 200	1620
3	15	80	185	90 500	209 800	87	163	15 490	14 870	8970	10 040
4	0	94	101	90 300	138 800	87	80	3000	70 500	> 19 770	829
5	22	0	15	40 000	287 800	253	43	142 400	114 500	11 540	4540
6	0	38	31	83 400	163 200	35	57	14 160	11 750	7160	6640
7	23	169	82	4670	18 350	23	212	10 700	7370	6060	1100
8	166	102	120	98 600	179 400	87	777	59 200	55 600	16 070	5050
9	2	36	23	14 420	91 800	96	32	39 600	52 500	11 890	2440
10	200	20	245	13 640	148 800	21	22	64 300	115 900	> 30 880	4140
11†	71	0	3	141 700	143 600	22	265	161 900	58 700	4850	790
12	87	10	48	14 720	164 100	8	210	115 700	69 500	12 500	849
13	NA	14	30	102 600	172 800	NA	30	130 900	59 800	8295	651
14†	NA	314	151	62 750	85 400	NA	142	135 700	58 700	11 360	4390
15	NA	43	0	2455	21 300	NA	149	7420	3855	2360	974
16†	NA	86	113	23 050	110 350	NA	50	58 800	225 500	3840	1436

IFN-γ (pg/mL) measured by ELISA, 1×10^5:1×10^5 effector:target cell 18-hour coculture.
NA indicates not available.
*Peptides, MART-1:27-35 and gp100:154-162 (positive, cognate peptide; negative, nonspecific peptide).
†Responding patient.

At the time of this cytokine surge, 29 of the 36 patients in the current trial exhibited a widespread erythematous skin rash that on biopsy showed prominent epidermal spongiosis and necrotic epidermal keratinocytes, with a dense infiltrate of $CD3^+$ T lymphocytes (predominantly $CD8^+$; Figure 6A). Surprisingly, there was destruction of epidermal melanocytes in all biopsies performed on 14 DMF5 patients and 13 gp100(154) patients, starting as early as day 5 after treatment (Figure 6A), although rare remaining melanocytes were sometimes observed around hair follicles. This loss of melanocytes coincided with the dermal and epidermal infiltration of lymphocytes as well as evidence of vitiligo in patients on later follow-up. This rash gradually subsided over several days without treatment in all patients. To elucidate the factors inciting the rash and the lymphocyte infiltration into skin, we studied 2 patients who presented with preexisting patchy vitiligo. These 2 patients developed a rash only in pigmented and not in vitiliginous skin (Figure 6B). Biopsies after treatment revealed diffuse infiltrates of $CD8^+$ cells in areas of pigmented skin with little or no lymphocytic infiltrate in vitiliginous skin (Figure 6B). These findings, along with the evidence of melanocyte destruction at a time when endogenous lymphocytes have been depleted, strongly suggested that the epidermal melanocytes were the targets of immune attack by the gene-engineered cells.

Because melanocytic cells expressing MART-1 and gp100 exist in the eye, all patients underwent ophthalmologic examination before and after treatment. None of the patients in the prior DMF4 TCR trial developed uveitis (cellular infiltrate into the eye). In contrast, 11 of 20 patients (55%) receiving the more reactive DMF5 TCR cells and 4 of 16 (25%) receiving the gp100(154) TCR cells developed an anterior uveitis, 2 of which were asymptomatic and 13 required the transient administration of steroid eye drops (Tables 1, 2; Figure 6C). Two patients developed synechiae of the iris that were asymptomatic (Figure 6C). In all patients, ocular findings reverted to normal. Sampling of the eye anterior chamber fluid in one patient with uveitis who received the DMF5-transduced cells revealed a predominance of MART-1 tetramer-positive $CD3^+$

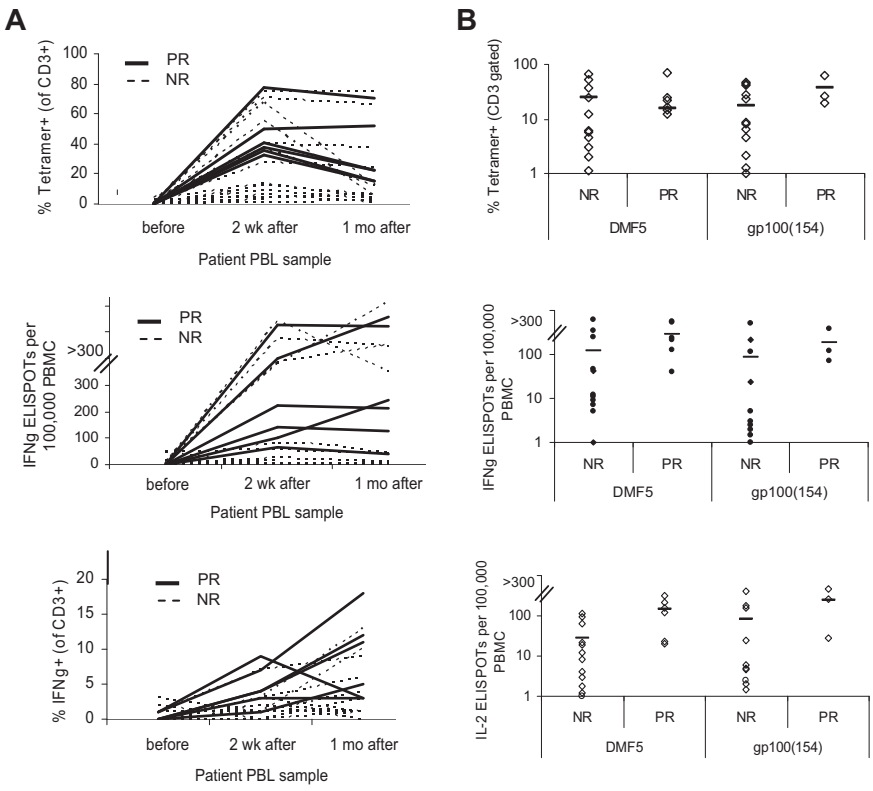

Figure 3. TCR-transduced cells from responding patients persisted and showed antitumor activity ex vivo. Blood samples were taken from patients' cells before and after TCR-transduced cell infusion. PBMCs were evaluated for persistence of infused cells in peripheral blood after treatment by specific tetramer staining and were also used directly in coculture assays with mel624 tumors (MART1$^+$, gp100$^+$, HLA-A2$^+$). Antitumor activity was evaluated by IFN-γ and IL-2 ELISPOT, and also by intracellular staining for IFN-γ production. (A) Persistence and activity of DMF5 patient treatment cells before, 2 weeks after, and 1 month after infusion. Responding (PR) and nonresponding (NR) patients are represented by solid and broken lines, respectively. (B) Comparison of PBMCs from patients treated with either DMF5 or gp100(154) TCR-transduced cells. Tetramer staining and ELISPOT analysis of IFN-γ and IL-2 production from nonresponding (NR) and responding (PR) patients at 1 month after treatment. All samples had less than 10 ELISPOTs (per 100 000 PBMCs) and less than 1% IFN-γ–positive cells, respectively, against the HLA-A*02$^-$ mel888 tumor (data not shown). Patients with objective clinical responses (PR) had higher numbers of antitumor IFN-γ ($P = .02$) and IL-2 ($P = .02$) secreting cells than nonresponders (NR).

T cells by FACS analysis (Figure 6D), indicating trafficking of gene-modified cells into the eye.

Melanocytic cells also exist in the striae vascularis of the inner ear. Although none of the patients in the prior DMF4 trial exhibited hearing loss, audiometric examinations revealed evidence of hearing loss in 10 of 20 of the DMF5 patients starting approximately 1 week after treatment (Table 1; Figure 6E), 7 of whom received intratympanic steroid injections. All patients improved, and the grade 3 hearing losses resolved or improved to a grade 1 or 2 with the exception of 2 patients who died of progressive metastatic melanoma before they were retested. Five of the 16 gp100(154) patients developed mild hearing loss (Table 2), only one of whom required treatment and continues to have mild residual changes. Nine of the 36 patients experienced inner ear–related dizziness that responded to treatment. This constellation of symptoms is similar to that observed in patients with Vogt-Koyanagi-Harada disease,[20] thought to result from autoimmune destruction of melanocytes located in the eye, the inner ear, skin, and hair. There were no off-target autoimmune effects observed in any patients.

DMF4 and DMF5 TCRs recognize the same MART-1:27-35 epitope and were derived from the same melanoma patient.[15] The improved ability of transduced cells expressing the DMF5 or gp100(154) TCR to recognize target antigens in the skin, eye, and ear and to persist in patients at levels up to 80% of circulating peripheral T lymphocytes for several months appears the result of the higher reactivity conferred by these improved TCR constructs compared with the prior DMF4 TCR.

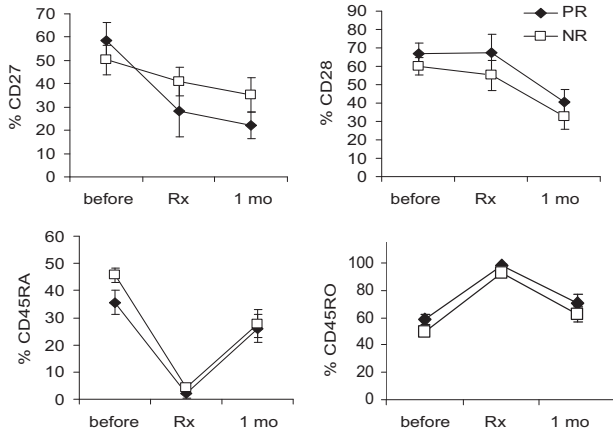

Figure 4. Phenotype of patient treatment cells before and after infusion. DMF5 patient PBMCs were stained by tetramers for TCR-recognizing MART1:27-35, and by mAb for CD3 and the activation and differentiation markers CD27, CD28, CD45RA, and CD45RO. Cell phenotype was evaluated by flow cytometry, gated on CD3$^+$ cells before treatment, and on CD3$^+$tetramer$^+$ cells for infusion (Rx) and 1 month after treatment (1 mo) samples. Error bars indicate mean ± SEM. Responding patients (PR) are represented by solid symbols, and nonresponding patients (NR) by open symbols.

Antitumor impact of the TCR-transduced cells

One patient who received DMF5-transduced cells underwent a series of sequential biopsies of subcutaneous metastatic lesions before and after treatment. Beginning at approximately 5 days after treatment, the biopsies revealed an increasing infiltration of CD8$^+$ T lymphocytes throughout the tumor that coincided with the progressive necrosis and partial regression of his tumors (Figure 7A-B). Growth of lymphocytes from resected lesions at days 9, 15, and 26 revealed more than 97% MART-1 tetramer-positive lymphocytes (Figure 7C). Although initially the majority of this patient's disease regressed, numerous new lesions developed after 2 months; and as such, this patient was a nonresponder.

Six of the 20 patients (30%) treated with DMF5 TCR and 3 of 16 (19%) treated with gp100(154) TCR experienced an objective antitumor response, as defined by RECIST criteria[17] (Tables 1-2).

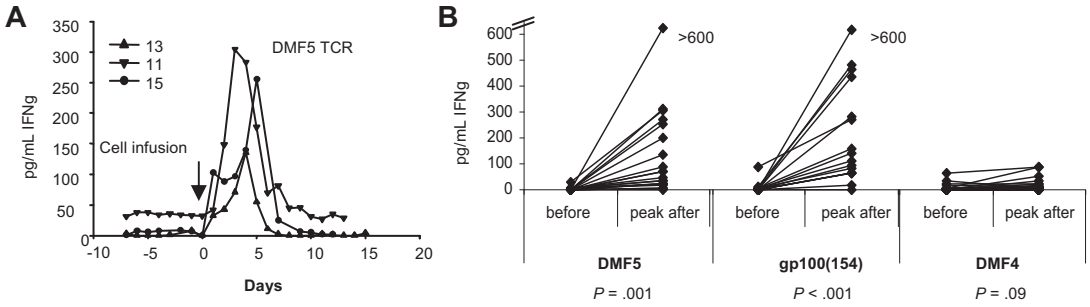

Figure 5. Patients treated with the highly reactive DMF5 or gp100(154), but not DMF4 TCR, had increased serum IFN-γ levels after treatment. Patient IFN-γ serum levels were measured daily before, during, and after cell infusion by ELISA. (A) Serum IFN-γ levels increased from baseline to a peak at 3 to 6 days after cell infusion (3 representative DMF5 patients are shown). (B) Peak IFN-γ levels in serum after treatment for patients treated with DMF5, gp100(154), and DMF4 TCR-transduced cells. Only patients treated with the highly reactive DMF5 or gp100(154) TCR demonstrated increased IFN-γ in serum ($P = .001$ and $P < .001$, respectively, compared with $P = .09$ for DMF4 TCR).

Tumors regressed in multiple organs, including the brain, lung, liver, lymph nodes, and subcutaneous sites (Figure 7D-F). There were no treatment-related deaths, and all patients recovered from treatment.

In analyzing the 36 patients, there was no correlation between the number of cells administered and the likelihood of a clinical response, with some responding patients receiving a log fewer cells than others (Table 1). There was also no correlation between clinical response and the duration the cells were grown ex vivo, or whether they received one or 2 OKT-3 stimulations. There was a correlation with clinical response and the persistence of administered cells at one month as assessed by ELISPOT analysis of both specific IFN-γ and IL-2 release (both $P = .02$; Figure 3B). Although all responding patients had more than or equal to 10% tetramer-positive cells persisting in blood at 1 month, this parameter did not correlate with response ($P = .4$; Figure 3B).

Discussion

The ability of genetically modified normal peripheral lymphocytes bearing highly avid TCR to destroy isolated antigen-expressing cells, such as individual melanocytes in the ear, the basal layer of the epidermis, and in immunoprivileged sites such as the eye, as well as melanoma metastases in visceral organs and the brain, has important implications for the development of cancer immunotherapy. This approach bypasses the need to identify and isolate antitumor effector cells from each patient. These gene-modified cells targeted only specific shared differentiation antigens and thus these findings validate the use of differentiation antigens as targets of cancer immunotherapy. It is of interest that cells expressing the murine gp100(154) TCR persisted at the same levels as those expressing the human DMF5 TCR, and also mediated cancer regression, suggesting that an immune reaction against the mouse TCR sequences may not be a limiting factor in TCR gene therapy in humans. The ability to use HLA-transgenic mice to raise TCRs against human cancer antigens represents a valuable approach to bypass the tolerance of patients to self-antigens and thus enable the generation of highly reactive antitumor TCRs.[21]

It was surprising that the percentage of PBLs staining positive for tetramer was always higher than the proportion of cells displaying antitumor activity by ELISPOT or intracellular cytokine staining (Tables 1-2; Figure 3). Part of this difference may be accounted for by the inclusion of all mononuclear cells in patient PBL functional assays, compared with the ability to gate on the $CD3^+$ T lymphocyte population by flow cytometry. However, this difference is small compared with the logs of difference between tetramer-positive cells by FACS (approaching 100% in treatment samples and up to 80% in posttreatment PBLs), and in ELISPOT results (generally $< 1\%$ of cells). It is also possible that, although tetramer staining identifies all lymphocytes expressing the tumor-reactive TCR, not all TCR^+ cells have equal antitumor function. As T lymphocytes can have an antitumor effector phenotype, they can also be anergic or have a suppressive phenotype, such as regulatory T cells. In the case of these latter phenotypes, antitumor functional response could be diminished or abrogated. Another potential explanation is the different sensitivities of direct tetramer staining for flow cytometry vs ELISPOT or intracellular cytokine staining.

The recognition of normal quiescent cells expressing the targeted cancer antigen raises obvious questions concerning the toxicity of this gene therapy approach. These results paralleled our findings in a murine melanoma model, which showed an association of ocular toxicity and antitumor activity using gp100 as the target antigen,[22] although clinically the local application of steroids attenuated normal tissue toxicity for patients treated with these melanocyte-specific TCRs. These findings emphasize the importance of the targeted tumor antigen. It may be highly effective to target differentiation antigens on cancers that arise in nonessential organs, such as the prostate, ovary, breast, and thyroid. Other cancers have higher expression of differentiation antigens, such as CEA and Muc-1, than are expressed in normal tissues, and this may create a therapeutic window to be exploited. The cancer-testes class of antigens not expressed on normal adult tissues may be ideal targets for this approach. In any case, the risks versus benefits of infusing highly reactive self-antigen specific TCRs must be carefully considered.

Although the low numbers in these clinical trials preclude statistical conclusions, the 30% and 19% objective cancer response rates seen with the DMF5 and gp100(154) TCR, respectively, were lower than the 51% to 72% response rates seen with the use of autologous TILs screened and grown individually from each patient's resected tumor.[7,8,11] Anterior uveitis and ototoxicity were seen in 6 of 93 (6.5%) and only one of 93 (1.1%) melanoma patients treated with TILs, respectively, in contrast with the 41.7% incidence of both toxicities seen in the current trial ($P < .001$), sometimes in the absence of tumor regression, implying that the shared melanoma/melanocyte antigens may not be the predominant targets of therapeutic TILs. Although many TILs do recognize the MART-1 and gp100 antigens, T-cell clones have been isolated from many clinically effective TILs that recognize mutated or unidentified antigens as well, and these may also be responsible for the antitumor effects of TIL transfer. Alternatively, differences in

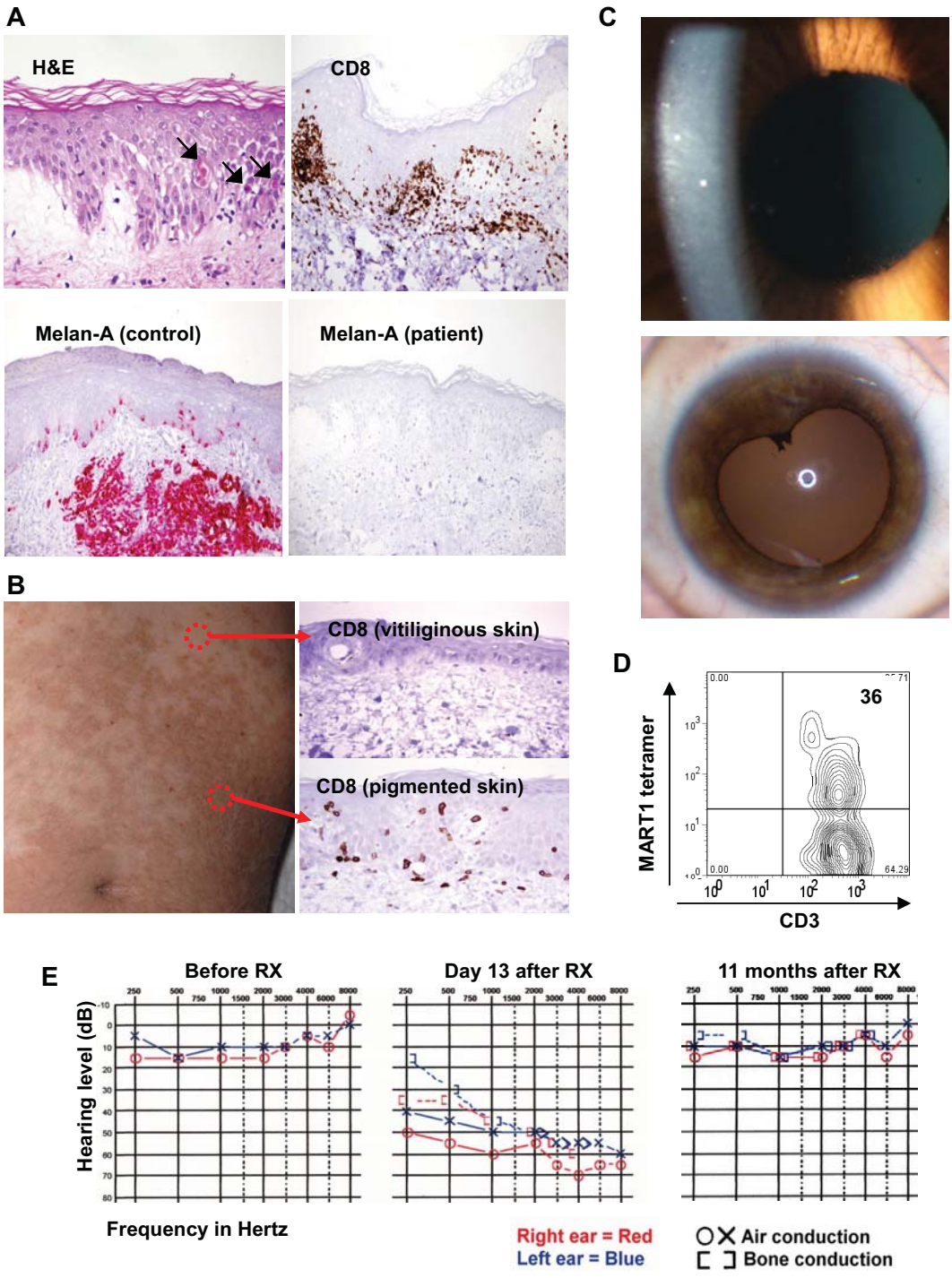

Figure 6. Tissue trafficking and target cell destruction in patients after infusion of TCR-transduced cells. (A) Biopsy of inflamed skin from DMF5 patient 2, day 5 after treatment, demonstrating spongiotic vesicles and necrotic/dyskeratotic keratinocytes (arrows), stained with hematoxylin and eosin, and immunohistochemically stained for CD8$^+$ cells or the Melan-A antibody recognizing MART-1 antigen. (Bottom left) Positive control staining for the anti-MART-1 Melan-A antibody showing normal epidermal melanocytes and a subcutaneous melanoma deposit (original magnification ×20). (B) One week after treatment, biopsies from gp100(154) patient with preexistent patchy vitiligo, demonstrating CD8$^+$ cellular infiltrate into the epidermis of pigmented, but not vitiliginous skin (original magnification ×20). (C) Slit-lamp ophthalmologic evaluation of DMF5 patient 5 eye, 2 weeks after treatment, demonstrating cloudy cellular anterior chamber infiltrate (above) and (below) with induced iris dilation 6 months after TCR treatment and steroid eye drop administration, demonstrating posterior synechiae (asymptomatic). (D) Cells present in ocular fluid from panel C (top), analyzed directly by staining and flow cytometry. (E) Audiologic examination of DMF5 patient 2 before TCR treatment, day 13 after treatment showing hearing loss, and 11 months after treatment, showing hearing recovery after intratympanic steroid treatment.

homing molecules or other phenotypic differences between blood and tumor-derived lymphocytes may account for this finding.

Several patients exhibited melanocyte toxicity without experiencing tumor rejection. Treating patients with low (DMF4) and high (DMF5) avidity TCR recognizing the same MART-1 antigen resulted in 13% (4 of 34) and 30% (6 of 20) objective tumor responses, respectively, suggesting that increased TCR avidity may improve tumor rejection. However, because of the small sample numbers, it is possible that the avidity of TCR in vivo is not a major determinant of tumor rejection. It is probable that factors, such as

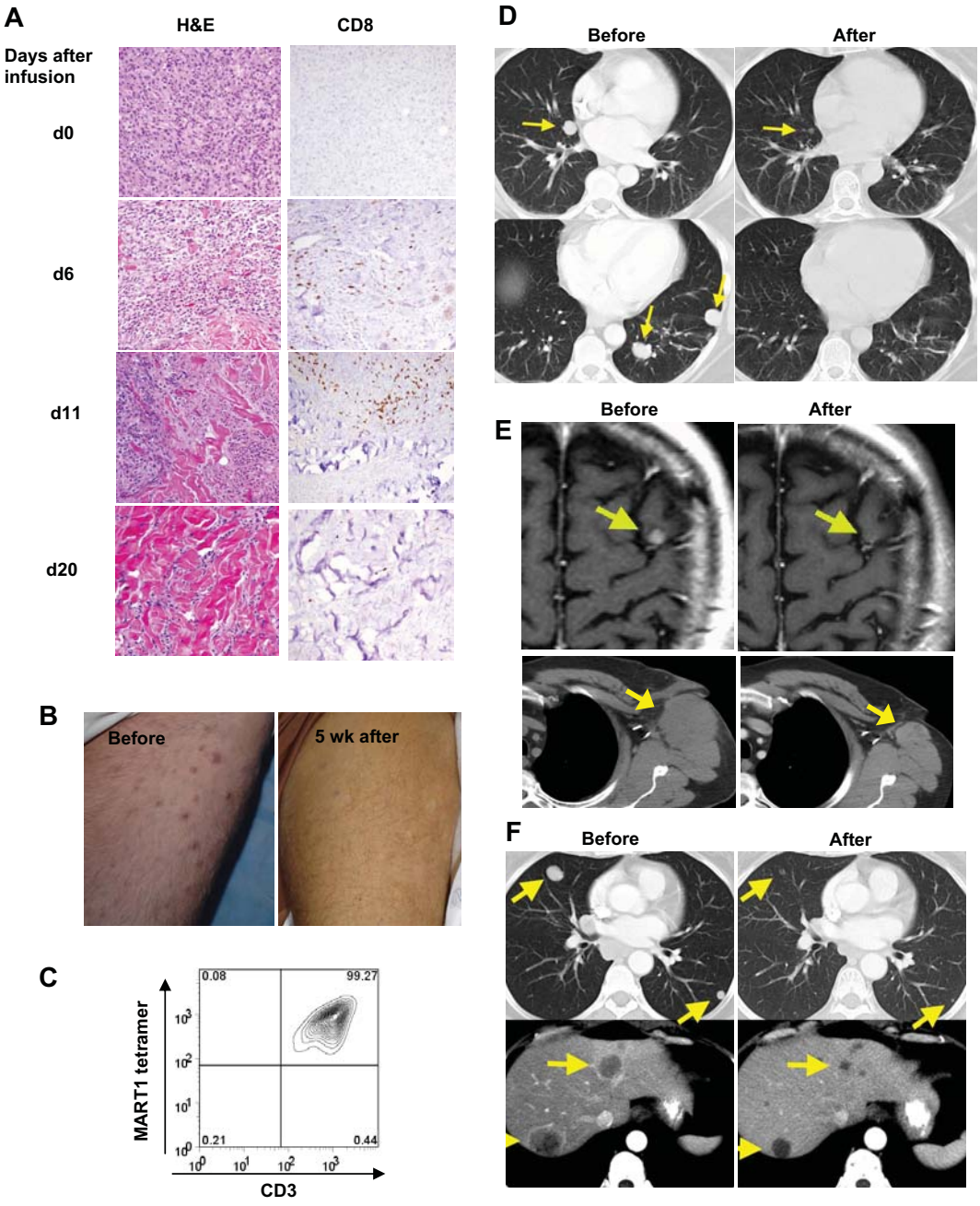

Figure 7. Highly reactive transferred cells traffic to and destroy melanoma tumors in patients. (A) Sequential biopsies of subcutaneous tumors from DMF5 patient 4 before (d0) and after treatment, stained with hematoxylin and eosin (left), or anti-CD8 (right). Original magnification ×20. (B) DMF5 patient 4 thigh covered with multiple subcutaneous melanoma lesions before treatment, and after partial tumor regression 5 weeks after treatment. (C) Flow cytometry of TILs grown from DMF5 patient 4 subcutaneous tumor resected 4 weeks after treatment. (D-F) Before treatment and after treatment CT scans of (D) DMF5 patient 2 lungs, (E) DMF5 patient 8 brain (top) and axilla (bottom), and (F) gp100(154) patient 14 lungs (top) and liver (bottom). Arrows represent location of melanoma metastases.

lymphocyte homing to normal vs tumor tissue, heterogeneity of antigen expression by tumor, T-cell exhaustion, suppression in the tumor microenvironment, or the need for polyvalent T-cell populations, may all need to be considered to improve tumor rejection in patients. Murine tumor models predict that intensifying the lymphodepletion before cell transfer and administering a vaccine to stimulate the transferred cells in vivo can improve the antitumor efficacy of this approach,[10,23] and we are currently implementing a clinical trial combining the DMF5 and gp100(154) TCR, with increased patient immunodepletion, followed by administration of a peptide vaccine.

Adoptive cell transfer therapy using gene-modified lymphocytes has also been shown to be effective in treating malignancies other than melanoma. In preclinical models, lymphocytes have been genetically modified to express costimulatory molecules designed to increase antitumor activity and T-cell survival, such as CD80 or 4-1BB ligand, or to abrogate the effects of inhibitory signals.[24] Genetically modified human lymphocytes expressing chimeric antigen receptors (CARs),[25] combining the antigen recognition of an antibody with T-cell signaling motifs, were able to eradicate human B-cell lymphoma by targeting CD19,[26] and human prostate tumors by targeting ErbB2/Her2[27] in xenogeneic SCID

mouse models. Using Epstein-Barr virus–specific human T cells transduced with CARs recognizing the Hodgkin lymphoma–specific CD30 antigen has also been shown to be effective in a xenogeneic mouse model.[28] Clinically, Epstein-Barr virus–specific cells expressing CARs that recognize the neuroblastoma tumor-specific diasialoganglioside GD2 have also recently been used to successfully treat patients with neuroblastoma.[29]

In an attempt to expand this TCR therapy to treat cancers other than just melanoma, we have now cloned the genes encoding high-avidity TCR that recognize a variety of antigenic epitopes, such as NY-ESO-1:157-165,[30] p53:264-272,[21,31] and CEA:691-699,[32] expressed on many common epithelial cancers.[9] To target tumors in a non–major histocompatibility complex-restricted fashion, we have also engineered lymphocytes to express chimeric receptors incorporating the antigen combining site of anti-Her2 or anti-CD19 mAb,[26] which may target tumor cells overexpressing these determinants on the cell surface.

Possible toxicities resulting from the expression of tumor-associated antigens on normal tissues need to be considered in the application of this approach. Our results, however, support the hypothesis that the administration, to cancer patients, of T cells transduced with highly reactive human or murine TCRs can mediate in vivo destruction of tissues that express the target antigen and suggest that cell transfer therapies can be a valuable adjunct to the treatment of patients with metastatic cancer.

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Authorship

Contribution: L.A.J. prepared the DMF5 patient treatment cells, conducted the laboratory experiments and analysis, and wrote the paper; R.A.M. prepared the gp100(154) patient treatment cells and conducted experiments; M.E.D., A.P.C., and J.R.W. prepared the patient treatment cells; S.L.S. performed experiments; L.C. and N.P.R. identified and isolated the gp100(154) TCR; J.C.Y., M.S.H., U.S.K., R.E.R., R.M.S., J.L.D., A.M., R.T.R., and S.A.R. were physicians and D.A.N. was a Research Nurse on the clinical protocols; R.A.M. constructed the retroviral TCR vectors; C.-C.R.L. conducted immunohistologic staining of patient biopsies; R.J.B. conducted patient ocular evaluations; H.K., C.C.B., S.F.R., and C.V.W. conducted patient auditory evaluations; C.M.L. provided support in clinical protocol preparation and reporting; S.A.R. supervised the clinical trials and contributed to data analysis and writing of the paper; R.J.B. provided information regarding patient ophthalmologic evaluations; H.K, C.C.B., S.F.R., and C.V.W. provided information regarding patient otolaryngologic evaluations; and L.A.J. and S.A.R. analyzed the data, to which all authors have access.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Cancer Regression in Patients After Transfer of Genetically Engineered Lymphocytes

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Cancer Regression in Patients After Transfer of Genetically Engineered Lymphocytes

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Through the adoptive transfer of lymphocytes after host immunodepletion, it is possible to mediate objective cancer regression in human patients with metastatic melanoma. However, the generation of tumor-specific T cells in this mode of immunotherapy is often limiting. Here we report the ability to specifically confer tumor recognition by autologous lymphocytes from peripheral blood by using a retrovirus that encodes a T cell receptor. Adoptive transfer of these transduced cells in 15 patients resulted in durable engraftment at levels exceeding 10% of peripheral blood lymphocytes for at least 2 months after the infusion. We observed high sustained levels of circulating, engineered cells at 1 year after infusion in two patients who both demonstrated objective regression of metastatic melanoma lesions. This study suggests the therapeutic potential of genetically engineered cells for the biologic therapy of cancer.

In the past two decades, fundamental advances in immunology have introduced opportunities for the development of cellular-based therapies for the treatment of cancer (*1*, *2*). After ex vivo expansion, transfer, and clonal repopulation in patients who have received lymphodepleting conditioning, autologous tumor-infiltrating lymphocytes (TILs) have been found to mediate objective cancer regression in a measurable proportion of patients with metastatic melanoma (*3*–*5*). A limitation of this approach is the requirement that patients have preexisting tumor-reactive cells that can be expanded ex vivo. In addition, in many cancer patients, especially those with cancers other than melanoma, it is difficult to identify these tumor-reactive lymphocytes. To overcome this limitation, we set out to develop an approach to cancer immunotherapy based on the genetic modification of normal peripheral blood lymphocytes (PBLs).

Tumor-associated antigens (TAAs) are recognized by the T cell receptor (TCR) on the T lymphocyte surface, which is composed of the TCR alpha and beta chains (*6*). The genes encoding the TCR that are specific for a variety of TAA have now been cloned, including the TCR-recognizing MART-1 and gp100 melanoma/melanocyte differentiation antigens, the NY-ESO-1 cancer-testis antigen that is present on many common epithelial cancers, and an epitope from the p53 molecule, which is expressed on the surface of approximately 50% of cancers of common epithelial origin (*7*–*12*). In each case, these antigens were detected by the TCR when they were presented as peptides by molecules encoded by the major histocompatibility complex protein human lymphocyte antigen (HLA)–A2. In vitro transcribed RNA from four TAA-reactive TCRs (recognizing MART-1:27–35, gp100:209–217, NY-ESO-1:157–165, and p53:264–272) were electroporated into $CD8^+$ PBLs, which were then cocultured with peptide-pulsed T2 cells. These transfected cells produced large amounts of interferon-γ (IFN-γ) upon stimulation with their respective peptides (Fig. 1A) and were able to recognize HLA-A2–matched tumors, including melanoma, lung cancer, and breast cancer (table S1). Furthermore, transduction with these TCR-encoding retroviral vectors converted normal PBLs into cells capable of specifically recognizing and destroying both fresh and cultured cells from multiple common cancers (such as sarcoma and breast, lung, esophagus, and liver cancers) in vitro (*9*–*12*).

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Table 1. Patient demographics, treatments received, and clinical outcome. Ln, lymph node; Cu, cutaneous; Sub, subcutaneous; Li, liver; Lu, lung; Ad, adrenal; Pa, pancreas; Br, brain; Hi, hilum. NR, no response; PR, partial response; MR, minor or mixed response.

Cohort	Patient	Age/sex	Total cells infused ($\times 10^{-9}$)	CD4/CD8 (%)	VB12 (%)	MART-1 cells infused ($\times 10^{-9}$)‡	Days in culture	Doubling time (days)†	IL-2 doses§	Sites of evaluable disease	Response (duration in months)‖
1	1	28/M	11.0	27/73	67	7.4	19	8.7	7	Ln, Cu	NR
	2a*	44/F	13.0	3/95	64	8.3	19	11.9	10	Ln, Cu	NR
	3	58/M	14.0	17/82	35	4.9	19	10.0	11	Cu, Sub	NR
2	4	52/M	1.0	50/50	42	0.5	6	1.4	9	Li, Sub	PR(21)
	5	50/M	12.0	18/82	17	2.2	8	1.0	7	Lu, Ln, Sub	NR
	6	55/F	7.0	37/72	51	3.6	7	1.3	8	Lu, Ln	NR
	7	56/M	9.0	75/21	40	3.6	7	1.0	5	Lu, Ln	NR
	8	37/M	6.1	68/40	32	1.9	7	1.3	12	Lu, Ln	NR
	9	53/M	4.2	72/24	41	1.7	7	2.0	9	Ln, Ad, Sub	MR
	10	45/M	8.6	53/30	34	2.9	6	0.6	5	Ln, Sub	
	11	45/M	6.3	7/92	45	2.8	6	0.8	5	Lu, Pa, Ln	NR
	12	32/F	4.7	30/60	61	2.9	6	0.7	5	Br, Sub	NR
	13	41/M	7.7	40/67	42	3.2	6	0.9	7	Lu, Sub	NR
	2b*	44/F	2.1	30/59	53	1.1	6	1.9	14	Ln, Cu	NR
3	14	30/M	86	11/60	40	34.4	18+9	0.9	5	Hi	PR(20)
	15	51/M	38	16/82	45	17.1	18+9	3.3	8	Lu	NR
	16	25/F	33	13/76	21	6.9	18+9	1.2	2	Lu, Li, Sub	NR
	17	20/F	23	17/78	30	6.9	17+8	1.1	3	Lu, Ln, Sub	NR

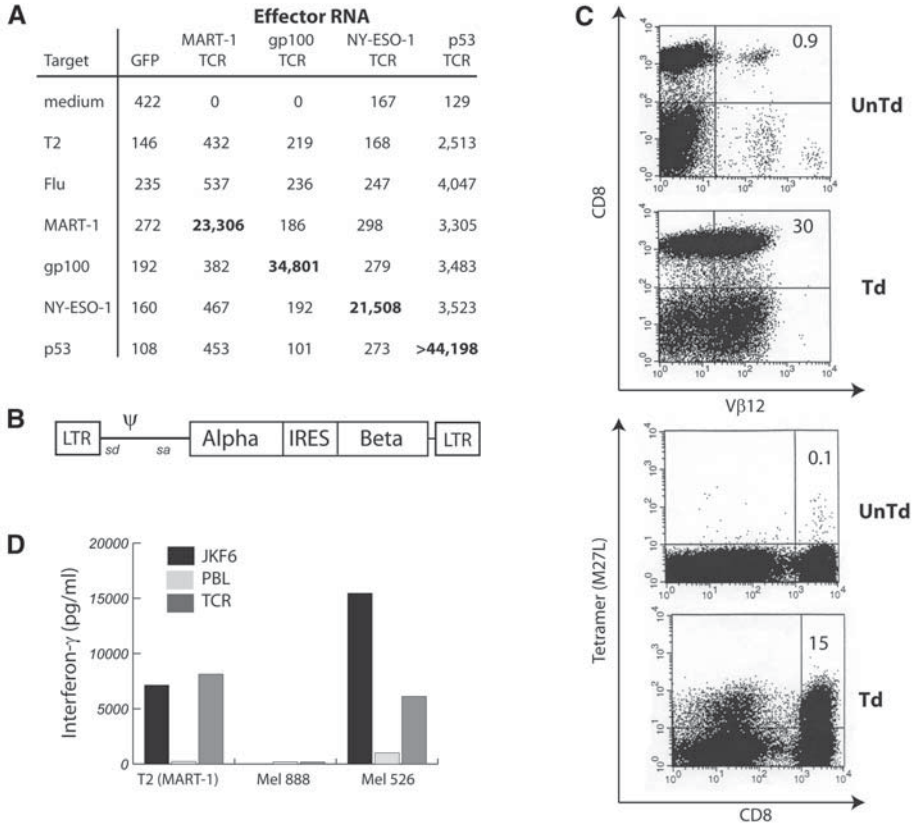
*This patient was treated twice; treatments were separated by 7 months. †Determined based on cell counts in the 2 days before infusion. ‡Total cells infused multiplied by %Vβ12. §720,000 international units/kg every 8 hours. All patients were previously refractory to treatment with IL-2 alone. ‖Based on RECIST criteria.

To investigate the ability of genetically engineered PBLs to recognize and destroy tumor cells in vivo, we transduced PBLs derived from patients with melanoma with the genes encoding the alpha and beta chains of the anti–MART-1 TCR. These genes were cloned from a TIL clone obtained from a cancer patient who demonstrated a near complete regression of metastatic melanoma after adoptive cell transfer (ACT) of TILs (*5*). A retroviral vector was constructed and optimized to express the MART-1 TCR alpha and beta chains (Fig. 1B) (*13*). Gene transfer efficiency, assessed by staining for the specific Vβ12 protein in this TCR, resulted in expression in 30% of the transduced $CD8^+$ cells (Fig. 1C), as compared with ~1% of untransduced control cell cultures (gene transfer was about equally divided between CD4 and CD8 cells). Fifteen percent of the transduced $CD8^+$ cells bound the MART-1 peptide-specific HLA-A*0201 tetramer (Fig. 1C and table S2). The TCR-transduced cells were biologically active, as demonstrated by the specific secretion of IFN-γ after coculture with both MART-1 peptide-pulsed cells and HLA-A2 positive melanoma cell lines (Fig. 1D).

To investigate the in vivo efficacy of these MART-1 TCR-engineered T cells, we selected 17 HLA-A*0201 patients with progressive metastatic melanoma (Table 1) for treatment. Cancers in all patients were refractory to previous therapy with interleukin-2 (IL-2). T cell cultures from all 17 patients were biologically reactive, with specific secretion of IFN-γ after coculture with either MART-1 peptide-pulsed T2 cells and/or melanoma cell lines expressing the MART-1 antigen (Fig. 1E). Gene transfer efficiencies measured by staining for Vβ12 expression in these lymphocytes ranged from 17 to 67% (42%, mean value) (Table 1 and table S2).

Patients received ACT treatments with MART-1 TCR-transduced autologous PBLs at a time of maximum lymphodepletion (*13*). Three patients in an initial cohort were treated with cells after an extended culture period of 19 days and had cell doubling times ranging from 8.7 to 11.9 days (Table 1, cohort 1, patients 1, 2a, and 3). In these patients, <10% of the transduced cells persisted across the time points tested during the first 30 days after infusion, and ≤2% of the cells persisted beyond 50 days (Fig. 2A). These first three patients showed no delay in the progression of disease.

In an effort to administer gene-modified lymphocytes that were in their active growth phase, the culture conditions were modified (*13*) to limit the ex vivo culture period to between 6 and 9 days after stimulation of cells with antibody to CD3 (Table 1, cohort 2, cell doubling times of ≤2 days). In another cohort, larger numbers of actively dividing cells for ACT were generated by performing a second rapid expansion protocol (*14*) after 8 to 9 days (Table 1, cohort 3, cell doubling times from 0.9 to 3.3 days). In contrast

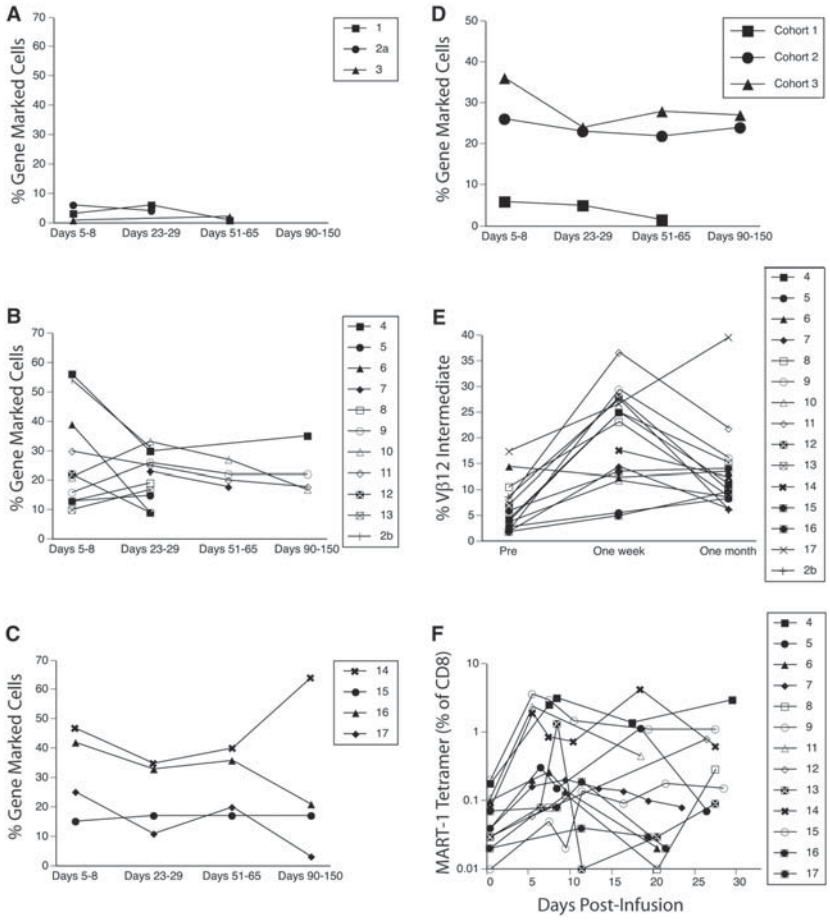


E

Cohort	Patient	Peptide Reactivity gp100	Peptide Reactivity MART-1	Tumor Reactivity 526	Tumor Reactivity 624	Tumor Reactivity 888	Tumor Reactivity 938
1	1	48	3,095	2,285	862	81	44
	2a	76	691	998	1,870	76	8
	3	66	1,238	345	474	121	50
2	4	588	6,315	3,072	2,200	183	135
	5	406	5,990	2,865	2,595	>2,882	274
	6	1,801	8,135	6,120	1,140	183	108
	7	126	1,698	610	199	142	185
	8	187	2,840	885	687	112	67
	9	113	5,680	751	433	100	91
	10	1,462	>22,045	9,150	7,495	724	656
	11	1,494	>23,975	7,745	4,805	327	275
	12	1,106	>23,450	8,190	4,315	168	131
	13	298	10,285	6,435	6,945	103	80
	2b	118	5,130	4,545	3,155	82	35
3	14	33	2,785	426	263	42	52
	15	213	3,115	964	453	122	115
	16	72	4,210	1,107	683	31	99
	17	148	4,600	1,035	848	50	57

Fig. 1. Transduction and analysis of TCR-engineered cells. (**A**) $CD8^+$ human lymphocytes were electroporated with RNA encoding control [green fluorescent protein (GFP)] or cloned TCRs reactive with HLA-A2 restricted epitopes from the human TAAs MART-1, gp100, NY-ESO-1, and p53. Effector T cells were cocultured with T2 cells pulsed with 1 μM of the indicated peptide (values are expressed as IFN-γ in pg/ml). Values demonstrating the specific release of cytokine are in bold. (**B**) Diagram of the recombinant retroviral vector MSGV1AIB used to engineer human lymphocytes. LTR, long terminal repeat; Ψ, extended packaging signal; sd, splice donor; sa, splice acceptor; Alpha, alpha chain; IRES, internal ribosomal entry site; Beta, beta chain. (**C**) Transduced (Td) lymphocytes were analyzed 5 days after transduction for the expression of Vβ12 and MART-1 tetramer [Ala^{27}→Leu^{27} (A27L)] in $CD8^+$ cells in comparison with untransduced (UnTd) cells. Numbers in the upper-right corners indicate the percentage of positive cells in that quadrant. (**D**) TCR vector-engineered cells from patient 6 (TCR) were cocultured with MART-1 peptide-pulsed T2 cells, HLA-$A2^-$ melanoma line (Mel 888), or HLA-$A2^+$ melanoma line (Mel 526), and the amount of IFN-γ produced was determined. Control effectors were untransduced cells (PBL) and the MART-1–reactive TIL JKF6 (JKF6). (**E**) Anti-melanoma properties of genetically engineered lymphocytes were determined for all patients before infusion. The production of IFN-γ (pg/ml) after coculture with peptide-pulsed T2 cells (Peptide Reactivity) and anti-melanoma activity (Tumor Reactivity) for HLA-$A2^+$ lines (526 and 624) and HLA-$A2^-$ lines (888 and 938).

Fig. 2. Persistence of gene-marked cells. DNA extracted from peripheral blood mononuclear cells (PBMCs) was subjected to real-time quantitative PCR to determine the percentage of vector-transduced cells in patient circulation at various times after infusion. Each line represents data from a separate patient. (**A**) Cohort 1; (**B**) Cohort 2; (**C**) Cohort 3. (**D**) Mean value of the percentage of gene-marked cells for all patients in each cohort at the given time interval after treatment. (**E**) The percentage of $CD8^+$/ $V\beta12^+$ cells in the intermediate gate (*13*) for patients in cohorts 2 and 3 is shown. (**F**) The percentage of $CD8^+$/MART-1^+ tetramer cells was determined for patients in cohorts 2 and 3 at the times shown. Pretreatment values for each patient are plotted as day 0 after infusion.



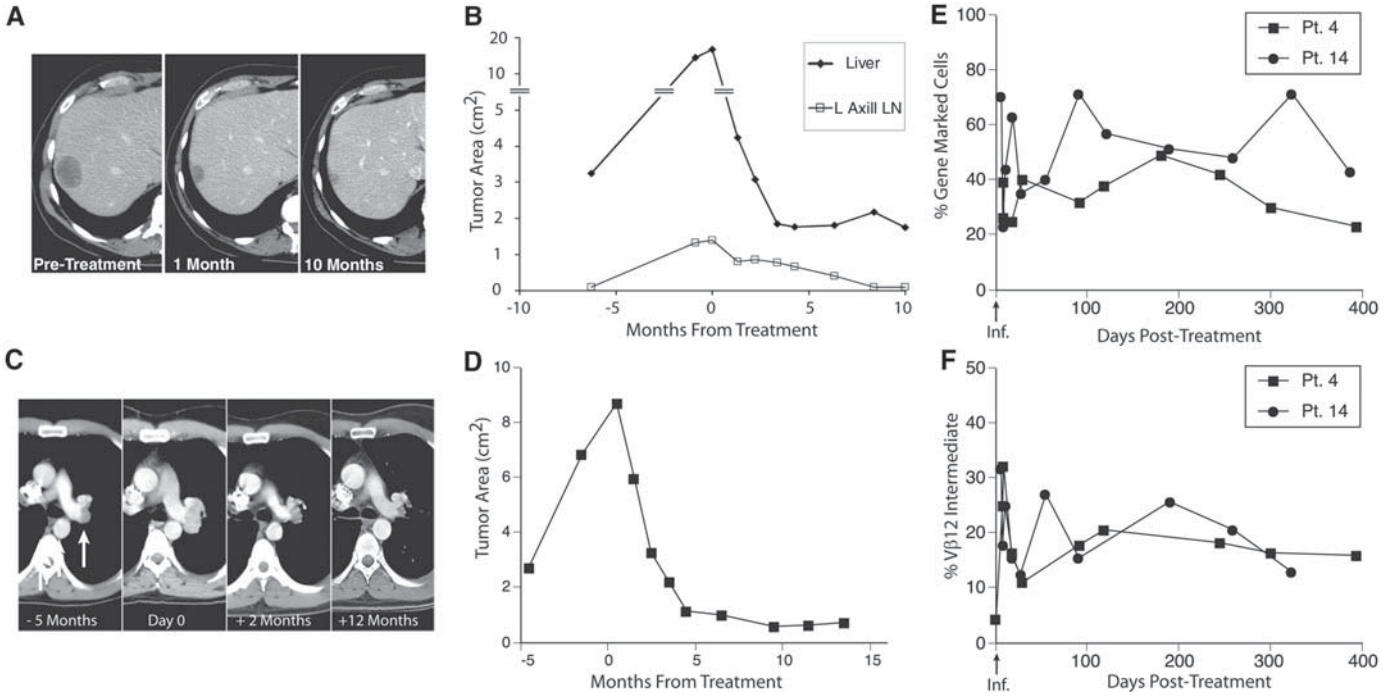


Fig. 3. Cancer regression in two patients. (**A**) Computed tomography (CT) images of liver metastasis in patient 4 taken at pretreatment, 1 month, and 10 months after treatment with TCR-engineered T cells. (**B**) Size of liver and axillary tumors and tempo of regression of tumor sites in patient 4. Day 0, beginning of treatment. L Axill LN, left axillary lymph node. (**C**) CT images of hilar lymph node metastasis in patient 14; pretreatment, day 0, and 2 months and 12 months after treatment. The white arrow indicates the mass in the lung hilum. (**D**) Size of tumor and tempo of regression in patient 14. (**E**) Quantitation of gene-marked cells in the PBMCs of patients 4 and 14 was determined by real-time quantitative PCR. Pt, patient. Day of infusion (Inf.) indicated by arrow. (**F**) The percentage of $CD8^+$/$V\beta12^+$ cells in the intermediate gate (*13*) in the circulation of patients 4 and 14.

to the lack of cell persistence seen in cohort 1 patients (Fig. 2A), patients in cohorts 2 and 3 (Fig. 2, B to D) all exhibited persistence of the transduced cells at >9% at 1 and 4 weeks after treatment (range, 9 to 56%). All eight patients who provided samples >50 days after treatment exhibited cell persistence of >17%, and this level of persistence was durable in seven patients during a >90-day monitoring period. In one patient (patient 14), >60% of circulating lymphocytes were positive for the gene-marked cells (Fig. 2C).

In 14 patient samples tested at one month after ACT, quantitative reverse transcription polymerase chain reaction (RT-PCR) assays revealed the presence of vector-derived RNA, confirming that gene expression continued (table S3). All but one of 15 patients analyzed had increased levels of $CD8^+/V\beta12$ cells at 1 week after treatment, and the levels of 11 of the 15 patients were higher at 1 month as compared to pretreatment levels (Fig. 2E). Of 13 patients that were examined, all had increased MART-1 tetramer-binding cells after treatment (Fig. 2F), and 11 of 14 had increased numbers of enzyme-linked immunosorbent spot–positive cells (table S4).

There was, however, a discordance between the mean persistence of transduced cells at 1 month in cohorts 2 and 3 measured by PCR (26%) as compared to the measurement of Vβ12-expressing cells (8.1%) and of MART-1 tetramer-binding cells (0.8%). This discordance may in part be due to mispairing of the introduced TCR chains with the endogenous chain, as well as to the different sensitivities of the assays. The reduced expression of the transgene in the persisting cells at ≥1 month may also be a function of the described decrease (*15*) in the transcription of retrovirally inserted trangenes and the decline in metabolic activity during the conversion of activated cells to memory cells. This decrease in expression of the retroviral transgene would be expected to affect the measurement of tetramers, which relies on the aggregation of multiple receptors, more heavily than the detection of Vβ12 cells directly by antibody staining.

Most important, two patients demonstrated a sustained objective regression of their metastatic melanoma assessed by standard criteria [response evaluation criteria in solid tumors (RECIST)] (*16*). Patient 4, a 52-year-old male, had previously received treatment with interferon-α (IFN-α), a lymph node dissection, an experimental vaccine, and high-dose IL-2. The patient then developed progressive disease in the liver (4.4- by 3.3-cm mass) and axilla (1.3- by 1.2-cm mass). After treatment with the ACT protocol described above, he experienced complete regression of the axillary mass and an 89% reduction of the liver mass (Fig. 3, A and B), at which time it was removed. He remains clinically disease-free at 21 months after treatment. Patient 14, a 30-year-old male, previously received treatment consisting of a lymph node dissection, IFN-α, and high-dose IL-2. He developed an enlarging 4.0- by 2.5-cm mass in the lung hilum. After ACT treatment, he underwent regression of the hilar mass and is now clinically disease-free 20 months later (Fig. 3, C and D). Thus, two patients with rapidly progressive metastatic melanoma showed full clinical regression of disease after the transfer of genetically engineered autologous PBLs.

In responding patients 4 and 14, the number of gene-marked cells in the circulation (assumed to be 1% of total body lymphocytes) increased by factors of 1400 and 30, respectively, as compared to the number of infusion cells. At 1 year after infusion, both responding patients had sustained high levels (between 20 and 70%) of circulating gene-transduced cells (Fig. 3E). This high level of gene-marked cells was confirmed in patient 4 by limiting dilution T cell cloning of circulating lymphocytes at 1 year after treatment, which revealed that 42% (33 out of 79) of T cell clones contained the transgene as assessed by the PCR assay. These two patients also displayed Vβ12 cells that were detectable by antibody staining between 12 and 16% for >300 days after treatment (Fig. 3F). Patients 4 and 14 were also two of four patients who had >1% of circulating tetramer positive cells detectable for >15 days after cell infusion (Fig. 2F), and these two patients demonstrated anti-TAA reactivity in ex vivo coculture assays (table S5). No toxicities in any patient were attributed to the gene-marked cells. Although the genetically modified transferred cells exhibited decreased expression of the transgene with time in vivo, the functional activity was apparently sustained at a level sufficient to mediate the tumor regression that was seen.

Approaches to increase the expression and function of the transgene are being studied, including the possible use of lentiviral vectors, the use of more powerful promoters specific to T cells, the use of higher-affinity TCRs that can mediate CD8 independent antitumor reactivity in CD4 cells, the further optimization of T cell transduction methods, and the production of higher titer clinical-grade viruses. Approaches to prevent chain mispairing may include modification of the TCR constant regions, the insertion of single-chain receptors (*17*), or the genetic modification of hematopoietic stem cells (*18*). Because tumor specificity can be conferred on bulk PBL populations with high efficiency, it may be possible to select subpopulations of PBLs that have distinct antitumor qualities. Further genetic modification of PBLs to insert cytokine or tissue-homing molecules may be beneficial. Mouse models predict that increased lymphodepletion, either by the addition of total body irradiation to the preparative regimen or by the administration of a vaccine containing the antigen recognized by the transduced TCR, can also enhance treatment effectiveness (*19*, *20*), and these modifications are currently being explored in clinical trials.

In human subjects, normal autologous T lymphocytes, transduced ex vivo with anti-TAA–TCR genes and reinfused in cancer patients, can persist and express the transgene for a prolonged time in vivo and mediate the durable regression of large established tumors. Although the response rate (2 out of 15 patients or 13%) seen in cohorts 2 and 3 is lower than that achieved by the infusion of autologous TILs (50%), this method has potential for use in patients for whom TILs are not available. Engineering PBLs to express high-affinity TCRs recognizing the NY-ESO-1 or p53 antigens (Fig. 1A and table S1) enables the in vitro recognition of TAAs expressed on a variety of common cancers, and the use of these genetically engineered cells for the treatment of patients with common epithelial cancers deserves evaluation.

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Supporting Online Material

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Gene Transfer of Tumor-Reactive TCR Confers Both High Avidity and Tumor Reactivity to Nonreactive Peripheral Blood Mononuclear Cells and Tumor-Infiltrating Lymphocytes[1]

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Cell-based antitumor immunity is driven by $CD8^+$ cytotoxic T cells bearing TCR that recognize specific tumor-associated peptides bound to class I MHC molecules. Of several cellular proteins involved in T cell:target-cell interaction, the TCR determines specificity of binding; however, the relative amount of its contribution to cellular avidity remains unknown. To study the relationship between TCR affinity and cellular avidity, with the intent of identifying optimal TCR for gene therapy, we derived 24 MART-1:27-35 (MART-1) melanoma Ag-reactive tumor-infiltrating lymphocyte (TIL) clones from the tumors of five patients. These MART-1-reactive clones displayed a wide variety of cellular avidities. α and β TCR genes were isolated from these clones, and TCR RNA was electroporated into the same non-MART-1-reactive allogeneic donor PBMC and TIL. TCR recipient cells gained the ability to recognize both MART-1 peptide and MART-1-expressing tumors in vitro, with avidities that closely corresponded to the original TCR clones ($p = 0.018–0.0003$). Clone DMF5, from a TIL infusion that mediated tumor regression clinically, showed the highest avidity against MART-1 expressing tumors in vitro, both endogenously in the TIL clone, and after RNA electroporation into donor T cells. Thus, we demonstrated that the TCR appeared to be the core determinant of MART-1 Ag-specific cellular avidity in these activated T cells and that nonreactive PBMC or TIL could be made tumor-reactive with a specific and predetermined avidity. We propose that inducing expression of this highly avid TCR in patient PBMC has the potential to induce tumor regression, as an "off-the-shelf" reagent for allogeneic melanoma patient gene therapy. *The Journal of Immunology,* **2006, 177: 6548–6559.**

In vivo, the antitumor immune response relies on $CD8^+$ CTL recognition of antigenic tumor peptides bound to MHC class I molecules (pMHC)[3] displayed on the tumor cell surface (1–3). Currently, an effective treatment for patients with metastatic melanoma involves obtaining tumor-reactive T cells from excised tumors, expanding these T cells in vitro and reinfusing them into a lymphodepleted patient (4, 5). This treatment, commonly referred to as adoptive cell transfer (ACT), achieves a 51% objective response rate as determined by Response Evaluation Criteria in Solid Tumors (6). Patients must satisfy several requirements to receive this ACT immunotherapy: they must have surgically resectable tumors, these tumors must generate viable tumor-infiltrating lymphocytes (TIL) in the laboratory, the TIL must be reactive against tumor Ags, and these tumor-Ag reactive TIL must expand in vitro to sufficient numbers for patient treatment. Fewer than half of patients chosen for ACT therapy meet these treatment requirements. Of those patients that do receive ACT, the ability of each TIL infusion to recognize and lyse tumor can vary dramatically, likely influencing the outcome of therapy (7–9). For many patients with cancers other than melanoma, it is difficult to obtain tumor-reactive TIL. A potential solution to these problems is the transduction of genes encoding tumor-reactive TCR into patient PBL to convert them into tumor-reactive T cells (10–13). This type of experiment has recently been reported in a mouse model, showing that T cells transduced with a retrovirus encoding a TCR against a self-expressed OVA Ag can persist and function in vivo in OVA transgenic mice (14).

Several groups have shown that highly avid T cells have superior function over low-avidity cells in $CD8^+$ CTL-mediated immunity against tumors (15–17) and viral infections (18, 19) in vivo. Recent results have linked high-affinity TCR with functional avidity in vivo in a mouse model (20), suggesting that expression of a high affinity TCR may confer high functional avidity to a T cell. Determination of TCR:pMHC affinity can be measured directly in vitro by surface plasmon resonance evaluation of binding interaction between the isolated proteins (21). In vivo, many factors contribute to the avidity of TCR-specific T cells interacting with target cells displaying cognate Ag bound to MHC. While TCR:pMHC binding is known to determine the specificity of interaction (22–24) and TCR affinity contributes to overall cellular avidity (25, 26), additional factors also affect T cell activation. The numbers and immune synapse clustering of TCR:pMHC interactions have been shown to play a role in T cell activation (27–31), and additional coreceptor:ligand interactions such as CD8/MHC H chain binding and CD28/CD80 and CD86 are also known to contribute to overall cellular avidity (8, 28, 32–35).

The substantial clinical response rate in ACT-based tumor immunotherapy demonstrates that T cells with TCR of sufficiently

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[3] Abbreviations used in this paper: pMHC, peptide bound to MHC class I molecule; ACT, adoptive cell transfer; CU, Cetus unit; STEM, Stemline medium; TIL, tumor-infiltrating lymphocyte; IVT, in vitro transcription.

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high avidity to eliminate tumors do exist in vivo. If these TCR were sufficient to determine cellular avidity, then the identification of an exceptionally high avidity TCR would provide an ideal candidate gene for allogeneic TCR gene therapy. Although it has been suggested that in vitro T cell binding of fluorescently labeled multimeric pMHC complexes may provide a surrogate measure of TCR affinity, a number of authors have reported a disconnect between mean fluorescence intensity of tetramer staining and TCR affinity (35–38). Several models of TCR:pMHC interaction are suggestive that naturally occurring T cell avidity may be primarily determined by TCR:pMHC affinity (25, 26); however, this hypothesis has yet to be tested empirically. Although there is no clear predictive relationship between tetramer binding intensity and functional avidity of T cells, it has recently been shown that high avidity T cells can be identified by their ability to bind pMHC tetramers independent of CD8 coreceptor binding (39).

In this study, multiple T cell clones reactive to the melanoma tumor Ag, MART-1:27-35 (MART-1) (40, 41) were isolated from TIL obtained from tumors from five different melanoma patients treated at the National Cancer Institute, Surgery Branch. MART-1-specific T cell clones from these TIL were characterized according to their ability to produce IFN-γ in response to coculture with MART-1 peptide or MART-1 expressing HLA-A*0201-positive melanoma tumor cells. Clones were then evaluated for their ability to bind MART-1:26-35(27L)/HLA-A*0201 tetramers in a CD8-dependent and independent fashion. Each clone was determined to be of high, medium, or low avidity based on their IFN-γ production in response to coculture with MART-1 peptide-pulsed targets and MART-1-expressing tumor cells, combined with an assessment of TCR affinity based on the ability to bind MART tetramer in a CD8-dependent or independent manner. The genes encoding the TCR α- and β-chains from multiple clones were then expressed in a common cell to study the degree to which the TCR affinity determines the overall avidity of effector T cells and to evaluate means to increase TCR expression and function.

Materials and Methods

Cell lines, TIL, and PBMC

All primary tissues and cells used in this study were obtained from patient samples at the Surgery Branch, National Cancer Institute.

Melanoma cell lines, mel526, mel624 (MART-1$^+$, HLA-A*0201$^+$), and mel888 and mel938 (HLA-A*0201$^-$) were generated from resected tumor lesions. T2 is a lymphoblastoid cell line deficient in TAP function, whose HLA-A*0201 molecules can be readily loaded by pulsing with exogenous peptide. Jurkat T3.5 is a CD4-restricted T cell line lacking surface expression of TCR and CD3 due to a defect in the TCR β-chain.

PBMC used were cryopreserved leukopheresis samples of donor blood. TIL and tumor digests were generated from resected patient tumors, and provided by the TIL lab (Surgery Branch, National Cancer Institute).

Cloning of MART-1-reactive TIL

Fresh melanoma tumors from three patients were digested and cultured in vitro for 0–14 days in presence of 6000 IU (1000 Cetus units (CU) of IL-2; Chiron) in a 1:1 mix of Stemline medium (Sigma-Aldrich) (STEM):RPMI 1640 (Invitrogen Life Technologies) culture medium plus 10% human AB serum (Gemini Bioproduct), HEPES, 2-ME, 100 U/ml penicillin, and 100 μg/ml Fungizone. Cells were costained with a panel of TCR VB Abs (Coulter Immunotech) and MART-1:26-35(27L)/HLA-A*0201 tetramer (Beckman-Coulter Immunomics) at day 0, 7, and 14. The widest diversity VB of MART-1-reactive T cells combined with the highest proportion of MART-1-reactive cells was observed between day 7–14 and varied between patients. TIL samples were taken from the time point at which MART-1-reactive T cell numbers and diversity were maximal and cloned by limiting dilution at two or five cells per well in 96-well plates in the presence of 400-fold excess 4000-rad irradiated allogeneic PBMC feeders, 30 ng/ml OKT-3, and 1000 CU of IL-2. Clones were sampled at day 14 for their ability to produce IFN-γ specifically in response to MART-1 peptide-pulsed target cells. These TIL clones were expanded with a second exposure to irradiated PBMC feeder cells and OKT-3 in 1000 CU of IL-2.

Evaluating cellular avidity

On day 14 of expansion, the clones were evaluated for their cellular avidity to MART-1 Ag by measuring IFN-γ secretion (ELISA paired Abs obtained from Pierce Endogen) in supernatant of 18-h cocultures with titrated MART-1 peptide on target cells or with MART-1 expressing HLA-A*0201 melanoma tumor cells. These assays were conducted with 1×10^5 effectors and 1×10^5 target cells in 0.2 ml of RPMI 1640 + 10% FBS (Invitrogen Life Technologies) in round-bottom 96-well plates at 37°C, 5% CO_2. TCR CD8 independence was evaluated by determining the ability of the clones to bind to MART-1:26-35(27L)/HLA-A*0201 tetramers generated either with an intact CD8-binding region (WT), or with this binding abrogated by inclusion of a double mutation in the HLA-A*A0201 molecule (mut). These tetramers were generated by the National Institute of Allergy and Infectious Diseases tetramer facility.

TCR gene isolation

RNA was purified from each clone using Qiagen RNEasy, and 5′ RACE was performed using BD SmartRace reagents and protocol, using the universal 5′ primer, and a 3′ gene-specific primer for the TCR α constant region, or C1 or C2 β constant regions. Results were run on a gel and appropriately sized bands (800–900 bp) were excised, subcloned into pCR2.1 (Invitrogen Life Technologies) vector, and sequenced.

In vitro TCR RNA transcription and expression in PBMC

Gene-specific oligonucleotide primers were generated for the production of in vitro RNA transcription (IVT). 5′ primer design included the T7 polymerase binding sequence, followed immediately by a Kozak sequence, a start codon and the next 19–15 bp of Vα or Vβ region for each TCR gene; JKF6 B28 fwd T7 gene-specific primer 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGG GAA TCA GGC TCC TCT GTC G-3′; M5 B6.5 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGA GCA TCG GCC TCC TGT GCT G-3′; M7 B19 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGA GCA ACC AGG TGC TCT GCT G-3′; M9/20 B4.1 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGG GCT GCA GGC TGC TCT GCT G-3′; #17 B11.2 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGG GCA CCA GGC TCC TCT GCT G-3′; DMF4 B10.3 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGG CAC AAG GTT GTT CTT C-3′; DMF5 B6.4 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGA GAA TCA GGC TCC TGT-3′. VA regions were either 12.2 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA GCA AAT CCT TGA GAG GTT TAC-3′; 12.3 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGA TGA AAT CCT TGA GAG TTT TAC-3′; OR 35 fwd 5′-TAA TAC GAC TCA CTA TAG GGA GAA CCG CCA TGC TCC TTG AAC ATT TAT TAA TC-3′. 3′ primers included 64T and 18–25 bp of the relevant α or β constant region sequence. Reverse primers were C-α 5′-(64)T TTC AAC TGG ACC ACA GCC TCA GC-3′; C1-β 5′-(64)T TTC ATG AAT TCT TTC TTT TCA CC-3′ or C2-β 5′-(64)T TCT AGC CTC TGG AAT CCT TTC TCT TG-3′.

For IVT, a PCR product was generated using the subcloned cDNA in pCR2.1 as a template with the above oligonucleotide primer sets. Resulting bands were gel purified and used for a second round of PCR amplification. PCR product was cleaned using Zymogen DNA purification columns. One to 3 μg of PCR product was used as template for IVT using Ambion T7 mMESSAGE MACHINE as per the manufacturer's instructions, followed by RNA clean up using Qiagen RNEasy. RNA quantity was measured at OD_{260} by spectrophotometer, and quality was determined by running 1 μg on a 2% agarose gel in denaturing loading buffer, following 5 min of incubation at 70°C.

In preparation for RNA electroporation, donor TIL or PBMC from phereses were stimulated in vitro with 50 ng/ml OKT-3, 50 CU of IL-2 in STEM:RPMI 1640 medium for 3 days, when CD8$^+$ or CD4$^+$ cells were positively selected using Miltenyi Biotec microbeads and magnetic columns. PBMC were then grown in vitro an additional 2–15 days in IL-2 containing medium before use. For TCR electroporation, 0.5–2.0 μg of RNA from each TCR gene was used per 1×10^6 cells (at 2.5×10^7 cells/ml in Opti-MEM serum-free medium (Invitrogen Life Technologies). Cells were rested for 2–16 h without IL-2 after electroporation before use in FACS staining or coculture experiments.

Evaluating T cell function

Cocultures were set up at 1×10^5:1×10^5 (E:T) cells in complete medium (RPMI 1640 + 10% FBS (Invitrogen Life Technologies)) in 96-well plates and incubated overnight at 37°C. All samples were run in duplicate, and RNA electroporations were repeated in PBMC or TIL from three different

donors. Supernatant was tested for cytokine secretion by IFN-γ ELISA (Pierce Endogen).

CTL assays coincubated decreasing ratios of effectors and ^{51}Cr-labeled target cells (E:T) in complete medium in 96-well plates at 37°C for 4 h. Lysis was measured by ^{51}Cr release in the medium: percent lysis = (sample release − minimum release)/(maximum release − minimum release) × 100%, average of duplicate samples.

Peptides used

Peptides used for coculture included MART-1:27-35, gp100:154-162, gp100:209-217, and gp100:280-288. Dilutions were made 1000× in DMSO before use. With the exception of MART-1, following 1 h pulsing, all target cells were washed three times before use; due to low solubility, MART-1 peptide was left in final coculture medium.

Flow cytometry

Cell surface expression of CD3 and CD8 was measured using FITC-conjugated Abs in conjunction with MART-1:26-35(27L)/HLA-A*0201 tetramers (Beckman Coulter) to measure surface MART-1 TCR on electroporated cells. Isotype controls were used to gate all samples. Cells were run on FACSCalibur or FACScan flow cytometers (BD Biosciences), with CellQuest software and analyzed using FlowJo software (Tree Star).

Results

Generating TIL clones directly from patient tumor digests reveals a diversity of MART-1-reactive T cells with varying cellular avidities

Surgically resected tumor specimens were obtained from three patients who had shown prior TIL reactivity against the HLA-A*0201 restricted melanoma tumor Ag MART-1. An array of MART-1-reactive TIL clones were generated by limiting dilution cloning after growth of the tumor digests in the presence of high-dose IL-2 in vitro for 10–14 days. A total of 576 individual clones (192 from each patient TIL) were isolated and assayed for reactivity to various melanoma tumor Ags, including MART-1, by coculturing with peptide-pulsed T2 target cells (42) and assessment of IFN-γ secretion following overnight incubation. Individual T cell clones were found that reacted with each of the melanoma Ag epitopes tested (Table I). Forty-one clones (7.1% per patient ± 0.3%) showed specific recognition of the native MART-1:27-35 Ag, 3 clones (0.5% per patient ± 0%) recognized gp100:209-217, 2 clones (0.3% per patient ± 0.3%) recognized gp100:154-162, and 31 (5.4% per patient ± 0.6%) responded to gp100:280-288. The prevalence of these specific tumor-Ag reactive cells was remarkably similar between patients. Cells were expanded by exposure to anti-CD3 Ab (OKT-3) in the presence of high-dose IL-2, and 21 MART-1 clones proliferated and retained Ag-specific reactivity.

Three additional CTL clones from two additional patients were included for analysis. A MART-1 reactive T cell clone, DMF4 (previously referred to as M1F12), was obtained from a clinically administered TIL that resulted in in vivo tumor regression (10). DMF5 was a second MART-1-reactive CTL clone from the same patient treatment TIL as DMF4. Additionally, clone JKF6, previously obtained from another patient TIL known to be highly MART-1 reactive in vitro, was also included in this study.

Table I. *Naturally occurring melanoma tumor-reactive TIL clones, prevalence by patient*

Patient (192 clones each)	No. of Reactive Clones[a]			
	MART-1: 27–35	gp100: 154–162	gp100: 209–217	gp100: 280–288
1-D	14; 7.3%	1; 0.5%	1; 0.5%	9; 4.7%
2-M	13; 6.8%	1; 0.5%	1; 0.5%	11; 5.7%
3-S	14; 7.3%	0; 0%	1; 0.5%	11; 5.7%
Total (576 clones)	41; 7.1%	2; 0.3%	3; 0.5%	31; 5.2%

[a] Clone reactivity was evaluated by secretion of IFN-γ in response to coculture with target cells pulsed with tumor peptides.

Functional avidity of these 24 T cell clones was evaluated by the production of IFN-γ in response to coculture with T2 target cells pulsed with diminishing amounts of MART-1 peptide and to naturally MART-1 expressing HLA-A*0201-restricted melanoma tumor cell lines. The clones DMF4, DMF5, and JKF6 were among the most highly avid CTLs, producing high amounts of IFN-γ even in response to low concentrations of MART-1 peptide or endogenous MART-1 on tumor cells (Fig. 1, *A* and *B*). Of all CTLs tested, DMF5 and JKF6 displayed the highest avidity for both tumors and exogenously supplied peptide. IFN-γ production in response to MART-1 peptide was compared with the response to MART-1-expressing tumors, and found to be strongly correlated ($p = 0.006$) (Fig. 1*C*), confirming that the Ag the clones recognized on tumors was MART-1, and that MART-1 peptide reactivity correlated with the ability to recognize MART-1 bearing tumors (Fig. 1*C*). In evaluating TCR for the potential use in antitumor allogeneic gene transfer, it is crucial that the receptor recognize a number of different HLA-A*0201-restricted melanoma tumors. In Fig. 1*D*, we show that the ability of any given T cell clone to recognize a MART-1-expressing melanoma tumor cell line was strongly correlated with its ability to recognize another MART-1 bearing melanoma cell line ($p < 0.0005$).

Tetramer binding of CTLs to pMHC complexes is accomplished by a combination of TCR:pMHC association and CD8 coreceptor interaction with the MHC class I H chain (27, 32). Clones were evaluated for their ability to bind pMHC complexes in a CD8-dependent or independent fashion using MART-1:26-35(27L)/HLA-A*0201 tetramers consisting of either nonmutated HLA H chains, or with an HLA H chain double-mutation eliminating CD8 binding (43). While most MART-1-reactive clones were capable of binding tetramer in the presence of CD8 coreceptor binding, only select clones (10 of 24) were able to do so in the absence of CD8 coreceptor binding (Fig. 2, Table II), indicative of a higher-affinity TCR.

To determine the degree to which TCR transfer can confer cellular avidity, clones were selected that showed low avidity (low IFN-γ production plus inability to bind tetramer in the absence of CD8 binding), medium avidity (high IFN-γ production in response to target cells but unable to bind tetramer with CD8-independence, or produced low IFN-γ in response to target cells but could bind tetramer with CD8-independence), or high avidity (high IFN-γ production combined with the ability to bind tetramer independently of CD8), the results of which are tabulated in Table II. While high TCR affinity often coincided with high production of IFN-γ in response to target cells, this was not always observed. Certain clones (M3, M19, M26, #18) were able to bind tetramer independent of CD8 coreceptor binding, yet produced only modest amounts of cytokine in response to target cell stimulus. The reason for this is not clear, but differing TCR affinities for the HLA-A*0201 molecule or the native MART-1:27-35 peptide on target cells vs the altered MART-1:26-35(27L) peptide used to stabilize tetramers may be a contributing factor. Alternatively, the overall capability of these T cell clones to produce IFN-γ may have been reduced in comparison to other clones. The highest-avidity IFN-γ-producing clones DMF5 and JKF6 were able to bind tetramer independent of CD8, as was one other highly avid clone, M5.

Analysis of TCR variable region diversity indicates a strong Vα gene restriction for recognizing MART-1 Ag

The ability of T cells to respond functionally to MART-1 Ag in both peptide- and tumor-associated Ag form was combined with

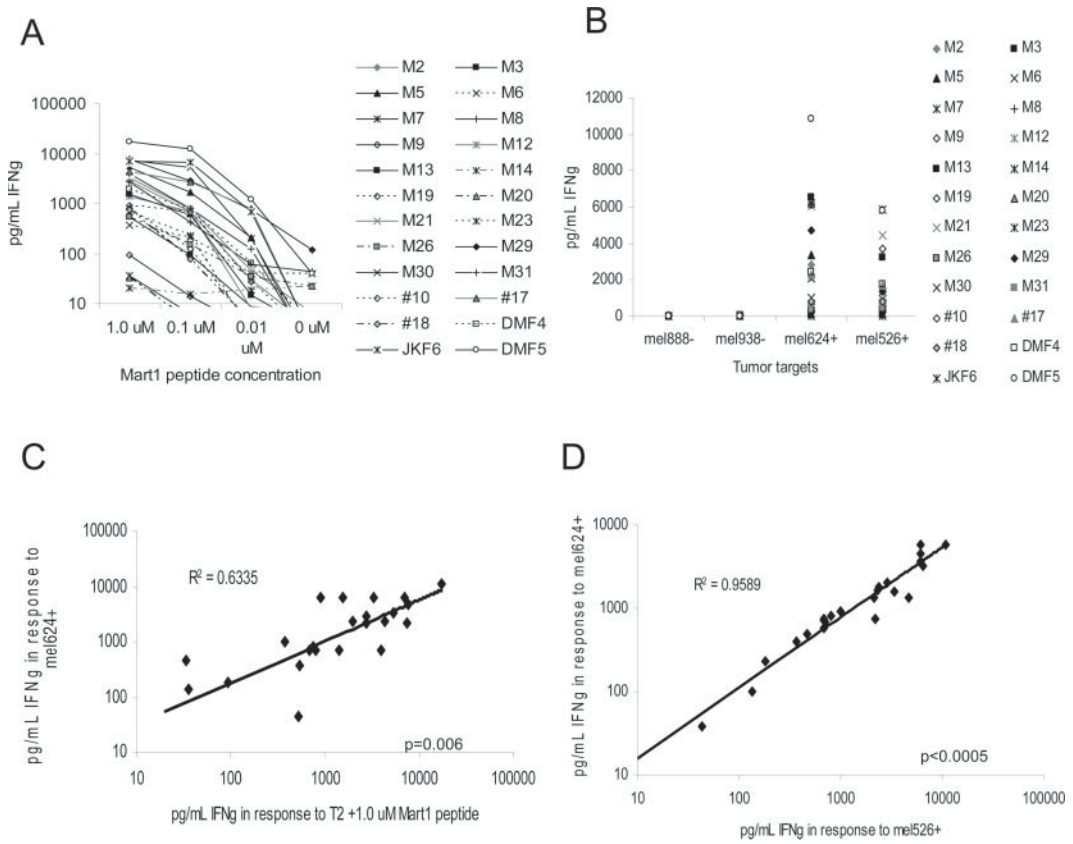

FIGURE 1. MART-1-reactive TIL clones derived from several patients display a wide diversity of peptide and MART-1 tumor reactivities. *A*, IFN-γ production by 24 MART-1 clones derived from five patients, coincubated with titered MART-1 peptide on T2 target cells. *B*, IFN-γ response of MART-1 clones following coculture with MART-1/HLA-A*0201-positive (mel526$^+$, mel624$^+$) and HLA-A*0201-negative melanoma tumors (mel888$^-$, mel938$^-$). IFN-γ release was evaluated from 18-h culture supernatant by ELISA. *C*, Correlation of IFN-γ produced by each clone in response to coculture with T2 cells pulsed with MART-1 peptide or mel624$^+$ MART-1-expressing tumor. *D*, Correlation of IFN-γ produced by each clone in response to coculture with two different MART-1/HLA-A*0201-expressing tumors. Statistical analysis by ANOVA regression analysis.

TCR affinity (estimated by mutated MART-1:26-35(27L)/HLA-A*0201 tetramer binding) to characterize T cell clones as high, medium, or low avidity-affinity (Table II). Representative clones were chosen from each category: three low (M7, M9, M20), two medium (#17, DMF4), and three high (M5, JKF6, DMF5). RNA was purified from each clone, and TCR α and β genes were isolated using 5′ RACE, subcloned, and sequenced. Of these eight MART-1-reactive clones, none exhibited similar β-chain variable regions, with the exception of clones M9 and M20, which were derived from the same patient and upon sequencing proved to be duplicate cells. This CTL clone (M9/M20) will be referred to as M9 throughout the remainder of this study. Five of seven clones showed Vα region 12-2 restriction, and a sixth clone consisted of the closely related Vα12-3 (Table III), confirming previous reports of a strong TCR α variable region restriction in recognition of MART-1 Ag (44–46).

TCR transfer is sufficient to confer overall cellular avidity to donor PBMC in an Ag-specific manner

RNA was generated by in vitro transcription of each pair of α and β TCR genes isolated from the seven clones indicated above. TCR RNA was electroporated into the TCR β-chain-deficient Jurkat T3.5 cell line (47, 48). As shown in Fig. 3*A*, CD3 Ab staining levels were similar on the surfaces of cells electroporated with each of the seven TCR pairs. This indicated that following electroporation of the same amounts of RNA, the α and β chains of each TCR were translated into similar amounts of TCR proteins within the cells, which in turn were able to form appropriate heterodimeric structures and recruit the CD3 portion of the TCR complex to the cell surface. Appropriate MART-1-reactive tertiary αβ TCR pairing was evaluated by the ability to bind MART-1:26-35(27L)/HLA-A*0201 tetramers (Beckman Coulter), analyzed by flow cytometry (Fig. 3*B*). Intriguingly, although TCR surface pairing assessed by CD3 staining was similar for all TCRs tested, there were marked differences in the ability of each TCR to bind MART-1 tetramer, correlating with the TCR affinity. RNA encoding these TCR was then electroporated into 7-day OKT-3-stimulated PBMC and stained for MART-1 TCR formation by tetramer staining (Fig. 3*C*). The tetramer staining of TCR electroporated CD8$^+$ PBMC was similar to that observed in Jurkat cells. Although PBMC bear the CD8 coreceptor, they also express endogenous TCR α- and β-chains to compete with the electroporated MART-1 TCR genes for heterodimer pairing. Notably, in both cell types, those TCR derived from high-avidity T cells (DMF5, JKF6, M5) were able to bind tetramer more readily than those with low avidity (M7, M9). Although RNA electroporation efficiency was observed to be >95% (by GFP controls, data not shown), only a fraction of the TCR-electroporated cells were able to bind MART-1 tetramer at all, suggesting that levels of TCR expression achieved by this method are still lower than TCR levels observed in endogenous TCR-expressing T cells.

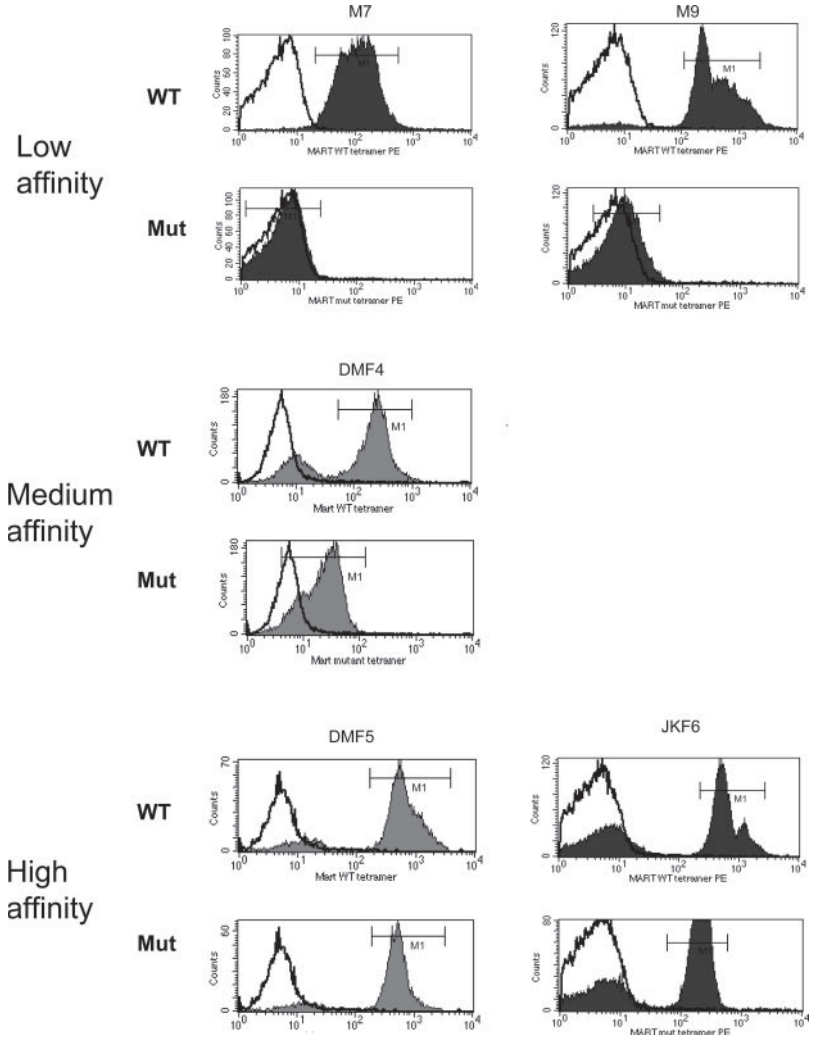

FIGURE 2. Different MART-1-reactive TIL clones vary in their ability to bind MART-1/HLA-A*0201 tetramer with CD8 independence. Each MART-1-reactive TIL clone was stained with MART-1:26-35(27L)/HLA-A*0201 (MART-1) unmodified tetramer (WT), or MART-1 tetramer with a HLA H chain double mutation abrogating CD8 binding to the HLA-A*0201 molecule (Mut). Representative graphs of each type of TCR binding to tetramer are shown: low-affinity clones were unable to bind tetramer in absence of CD8 coreceptor binding, medium-affinity clones bound CD8-independent tetramer at low levels, and high-affinity clones bound tetramer at high levels in absence of CD8 coreceptor binding. In each graph the isotype control is shown (white) as well as MART-1 tetramer binding (shaded).

Ag-specific response by the TCR-electroporated T cells was evaluated by coculture with MART-1 peptide-pulsed T2 targets, or MART-1-expressing melanoma tumor cells, and assayed for supernatant IFN-γ (Fig. 4, *A* and *B*). The TCR-electroporated cells were capable of generating an Ag-specific response, and this response varied in intensity. Three TCR (DMF5, JKF6, and M5) were able to generate high amounts of IFN-γ, two (DMF4, #17) generated mid-level responses, and two (M7, M9) generated only negligible responses to MART-1 Ag. In order of diminishing functional avidity, the response induced by each TCR was DMF5 > JKF6 > M5 > #17 > DMF4 > M7 = M9. These cells retained the same respective avidities observed for the original TIL clones, and this pattern was repeated regardless of the amount of TCR RNA electroporated (2–0.5 μg RNA per million cells, data not shown). Statistical correlation (ANOVA regression analysis) between IFN-γ production of the original TIL clones and corresponding TCR RNA electroporated PBMC in response to MART-1-bearing target cells was evaluated (Fig. 4*C*) and found to be highly significant ($p = 0.018$ for 1.0 μM MART-1 peptide; $p = 0.0003$ and $p = 0.009$ for mel624 and mel526 tumor targets, respectively). CD8$^+$ PBMC electroporated with MART-1 TCR were also able to induce specific target cell lysis of peptide-pulsed targets or MART-1-bearing tumors in chromium release assays (Fig. 5*A*), at levels consistent with each relative TCR avidity: DMF5 > JKF6 > M5 > DMF4 > #17 > M7 = M9.

PBMC expressing high-avidity TCR are capable of functional recognition of MART-1 target cells, even in the absence of CD8

Since these high-avidity TCRs were chosen in part for their ability to bind MART-1 tetramer independently of CD8, we next determined if this TCR avidity was strong enough to confer CD8-independent recognition of MART-1 Ag on target cells. TCR RNA from the high-avidity receptors DMF5, JKF6, and M5 and medium-avidity DMF4 was electroporated into CD4$^+$-enriched (>95% pure) OKT-3-activated donor PBMC. As assessed by IFN-γ production in response to coculture with titered MART-1 peptide on T2 target cells, each MART-1 TCR in CD4$^+$ cells was able to recognize MART-1 peptide. DMF5 showed the highest response, followed by JKF6 and M5, then DMF4 (Fig. 5*B*), although the overall levels were lower than those observed in CD8$^+$ T cells from the same donor electroporated with the same TCR. In response to MART-1 tumor Ag naturally presented on tumor cells, only those CD4$^+$ cells that expressed the highest-avidity DMF5 or JKF6 receptors were able to recognize and respond to tumors, with DMF5 generating the highest response (Fig. 5*C*).

Nonreactive TIL can be made tumor reactive upon RNA electroporation with a high-avidity MART-1 TCR

There are many differences between the T cells found in TIL and those circulating in PBMC (8). Currently, it is not known whether

Table II. *Avidity and affinity characterization of MART-1-reactive TIL clones*

Clone	Tetramer MFI			IFN-γ Produced (pg/ml)					Avidity-Affinity
	gp100: 209	WT MART-1	mut MART-1	1.0 μM MART-1	1.0 μM gp100	mel526$^+$	mel624$^+$	mel888$^-$	
M2	5	51	6	2,777	10	2,070	2,864	2	Medium
M3	6	365	106	528	15	38	43	2	Low/Medium
M5	5	621	98	5,381	50	1,554	3,346	1	High
M6	5	5	5	377	47	935	990	20	Medium
M7	5	109	6	35	23	102	135	4	Low
M8	5	31	5	2,709	48	759	2,171	41	Medium
M9	5	316	9	92	64	236	182	3	Low
M12	5	246	5	1,403	14	709	687	1	Medium
M13	6	372	6	1,531	59	3,207	6,522	4	Medium
M14	5	5	5	20	24	13	8	11	N/A
M19	5	799	279	794	4	581	679	1	Medium/High
M20	7	271	7	34	27	489	465	3	Low
M21	6	84	6	3,303	34	4,428	6,096	19	Medium
M23	10	378	10	686	3	682	699	10	Medium
M26	6	594	302	541	102	405	367	12	Medium
M29	5	193	5	7,789	557	1,352	4,704	17	Medium
M30	5	294	6	7,340	2	1,364	2,111	14	Medium
M31	6	352	7	3,974	48	735	692	19	Medium
#10	6	259	6	897	105	3,703	6,158	13	Medium
#17	5	533	84	4,330	633	1,678	2,322	9	Medium/High
#18	6	649	118	751	23	815	796	16	Medium/High
JKF6	6	537	207	6,917	122	5,896	6,224	15	High
DMF4	5	308	25	1,987	45	1,780	2,397	24	Medium
DMF5	5	538	474	17,161	80	5,806	10,865	29	High

TIL have unique properties that may make them more effective in treating tumors in vivo than T cells derived from PBMC. To investigate whether it is possible to convert nonreactive TIL to recognize and lyse tumors, we selected previously generated TIL obtained from melanoma patient samples with determined activity only against autologous tumor and not against any known shared tumor Ags such as MART-1. Patient-matched PBMC samples were obtained, and both PBMC and TIL were stimulated similarly, using OKT-3 Ab and irradiated allogeneic feeder PBMC in the presence of high-dose IL-2. CD8$^+$ T cells were isolated and electroporated with TCR RNA from the highest-avidity DMF5 TCR. MART-1 reactivity was assayed by coculture of RNA electroporated T cells with MART-1 peptide-pulsed target cells and tumors, and evaluated for IFN-γ production and CTL target cell lysis (Fig. 6, *A* and *B*). It was possible to convert non-MART-1-reactive TIL into reactive TIL using this high-avidity TCR. These RNA electroporated TIL were able to recognize and lyse MART-1-expressing tumors, albeit at lower levels than matched RNA electroporated PBL obtained from the same patient, even though surface MART-1 TCR expression was comparable (based on tetramer staining, data not shown).

Avidity of DMF5 TCR-engineered T cells can be further improved by replacing the endogenous TCR constant region with a murine version

One caveat to using TCR gene transfer therapy in T cells is the inherent competition with endogenously expressed α and β TCR genes for appropriate αβ TCR heterodimer pairing. In an attempt to increase the pairing of functional antitumor TCR αβ chains on allogeneic PBMC, modifications were made to the transmembrane constant regions of each TCR chain, replacing them with constant regions derived from the mouse genome (49). These small portions of the TCR vary enough from the human sequence to facilitate preferential pairing, and improve CD3ζ chain recruitment, yet are similar enough that potential immunogenicity should be minimized. We have modified the DMF5 TCR α- and β-chains with these murine constant regions, and evaluated their ability to confer increased cellular activity against MART-1 peptide-pulsed targets or melanoma target cells. As previously reported for the DMF4 TCR (49), replacing the DMF5 constant regions with the murine sequence (mDMF5) likewise shows an increase in TCR pairing at the cell surface, demonstrated by increased MART-1 tetramer binding by RNA electroporated CD8$^+$ PBMC (Fig. 6*C*). Functional avidity was also increased, as demonstrated by increased IFN-γ production upon coculture with MART-1$^+$ target cells (Fig. 6*D*). This increased activity was particularly apparent when measured against melanoma tumor cells, which normally result in only low TCR-engineered T cell reactivity compared with peptide-pulsed target cells. This modified mDMF5 TCR conferred the highest avidity of any TCR tested, in both CD4$^+$ and CD8$^+$ T cells, against all targets (Fig. 6*D*).

Discussion

The present study was undertaken to determine the relationship between the functional avidity of multiple MART-1-reactive clones, to the properties of the clonal TCR, and to use this information to select TCR that confer high Ag recognition for use in

Table III. *TCR variable-region restriction of MART-1-reactive clones of various avidities*

Mart Clone	TCR Vα	TCR Vβ	Clone Avidity/Affinity (IFN-γ/CD8 independence)
DMF4	35	10-3	Medium/Medium
DMF5	12-2	6-4	High/High
JKF6	12-2	28	High/High
#17	12-2	11-2	Medium/High
M5	12-2	6-5	High/High
M7	12-2	19	Low/Low
M9[a]	12-3	4-1	Low/Low
M20[a]	12-3	4-1	Low/Low

[a] Clone M9 and M20 are the same clone, derived from the same patient.

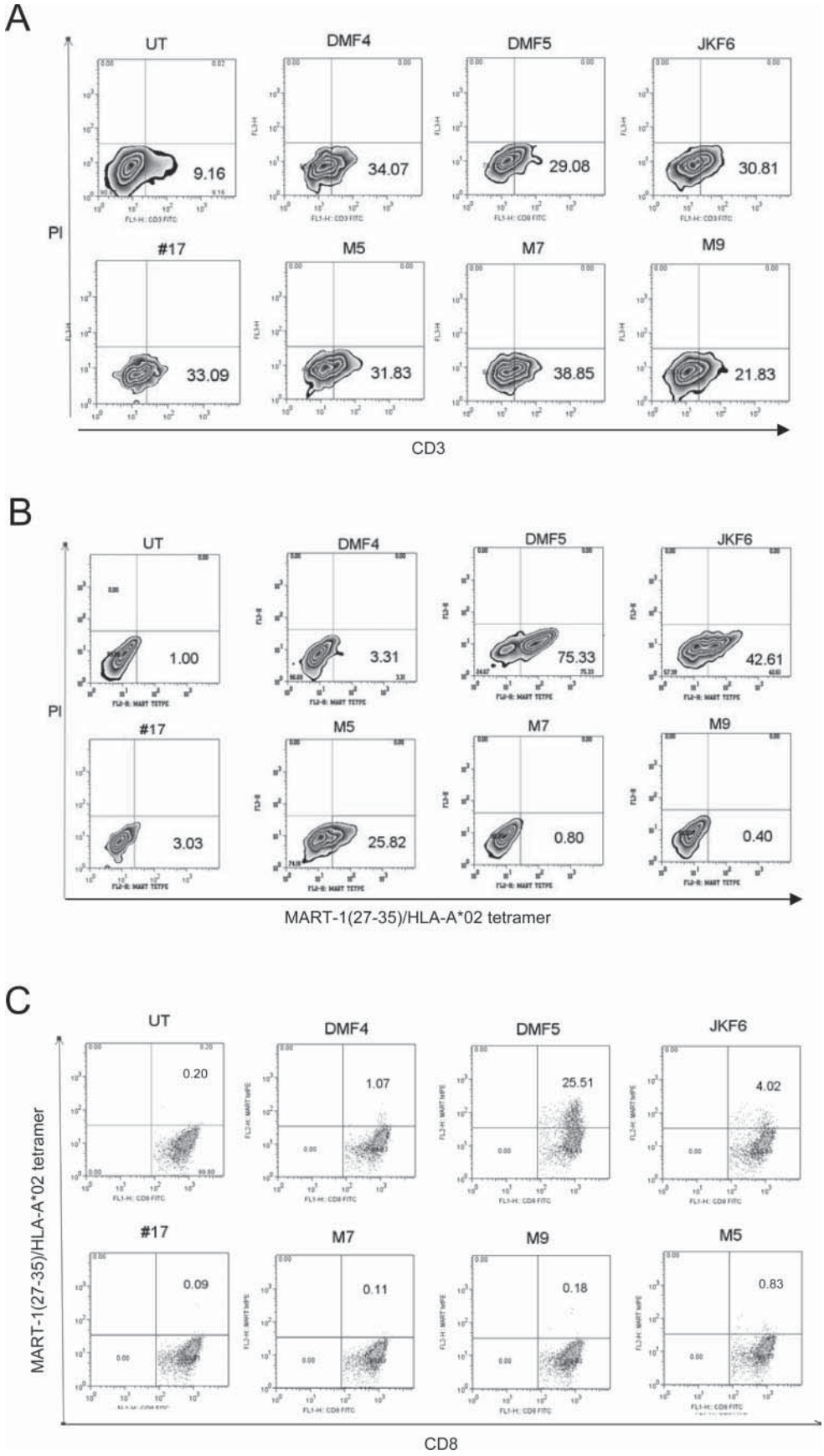

FIGURE 3. RNA electroporation of TCR derived from MART-1 clones into Jurkat and donor $CD8^+$ PBMC resulted in appropriate $\alpha\beta$ TCR translation and surface expression. Percentage of live cells staining positive is indicated. *A* and *B*, Two micrograms per 1×10^6 cells in vitro transcribed RNA from TCR α- and β-chains of each MART-1 TIL clone was electroporated into TCR-deficient, CD8-negative Jurkat T3.5 cells that were then stained for CD3 cell surface expression (*A*) or MART-1:26-35(27L)/HLA-A*0201 tetramer (*B*), and evaluated by flow cytometry. *C*, Flow cytometric evaluation of $CD8^+$ donor PBMC electroporated with 2 μg per 1×10^6 cells TCR RNA, costained with CD8 and MART-1:26-35(27L)/HLA-A*0201 tetramer. UT = untreated cells.

TCR transduction gene therapy protocols. Clones were generated by limiting dilution cloning of day 7–14 in vitro tumor digests in the presence of high-dose IL-2. The properties of the TCR were evaluated by functional assays for IFN-γ secretion in response to MART-1-bearing target cell coculture, combined with the ability of the TCR to bind mutated MART-1:26-35(27L)/HLA-A*0201

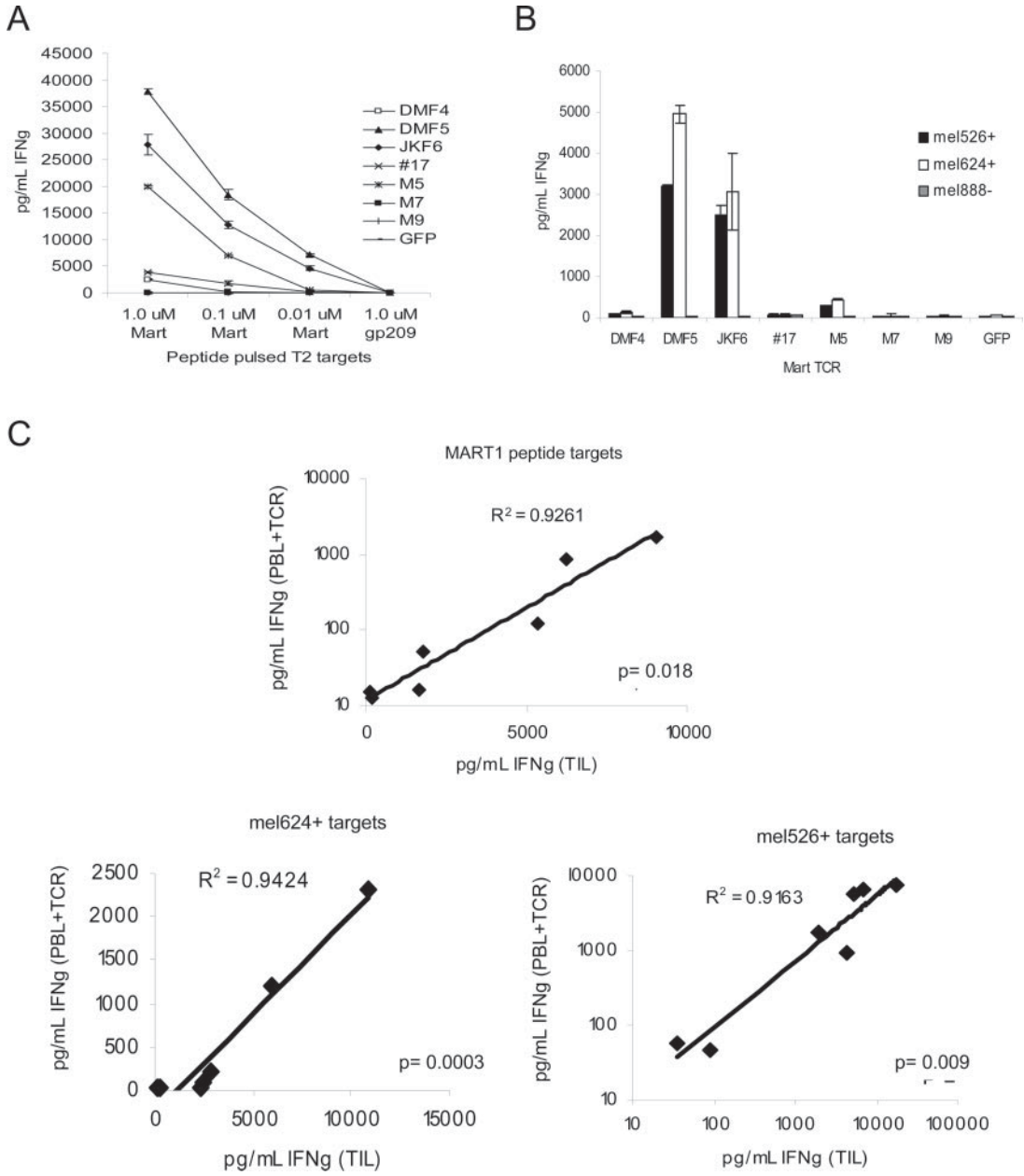

FIGURE 4. RNA electroporation of MART-1 TCR derived from clones of varying avidities into activated donor CD8$^+$ PBMC resulted in similar Ag-specific functional avidities. *A* and *B*, Donor CD8$^+$-enriched PBMC previously activated with OKT-3 Ab were electroporated with 2 μg of RNA from each TCR chain per 1×10^6 cells, and cocultured overnight with MART-1 peptide pulsed T2 target cells (*A*) or MART-1/HLA-A*0201-expressing (mel526$^+$, mel624$^+$) or nonexpressing (mel888$^-$) melanoma tumors (*B*). IFN-γ production was measured in supernatants by ELISA. Values shown are the average of duplicate samples, $\pm$SEM. *C*, Correlation of IFN-γ produced by native TIL clones and donor CD8$^+$ PBMC electroporated with matched TCR RNA. Responses to cocultures with T2 cells pulsed with MART-1 peptide, mel526$^+$, and mel624$^+$ MART-1-expressing melanoma tumors are shown. The *p* values were obtained by ANOVA regression analysis.

tetramers independently of CD8 coreceptor help. α and β TCR genes were isolated from representative clones of high, medium and low avidity using 5′ RACE, and RNA electroporated into activated PBMC T cells from a common donor. IFN-γ production and lytic activity in response to target cell cocultures demonstrated a direct correlation of avidities to those observed in the original CTL clones. Thus, the TCR was the main determinant of cellular avidity during Ag-specific T cell:target cell interaction, and this avidity could be conferred upon allogeneic cells by TCR transfer.

It has recently been shown both in vitro and in an animal model that high-affinity TCR give rise to high avidity T cells and that only high-avidity cells were able to elicit autoimmunity in lymphocytic choriomeningitis virus-infected TCR transgenic mice expressing lymphocytic choriomeningitis virus epitope as "self" in pancreatic islet cells (20). Other animal models have similarly shown that only high-avidity T cells have the ability to mediate tumor regression in spontaneously arising pancreatic tumors in RIP-Tag2-InsHA transgenic mice (16, 17, 50), or through ACT to treat metastatic B16 melanoma tumors in C57BL/6 mice (15) or HLA-A*0201 transgenic mice engrafted with human melanoma (51). These combined data suggest that to achieve an antitumor immune response in vivo, T cells recognizing tumor Ags with the highest avidity and bearing the highest-affinity TCR provide the most potential to induce an in vivo antitumor response.

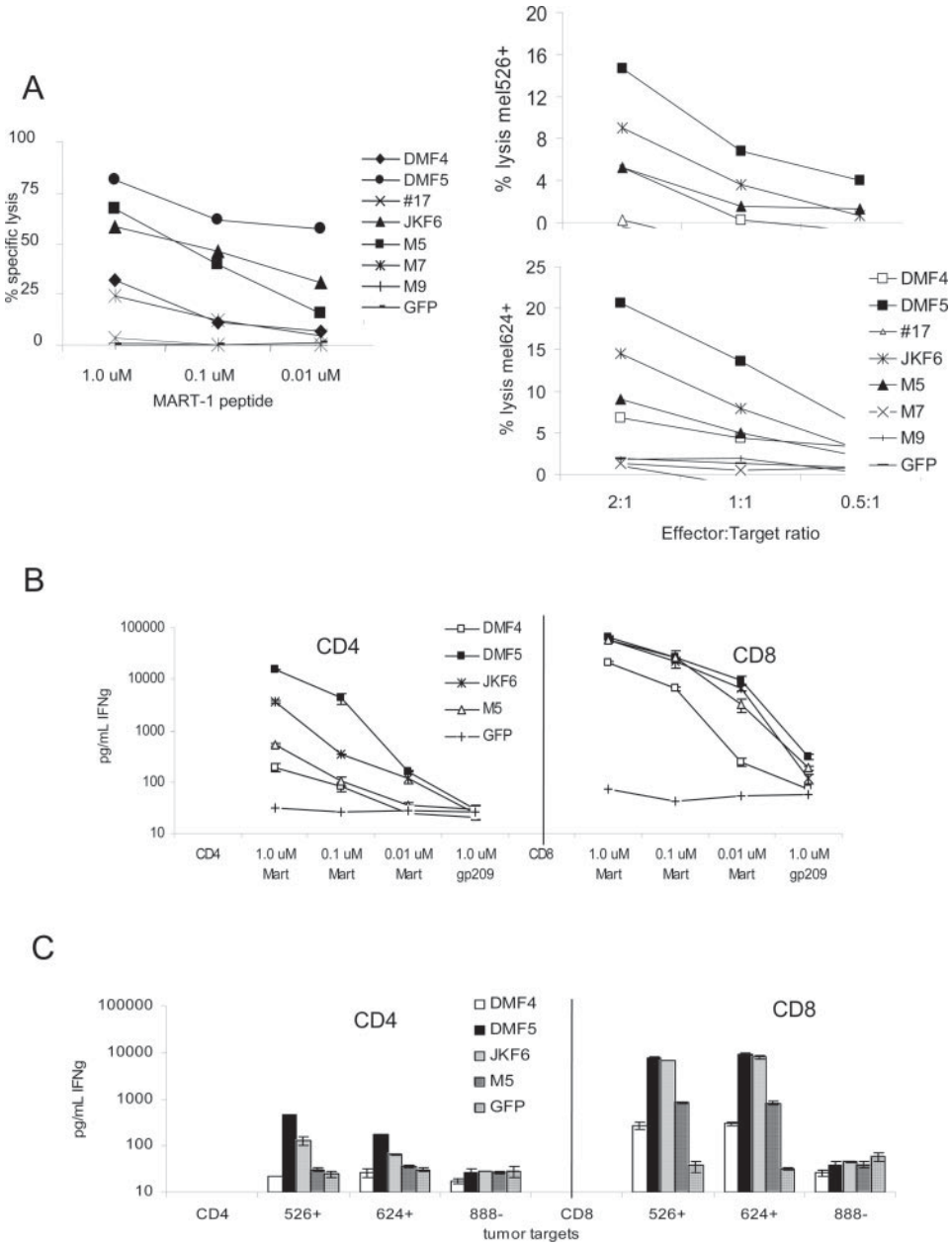

FIGURE 5. TCR transfer of a high-avidity TCR in vitro can confer antitumor CTL activity and IFN-γ production in a CD8-independent fashion. *A*, OKT-3 stimulated CD8$^+$ PBMC RNA-electroporated with different avidity MART-1 TCR were evaluated for their ability to lyse ^{51}Cr-labeled target cells in a 4-h assay. CTL were coincubated with (*left*) MART-1 peptide-pulsed T2 target cells at 2:1 E:T ratio, or (*right*) at varying E:T ratios against MART-1 expressing tumor cells. *B* and *C*, OKT-3-stimulated donor PBMC were separated into CD4$^+$ and CD8$^+$ populations by positive selection using magnetic beads and each of the four highest avidity MART-1 TCR were expressed in each population by RNA electroporation (2 μg/1 $\times$ 10^6 cells). Functional antitumor response was evaluated by coculture with either MART-1-pulsed T2 target cells (*B*) or MART-1-expressing tumors (*C*). IFN-γ production was measured in supernatants by ELISA. Values shown are the average of duplicate samples, $\pm$SEM.

Although there is no clear predictive relationship between tetramer binding intensity or tetramer dissociation and functional avidity of T cells (35), it has recently been shown that high avidity T cells can be identified by their ability to bind pMHC tetramers independent of CD8 coreceptor binding (39). In this current study, we used CD8-independent binding of tetramer as a tool to identify higher-affinity TCR for potential clinical TCR gene transfer immunotherapy. The ability to engage pMHC complexes independent of CD8 coreceptor is desirable for TCR destined for transfer into CD4 donor PBMC, or T cells with varying expression levels of CD8. A high-affinity TCR for gene transfer is particularly desirable to compensate for lowered levels of appropriate $\alpha\beta$ TCR pairing due to endogenously expressed TCR genes, because overall avidity is determined by a combination of the number of TCR/pMHC interactions as well as the affinity of each interaction. Several groups have used a measure of "dwell-time" between T cells and tetramer complexes in relation to T cell functional avidity, with varying results (52–54). Invoking point mutations in the pMHC-binding region of a vesicular stomatitis virus-specific murine TCR, Kalergis et al. (53) have demonstrated that tetramer dissociation rates are not directly correlated with cellular avidity and that in fact there appears to be an optimal tetramer dissociation rate specific to each TCR/pMHC tetramer combination. TCR mutations that altered this rate above or below a certain level correlated with reduced cellular function, suggesting an optimal level of TCR binding for CTL function. This idea of an "ideal" TCR affinity supports additional recent work demonstrating that HIV-specific CD8$^+$ T cells with a longer TCR:tetramer dwell-time were actually less effective against target cells pulsed with low concentrations of HIV peptide or displaying endogenous HIV Ag, than cells with a shorter TCR:tetramer interaction (52).

Although many high-avidity T cells with high-affinity TCR exist against foreign Ags, thymic selection of CD8$^+$ T cells ensures that no extremely high-affinity T cells against self-Ags are released into the periphery (55). This poses a problem for the development of immunotherapy approaches to treat cancer since many tumor Ags are derived from self proteins (3). The substantial clinical response rate in ACT-based tumor immunotherapy demonstrates human

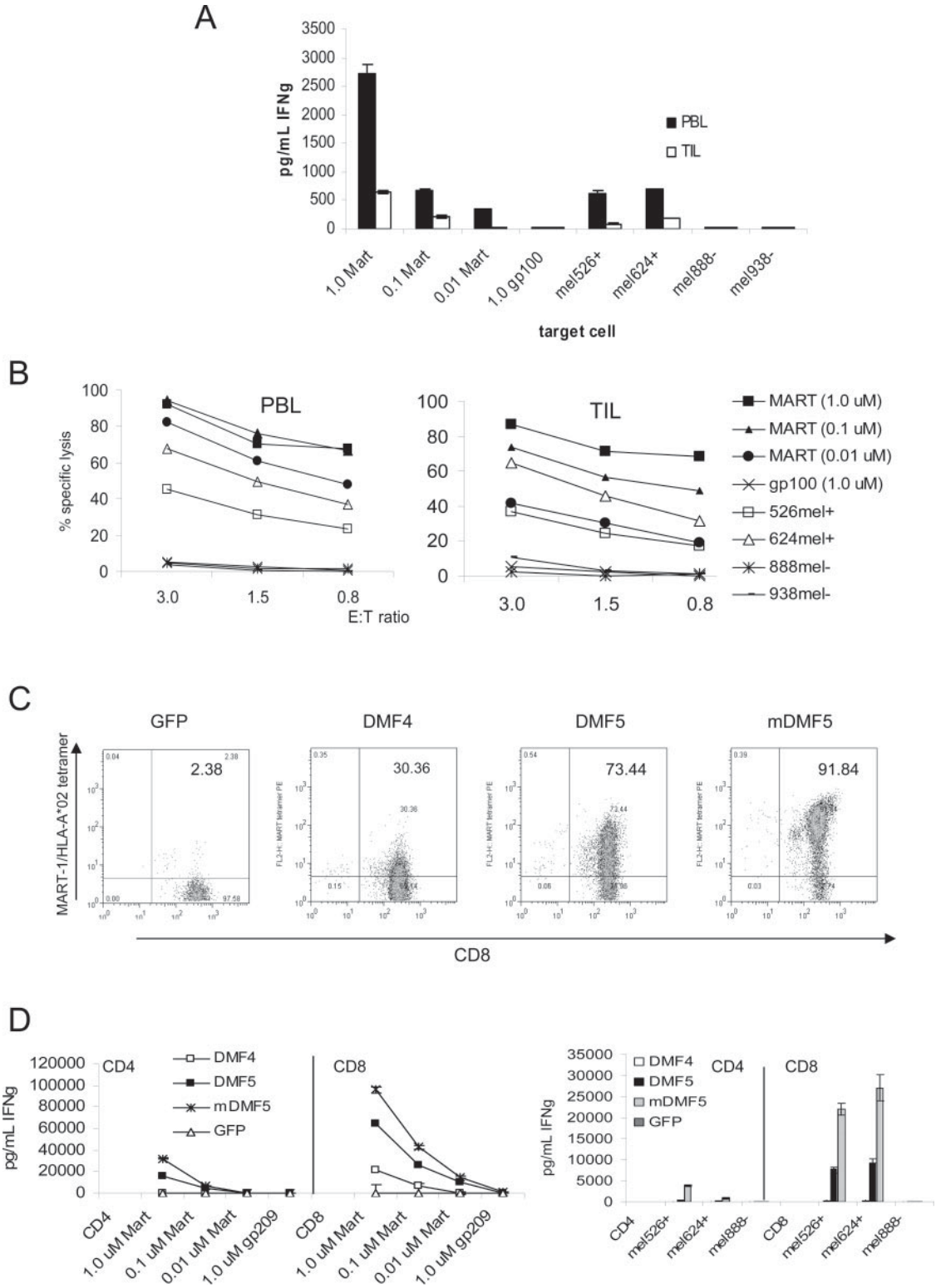

FIGURE 6. Nonreactive TIL can also be rendered Ag reactive by TCR transfer of the high-avidity TCR DMF5 in vitro, and this TCR can be altered to generate even higher cellular avidity. *A* and *B*, Non-tumor-reactive PBL and TIL from the same patient were stimulated using OKT-3 and irradiated feeder cells in the presence of high-dose IL-2 for 3 days before $CD8^+$ T cell enrichment using magnetic beads. These $CD8^+$ T cells were then electroporated with 2 μg/1 $\times$ 10^6 cells TCR RNA from high-avidity DMF5, and cocultured with MART-1 peptide-pulsed T2 target cells or MART-1/HLA-A*0201-expressing melanoma tumors ($mel526^+$, $mel624^+$) or non-HLA-A*0201-expressing tumors ($mel888^-$, $mel938^-$). *A*, IFN-γ production was measured in supernatants by ELISA. Values shown are the average of duplicate samples, $\pm$SEM. *B*, TIL and PBL electroporated with DMF5 TCR RNA as in *A* above, were cocultured against MART-1 peptide pulsed T2 cells or tumors in a 4-h ^{51}Cr release assay. Results are representative of four patient samples tested. *C* and *D*, The DMF5 TCR was altered by replacing the constant region with murine TCR constant regions (mDMF5) and compared with the naturally occurring DMF5 and DMF4 TCRs by RNA electroporation in activated $CD4^+$ or $CD8^+$ PBMC. *C*, CD8 and MART-1/HLA-A*0201 tetramer staining of OKT-3-stimulated, TCR RNA-electroporated $CD8^+$ PBMC. Percentage of live lymphocytes staining double positive is indicated. *D*, IFN-γ production by 18-h coculture of $CD4^+$ and $CD8^+$ TCR RNA electroporated PBMC with MART-1 peptide pulsed T2 targets (*left*) or tumor target cells (*right*). Values shown are the average of duplicate samples, $\pm$SEM.

TIL do contain TCR of sufficiently high avidity to eliminate tumors in vivo. In contrast to T cells responding to infection by foreign pathogens, individuals with cancer are unlikely to have naturally occurring antitumor T cells displaying TCR of such high affinity to be functionally detrimental; rather the problem lies in isolating T cells of high enough avidity to induce tumor regression. We have shown here that once isolated, the TCR from these high-avidity T cells can confer not only Ag specificity, but also functional avidity to donor cells.

Of 24 MART-1-specific CTL clones obtained from 5 different patients' TIL, a diverse variety of cellular avidities was observed, with substantial restriction of the TCR α-chain to variable region 12-2. This Vα TCR restriction was independent of cellular avidity, with both high- and low-avidity CTL displaying Vα 12-2. One particular MART-1-reactive TCR, DMF5, derived from a TIL infusion with demonstrated ability to cause in vivo tumor regression (10), repeatedly exhibited the highest activity against MART-1-expressing tumors in vitro, whether expressed by the native TIL clone, or by gene transfer into donor PBMC. It was possible to improve upon the already high avidity provided by the DMF5 TCR by exchanging the TCR α- and β-chain constant (C) regions with sequences derived from murine TCR C-regions, resulting in increased TCR surface pairing and functional avidity over the naturally occurring TCR sequence. Although RNA electroporation and gene expression efficiency in PBMC was consistently >95% by GFP controls (data not shown), tetramer staining indicated lower levels of surface TCR expression compared with the original clones. For use in gene therapy, the TCR genes would be incorporated into a retroviral expression system and used to transduce PBMC ex vivo, before reinfusion. The prolonged endogenous expression induced by retroviral transduction would also be likely to increase TCR gene expression levels.

The highest-avidity TCR we identified, CD8-independent DMF5, holds promise as a candidate for allogeneic TCR gene therapy in metastatic melanoma patients. This TCR was sufficient to transform nonreactive donor $CD8^+$ and $CD4^+$ PBMC as well as TIL to recognize MART-1-expressing tumors, produce high levels of multiple immunologically relevant cytokine, and lyse tumor cells in vitro. The only way to determine whether high-avidity TCR have the same effect on human tumors in vivo as they do in mouse models is to test this TCR in a clinical trial, with the goal of inducing tumor regression in patients, and such a clinical trial is being planned.

The patient TIL infusion from which DMF5 was derived resulted in clinical tumor regression, and was comprised primarily of two $CD8^+$ MART-1-reactive T cell clones, DMF4 and DMF5. DMF4 was found to persist in the patient's peripheral blood at very high levels at more than 1 year after treatment (10). DMF5 was also found in the blood following treatment, but at much reduced levels compared with DMF4. It has recently been shown that the telomere length of TIL is strongly correlated with its ability to persist long-term in vivo following adoptive transfer (9, 56). Further examination of the treatment cells originally administered to the patient revealed that DMF4 cells had long telomeres (8.91 kb) compared to DMF5 with shorter telomeres (4.61 kb), indicative of approaching cell senescence (9). Therefore, although we have shown that the DMF5 TIL had a higher-affinity TCR than DMF4, the actual DMF5 infusion cells may have undergone rapid proliferative exhaustion in response to antigenic tumor stimulation and therefore were unable to persist long-term in vivo. It remains unknown which of these cells (if either) were responsible for the patient's tumor regression.

Identification of the high avidity TCR DMF5, along with the determination that TCR transfer is sufficient to confer cellular avidity as well as Ag recognition specificity, holds the potential to overcome problems presented by conventional ACT therapy. TCR gene immunotherapy has the potential for use in many types of cancer besides melanoma. Indeed, TCR recognizing the cancer testis Ag NY-ESO1, and p53, expressed in many types of tumors, including melanoma, breast cancer, lung cancer, prostate cancer, and sarcomas, have recently been isolated (11, 12, 49, 57). Since the Ags recognized by antitumor TCR are often comprised of self-peptides, selecting high-avidity TCR may contribute to the induction of autoimmunity; vitiligo and uveitis have been observed in some patients in prior cell transfer trials (4). The use of highly reactive, high-affinity tumor-Ag specific TCR to transduce T cells from cancer patients with poorly reactive or unavailable TIL may have the potential to recognize and eliminate tumors in vivo. The use of autologous cells minimizes any risk of graft vs host disease or cell rejection. Retroviruses encoding antitumor TCR offer an off-the-shelf reagent for use in the immunotherapy of patients with cancer.

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Disclosures

The authors have no financial conflict of interest.

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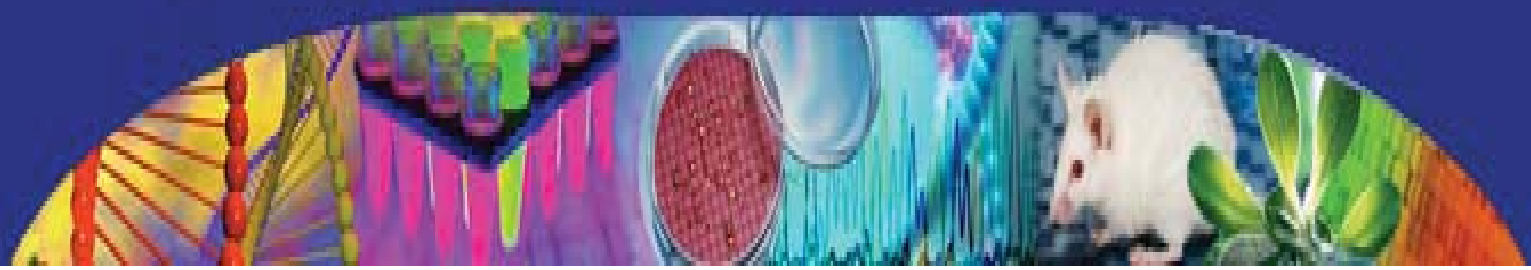
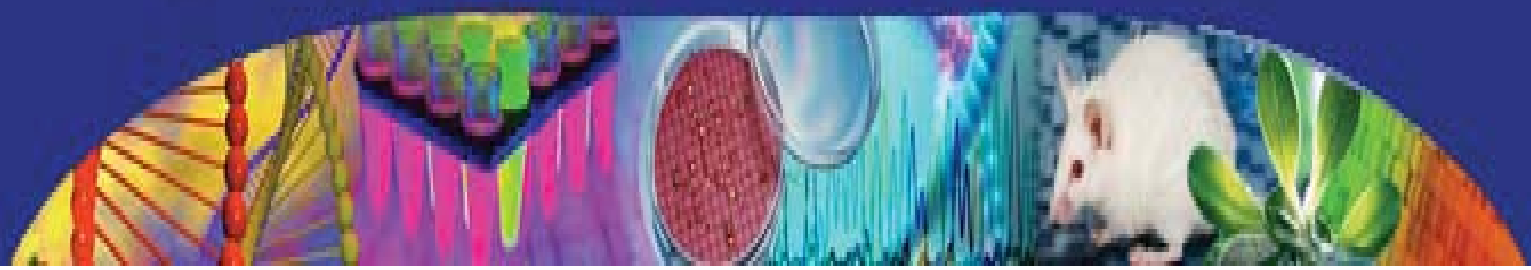


ESO-1
Protocols

Antigen Target	**ESO-1**
Antigen Expression	**NY-ESO-1 protein has been observed in approximately one third of melanoma, breast, prostate, lung, ovarian, thyroid and bladder cancer, but is limited in normal tissues to germ cells and trophoblasts.**

	Gene Engineered T cell Receptors Trial Registered with OBA
Title	**Protocol # 886: Phase II Study of Metastatic Cancer that Expresses NY-ESO-1 Using Lymphodepleting Conditioning Followed by Infusion of Anti-NY-ESO-1 TCR-Gene Engineered Lymphocytes**
PI	**Steven A. Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/NY-ESO-1 alpha and beta chains**
Status	**Active**
Doses Proposed	**$1x10^7$ – $1x10^{11}$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m^2**
Subject Enrolled	**22**
Summary of Unexpected and Related Events	**Adverse events observed have mostly been those expected for the nonmyeloablative chemotherapy regimen and high dose aldesleukin administration.**
Summary of Results	**Four subjects with synovial sarcoma were treated and 3 of the 4 had a partial response but recurred at 10, 6 and 4 mos. An additional subject treated by compassionate exemption had objective response but recurred by 3 months. Eight evaluable metastatic melanoma subjects showed one complete response ongoing at 10 months and 3 with a partial response with one ongoing at 8 months and 3 recurrences at 8 and 3 months. Two breast cancer subjects were treated (one on compassionate exemption) and one had PR but recurred at 2 months. Thus, of the 15 subjects treated, there was a 60% response rate but 7 of the 9 responders recurred within 2-10 months. The protocol is being amended to add the ALVAC encoded TRICOM vaccine and to allow subjects who do not response to unselected anti ESO-1 TCR transduced cells and vaccine to be eligible for retreatment with CD8+ enriched anti ESO-1 TCR transduced cells and ALVAC vaccine.**

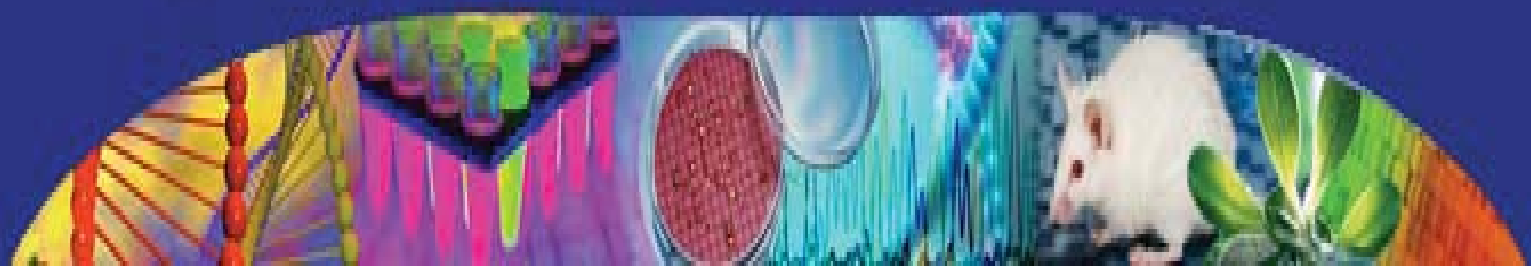


p53 Protocols

Antigen Target	**P53**
Antigen Expression	**Over expressed in more than 50% of all tumors. P53 is also present in most lymphoid and human epithelial cells in a cytoplasmic-perinuclear localization pattern.**

	Engineered T Cell Trials Registered with OBA
Title	**Protocol # 759: Phase II Study of Metastatic Cancer That Overexpresses p53 Using Lymphodepleting Conditioning Followed by Infusion of Anti-p53 TCR-Gene Engineered Lymphocytes**
PI	**Steven A. Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/TCR alpha and beta**
Status	**Completed**
Doses Proposed	**At least $5x10^8$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m^2**
Subjects Enrolled	**12**
Summary of Unexpected and Related Events	**Adverse events were those expected for the nonmyeloablative chemotherapy regimen and high dose aldesleukin administration.**
Summary of Results	**12 patients enrolled in the protocol (2 in arm 1 metastatic melanoma and ten in arm 2 (other histologies)). Eleven subjects were treated and one patient sustained a partial response for 6 months. Of the 10 patients whose PBLs were tested for persistence at one month, the mean CD4+ and CD8+ T cell persistence were 2.6 and 2.5%, respectively. The range of specific CD4+ persistence values was from 0% to 9.92%. For CD8+ T cells, this range was from 0.05 to 4.72%. Only one patient has been tested beyond 2 months, and at 79 days post cell transfer 16.52% and 0.52% were transduced CD4+ and CD8+ T cells respectively. The evidence that transduced cells persist for longer periods of time comes from one subject's retreatment evaluation. Cells were collected approximately 7 months after first cell product administration and were analyzed by FACS. On day 4 after standard OKT3 stimulation, upon CD3+ cell gating, 20.64% of the cells were CD4+ transduced TCR positive and 1.46% of the cells were CD8+ transduced TCR positive.**

Title	**Protocol # 876: Phase II Study of Metastatic Cancer that Overexpresses p53 Using Lymphodepleting Conditioning Followed by Infusion of Anti-P53 TCR-Gene Engineered Lymphocytes and Dendritic Cell Vaccination**
PI	**Steven A. Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/TCR alpha and beta**
Status	**Closed**
Doses Proposed	**$1x10^8$ – $5x10^{10}$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m2**
Subjects Enrolled	**3 (2 with metastatic melanoma and 1 with breast cancer)**
Summary of Unexpected and Related Events	**Adverse events observed were those expected for the nonmyeloablative chemotherapy regimen and high dose aldesleukin administration.**
Summary of Results	**No clinical responses were observed.**



CEA Protocols

Antigen Target	**CEA**
Antigen Expression	**Expressed on normal epithelium and upregulated in malignant epithelium.**

	Engineered T Cell Trials Registered with OBA
Title	**Protocol # 925: Phase I/II Study of Metastatic Cancer that Expresses Carcinoembryonic Antigen (CEA) Using Lymphodepleting Conditioning Followed by Infusion of Anti-CEA TCR-Gene Engineered Lymphocytes**
PI	**Steven Rosenberg, M.D., Ph.D.**
Vector	**Retrovirus/TCR alpha and beta chains**
Status	**Not Currently Enrolling**
Doses Proposed	**$1x10^8$ – $5x10^{10}$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m^2**
Subjects Enrolled	**3**
Summary of Unexpected and Related Events	**There were three subjects who experienced events of colitis which occurred within one week of cell infusion. Of these, one subject experienced a dose limiting toxicity with fever, tachycardia, low oxygen saturation and diarrhea.**
Summary of Results	**The subject that experienced a dose limiting toxicity received a dose of $2x10^8$ cells as a protocol deviation as the lowest dose originally specified was $1x10^{10}$ cells. Following these events, the dosing in the protocol was modified. The lowest dose that will be administered now is $1x10^8$ and the highest will be $5x10^{10}$. In addition, based on studies that demonstrate reduced toxicity with CD8-enhanced cells compared with PBL containing $CD8^+$ and $CD4^+$ cells, the infusions will now be $CD8^+$ enhanced.**

	Chimeric Antigen Receptors Trials Registered with OBA
Title	**Protocol # 249: Phase I Study of T Cells Modified with Chimeric AntiCEA Immunoglobulin-T Cell Receptors (IgTCR) in Adenocarcinoma**
PI	**Richard P. Junghans, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/CD3 zeta**
Status	**Closed**
Doses Proposed	**$1x10^9$ – $1x10^{11}$ cells**
Lymphodepletion	**None**
Subjects Enrolled	**7**
Summary of Unexpected and Related Events	**One subject with coronary artery disease s/p CABG had a heart attack shortly after the 5^{th} infusion of cells. The subject had no symptoms during infusion and autopsy did not reveal a T cell infiltrate.** **One subject experienced Grade 2 fever and rigors after infusion and then Grade 4 supraventricular tachycardia (baseline EKG with Right Bundle Branch Block). The subject had a history of atrial fibrillation. The subject also developed grade II diarrhea, non-bloody. No T cell infiltration was found in the biopsy.**
Summary of Results	**Pharmacokinetic studies were conducted by measuring the number of IgTCR+ T cells in patient blood samples. Only a small fraction of the total number of cells infused could be detected in blood samples taken immediately after the infusions (l-10% of activated T cells recovered from blood). This was true for all but one dose that was administered. In some cases, the number of detectable T cells was increased slightly by 24 hours after the infusion, but then declined to undetectable levels by day 2-3. These results demonstrate that the majority of infused cells are rapidly absorbed into peripheral tissues, most likely in the lungs, liver, and spleen.**

	Junghans, R. P., *et. al.* (2000). Preclinical and Phase I Data of Anti-CEA Designer T Cell Therapy for Cancer: A New Immunotherapeutic Modality (Abstract) *Proceedings of the American Cancer Association for Cancer Research* 41: 543.
Title	**Protocol # 564: Phase Ia/Ib Trial of 2nd Generation Anti-CEA Designer T cells in Gastric Cancer**
PI	**Richard P. Junghans, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Active**
Doses Proposed	**$1x10^9$ – $1x10^{10}$ cells**
Lymphodepletion	**Yes**
Subjects Enrolled	**6**
Summary of Unexpected and Related Events	**None**
Summary of Results	**One subject with a minor reduction in the size of brain and lung metastases and one subject with stable disease for greater than one year.**
Title	**UK Study: Anti-CEA T cells in Colorectal Cancer**
PI	**Robert E. Hawkins, Ph.D., FRCP**
Vector	**Retrovirus/CD 3 zeta**
Status	**Not currently enrolling**

Doses Proposed	**$1x10^8$ – $5x10^{10}$ cells**
Lymphodepletion	**Yes**
Subjects Enrolled	**4**
Summary of Unexpected and Related Events	**Toxicities that were experienced were related to IFNgamma or IL-2.**
Summary of Results	**Tumor biopsies post infusion demonstrated engineered T cells with selective accumulation.**

sion of p53 4-fold in both non-EAF and EAF hepatocytes, whereas, as expected, DEN treatment induced p53 accumulation in non-EAF hepatocytes only. Incorporation of thymidin was inhibited in both non-EAF and EAF hepatocytes by $CoCl_2$-treatment, indicating that the p53 response was functional. When DEN and $CoCl_2$ was added to the same culture no additivity in the p53 response was observed in EAF hepatocytes, whereas PSI (an inhibitor of p53 degradation) and $CoCl_2$ were additive. Nanomolar concentrations of wortmannin, an inhibitor of the p53-phosphorylating kinases DNA-PK and ATM, inhibited the DEN-induced p53 response in non-EAF hepatocytes. In contrast, wortmannin did not inhibit the $CoCl_2$-induced response. Similar results were obtained with another kinase inhibitor, i.e., caffeine. These data suggest that in EAF hepatocytes the p53 response to DNA damage is altered, whereas the respone to hypoxia remains unchanged. This selective attenuation of the p53 response to DNA damage may confer growth advantages to EAF hepatocytes when exposed to genotoxic carcinogens, but it may also increase the risk for cancer development.

CLINICAL RESEARCH 20: Clinical Evaluation of Novel Therapies

#3459 GENE THERAPY WITH INTRATUMOR (IT) INJECTION OF AN ADENOVIRUS EXPRESSING THE IL2 GENE (RAD-IL2) IN LUNG CANCER: RESULTS OF A PHASE I STUDY. Bernard Escudier, T Le Chevalier, E Angevin, F Griscelli, P Opolon, F Mami-Chouaib, B Acres, P Squiban, and T Tursz, *I G Roussy, Villejuif, France, and Transgene, Strasbourg, France*

Once a single IT injection of rAd-bGAL was proven safe, we performed a phase I study using a rAd-IL2. Nine patients (pts) with inoperable NSCLC received a single IT injection of rAd-IL2 (10^7, 10^8 and 10^9 pfu, 3pts at each dose) without concomitant- therapy. Virus shedding (293 cell culture and rIL2PCR) was verified in body fluids after injection. Control biopsies were performed at days 7,14 and 28 to detect the presence of rAd-IL2, and to measure both native and recombinant IL-2 mRNA expression using a highly sensitive quantitative RT-PCR method. Anti-adenovirus neutralizing antibodies (nAd-Abs), T-cell antiadenoviral immune responses and cytokine levels were measured at days 0,7,14 and 28. Results: Tolerance of rAd-IL2 was excellent. Virus was detected by culture, only in pts who received 10^9 pfu (blood 2/3, feces 3/3). Vector-derived sequences were also detected by PCR in blood, feces (up to day 4) and sputum. In tumor biopsies, rAd-IL2 was still detected at day 28, with a dose-effect relationship. A rIL2 mRNA expression was detected in 2/3 pts at 10^9 pfu. Significant pre-existing nAd-Ab titers were detected in 5/9 pts, and all but one pt developed high titers at day 14. Strong proliferative and cytotoxic antiadenoviral responses were observed at days 14 and 28 in pts with low nAd-Ab titers at baseline. Transient tumor necrosis was observed at day 14 in 6/9 pts at histological analysis. No antitumor effect was observed. Conclusions: A single IT injection of rAd-IL2 is safe and induces prolonged local expression of IL2. Immune responses appear to be lower in pts with pre-existing nAbs. Further studies are necessary to better determine if such pts should be excluded from gene therapy trials.

#3460 GENE THERAPY OF HUMAN GLIOMAS WITH TGF-β2 ANTISENSE GENE MODIFIED AUTOLOGOUS TUMOR CELLS. A PHASE I CLINICAL TRIAL. H Fakhrai, J Mantil, S Gramatikova, G Nicholson, C Murphy-Satter, G Kraus, R Poelstra, J Ruppert, P Sequeira, M Satter, and C Kruse, *Advanced Biotherapies, San Diego, CA, Univ of Colorado Health Sci Ctr, Denver, CO, and Wallace-Kettering Neuroscience Institute, Kettering, OH*

Transforming Growth Factor-β2 (TGF-β2) levels are usually elevated in glioma patients. One action of TGF-β2 is to suppress induction of the T cell response and thus it may be responsible for the immunosuppression exhibited by these patients. In animal tumor models we have shown that multiple subcutaneous injections of intracranial tumor-bearing animals with TGF-β antisense gene modified tumor cells generates systemic immunity that eradicates previously implanted tumors. In a Phase I clinical trial we have used TGF-β antisense gene therapy in patients with either recurrent glioblastoma multiforme or gliosarcoma. Gene modification resulted in a 55-98% reduction in TGF-β2 secretion by the vaccines. Five patients, inoculated 3–7 times at 3–4 week intervals with either 5×10^6 or 1×10^7 autologous TGF-β2 antisense gene modified tumor cells did not have greatly exaggerated erythematous reactions at the injection site. At either dose, skin biopsies of the patients indicated an endogenous immune response in the form of increased immune infiltrates at the injection site (both CD4+ and CD8+ cells) after the second injection. Tumor biopsy and autopsy data subsequent to treatment showed necrosis of the tumor and prominent lymphocytic infiltrates in the tumor bed. MRIs suggested tumor progression in two patients, a transient response in one, and tumor necrosis and regression in two patients.

#3461 PRECLINICAL AND PHASE I DATA OF ANTI-CEA DESIGNER T CELL THERAPY FOR CANCER: A NEW IMMUNOTHERAPEUTIC MODALITY. Richard P Junghans, Mazin Safar, Mark S Huberman, Qiangzhong Ma, Robin Ripley, Shi-On Leung, and Edward J Beecham, *BIDMC, Boston, MA, Harvard Med School/Beth Israel Deaconess Med Ctr, Boston, MA, Hms/Bidmc, Boston, MA, and Immunomedics, Inc, Morris Plains, NJ*

Carcinoembryonic antigen (CEA) is expressed as a tumor-associated antigen in cancers of the colon and rectum, breast, lung and others, with CEA+ malignancies accounting for 150,000 deaths each year in the U.S. alone. Patients T cells are transduced with chimeric immunoglobulin-T cell receptor (IgTCR) genes to create new designer lineages of immune effector cells. These designer T cells bind specifically to CEA+ tumor cells, with activation of IL2 secretion and efficient tumor cell lysis and T cell recycling over a period of at least several days, and are not inhibited by high levels of soluble CEA. Therapy consists of collecting patient lymphocytes by leukopheresis, modifying the T cells by retroviral-mediated insertion of the chimeric IgTCR genes, and expanding the transduced T cells which are now specific for the CEA tumor antigen. These cells are then reinfused back into the patients. The phase I study includes a dose escalation to span the range of 1×10^9 to 1×10^{11} T cells on two study arms, without and with co-administration of continuous infusion IL2. At the present study mid-point, seven patients have received a total of 24 doses of anti-CEA designer T cells. Toxicities have been modest and positive responses obtained.

#3462 ARSENIC TRIOXIDE (AS_2O_3): A DOSE-RANGING AND CLINICAL PHARMACOLOGIC STUDY IN PATIENTS WITH ADVANCED HEMATOLOGIC CANCERS. Steven L Soignet, Steven Novick, Bryan Bienvenu, Suzanne Chanel, Raymond Ho, David Spriggs, Ralph Ellison, and Raymond P Warrell Jr., *Memorial Sloan-Kettering Cancer Ctr, New York, NY, and PolaRx Biopharmaceuticals, Inc, New York, NY*

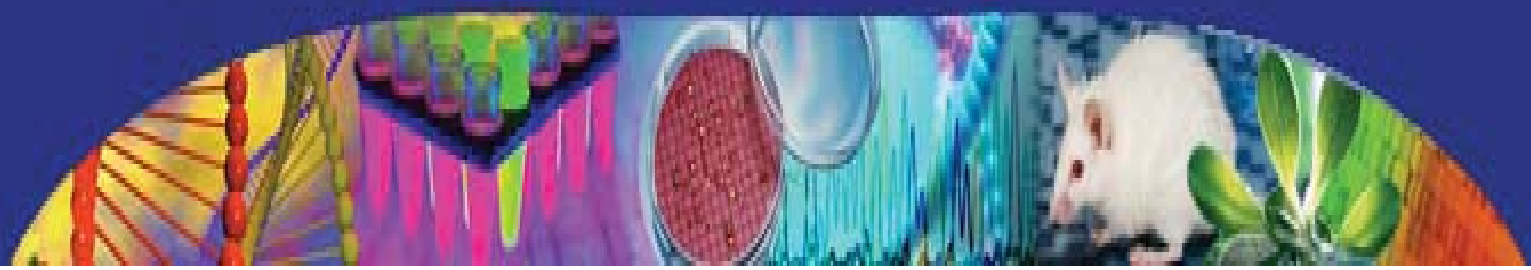
As_2O_3 has been shown to be safe and effective treatment for acute promyelocytic leukemia (APL) and the drug also inhibits growth of other hematologic cancer cell types *in vitro*. We initiated the first dose-ranging study of this drug in patients with advanced hematologic cancers. Pts received 1–2 hour infusions of As_2O_3 for a cumulative total of 25 days, repeated every 3–5 weeks. Cohorts of 3–6 pts were treated at 0.05 increments of doses ranging from 0.1 to 0.30 mg/kg/d. To date, 23 pts with acute and chronic leukemias, Hodgkin's disease, non-Hodgkin's lymphoma (NHL), myeloma, or MDS have been acrued. One response was observed in a pt with NHL, and 1 pt with multiple myeloma had a > 30 % reduction in serum IgG level after 1 cycle. One pt with MDS had stable disease for 3 cycles. Two of the 3 APL pts who had relapsed from lower As_2O_3 doses showed rapid progression of disease; the other pt achieved a partial response after 60 days of therapy. PK analysis for elemental arsenic concentration revealed a distribution in both plasma and RBC compartments of whole blood. Parallel elimination curves suggested that these 2 compartments were freely exchangeable. Mean plasma C_{max} and $AUC_{0-24\ hr}$ values appear to be dose-proportional. Plasma levels decayed from peak value with an initial $T_{½}$ of>4 hrs and a terminal half-life of <tilde minus> 115 (± 28) hours. Adverse effects include skin rash, fluid retention, fatigue, diarrhea, mild nausea, neuropathy and QTc prolongation on EKG. Based on results to date, we expect the MTD on this schedule will be <tilde minus> 0.30 mg/kg/d; daily doses of 0.25 to 0.30 mg/kg can be recommended for disease oriented studies. Given the preliminary responses, myeloma and lymphoma should represent priorities for extended evaluation.

#3463 AUTOLOGOUS TUMOR-DERIVED HEAT-SHOCK PROTEIN PEPTIDE COMPLEX-96 (HSPPC-96) IN PATIENTS (PTS) WITH METASTATIC MELANOMA. Omar Eton, Mary Jo East, Merrick I Ross, Cherylyn A Savary, Stephen P Tomasovic, Dirk Reitsma, Elma S Hawkins, and Pramod K Srivastava, *Antigenics, LLC, New York, NY, Univ of Connecticut, Farmington, CT, Univ of Texas, MD Anderson Cancer Ctr, Houston, TX,*

Gp96, a non-polymorphic heat shock-protein, associates with intracellular peptides. Autologous tumor (AT) derived HSPPC-96 can elicit potent tumor-specific T cell responses and protective immunity in animal models. This Phase I trial sought to evaluate the safety of HSPPC-96 given ID @ 2.5, 25 or 100 μg/dose weekly x 4 and to select a dose based on induction of anti-tumor immunity and clinical activity. Since 1/98, 36 pts were enrolled after routine resection of a > 3 cm melanoma metastasis in the Stage IV (26 pts) or advanced Stage III (10 pts) setting. 16 pts had indicator lesions. 30 pts had prior systemic therapy. HSPPC-96 was prepared from specimens as small as 2 gm. There were no serious toxicities. Lack of DTH response to DNCB in 11 pts (31%) correlated with presence of indicator lesions ($p<0.01$) but not with clinical stage. There was no DTH reactivity through week 8 to either HSPPC-96 or 10^5 irradiated AT cells. One pt at each dose level had stabilization or mixed response after initial progression in nodal or lung metastases, all lasting 7+ mo. Eleven of 12 Stage IV adjuvant pts (8 Stage IVM1B)remain free of disease a median of 11+ mo. With 9 mo median f/u, 19 pts remain on study with 29 (80%) alive. Ongoing *in vitro* studies to confirm induction of anti-tumor immunity will be required to determine the dose range for future clinical trials with HSPPC-96.

#3464 NEW BIOTHERAPEUTIC AGENTS EFFECTIVE IN CHEMOTHERAPY - REFRACTORY HAIRY CELL LEUKEMIA: RECOMBINANT IMMUNOTOXINS TARGETING CD25 OR CD22. Robert J Kreitman, W H Wilson, I Margulies, M Stetler-Stevenson, M Raggio, D J FitzGerald, T A Waldmann, and I Pastan, *National Cancer Inst, Bethesda, MD*

An increasing number of patients with hairy cell leukemia (HCL) are relapsing after purine analog therapy with chemotherapy-refractory disease. HCL cells are CD25+ in 80% of cases and CD22+ in ~100%, and the $CD25^-/CD22^+$ phenotype carries a poor prognosis. Eleven patients with purine analog-refractory HCL

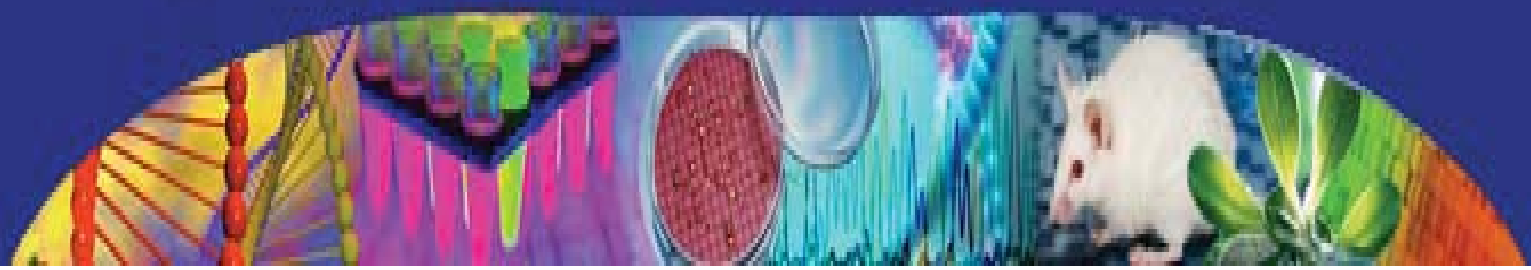


PSMA Protocols

Antigen Target	**PSMA**
Antigen Expression	**In prostate cancer cells, PSMA is expressed at 1000-fold higher levels compared with normal prostate epithelium. Low levels expressed in the brain, duodenal epithelium and proximal renal tubular cells.**

	Chimeric Antigen Receptors Trials Registered with OBA
Title	**Protocol # 681: Phase Ia/Ib Trial of Anti-PSMA Designer T Cells in Advanced Prostate Cancer after Non-Myeloablative Conditioning**
PI	**Richard P. Junghans, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/CD3 zeta**
Status	**Active**
Doses Proposed	**$1x10^9$ – $1x10^{11}$ cells**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m^2**
Subjects Enrolled	**5**
Summary of Unexpected and Related Events	**None**
Summary of Results	**Two subjects had PSA reductions (50% and 70%) and improved bone scans. There were low levels of IL2 in non-responders and high levels in the 2 responders.**
Title	**Protocol # 965: Adoptive Transfer Of Autologous T Cells Targeted To Prostate Specific Membrane Antigen (PSMA) For The Treatment Of Castrate Metastatic Prostate Cancer (CPMC).**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**

PI	**Susan Slovin, M.D., Ph.D.**
Status	**No information**
Doses Proposed	**$1x10^7$ – $1x10^8$ cells/kg**
Lymphodepletion	**Cyclophosphamide 300 mg/m^2**
Subjects Enrolled	**No information**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**



Carbonic Anhydrase IX Protocols

Antigen Target	**Carbonic Anhydrase IX**
Antigen Expression	**Over expressed in renal cell carcinoma and bile duct epithelial cells.**

	Chimeric Antigen Receptor Trial
Title	**Treatment of Metastatic Renal Cell Carcinoma with Autologous T Lymphocytes Genetically Retargeted Against Carbonic Anhydrase IX (CAIX)**
PI	**Egbert Osterwijk, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD3 zeta**
Status	**Ongoing**
Doses Proposed	**$2x10^7$ – $2x10^9$ cells**
Lymphodepletion	**No**
Subjects Enrolled	**3**
Summary of Unexpected and Related Events	**Liver toxicity (grade 2-4) in the first three subjects most likely due to the reactivity of transduced T cells against the target antigen expressed on bile duct epithelium. Liver biopsy was significant for T cell infiltrate around the bile duct.**
Summary of Results	**The protocol has been amended to administer antibody to cG250, the epitope that recognized CAIX, three days before infusion. The rationale is that administration of cG250 has been shown to be clinically safe and well tolerated in more than 200 subjects. Infusion should protect bile duct epithelium from scFv (G250).** **Oosterwijk, E., *et. al*. (2006). Treatment of Metastatic Renal Cell Carcinoma with Autologous T-Lymphocytes Genetically Retargeted Against Carbonic Anhydrase IX: First Clinical Experience. *Journal of Clinical Oncology* 24: 20-22**

Treatment of Metastatic Renal Cell Carcinoma With Autologous T-Lymphocytes Genetically Retargeted Against Carbonic Anhydrase IX: First Clinical Experience

TO THE EDITOR: Adoptive transfer of autologous T-lymphocytes that are gene transduced to express antigen-specific receptors represents an experimental therapy to provide tumor-specific immunity to cancer patients. We studied safety and the proof of this concept in patients with metastatic renal cell carcinoma (RCC), and have encountered toxicity that is likely to be antigen specific.

We have constructed a single-chain antibody-type (scFv) –receptor based on murine monoclonal antibody (mAb) G250.[1] This mAb recognizes an epitope on carboxy-anhydrase-IX (CAIX), which is frequently overexpressed on clear cell RCC. Following retroviral introduction of the *scFv(G250)* transgene into primary human T-cells, the scFv(G250) receptor is expressed on the surface of these cells, which enables them to recognize CAIX and to exert antigen-specific effector functions, such as cytokine production after exposure to CAIX and the killing of $CAIX^+$ RCC cell lines.[2,3]

We treated patients with scFv(G250)-transduced T-cells in an inpatient dose-escalation scheme of intravenous (IV) doses of 2×10^7 cells at day 1; 2×10^8 cells at day 2; 2×10^9 cells at days 3 through 5 (treatment cycle 1); and 2×10^9 cells at days 17 to 19 (treatment cycle 2), in combination with human recombinant interleukin-2 (IL-2; Chiron Corporation, Amsterdam, the Netherlands), subcutaneously, 5×10^5 U/m^2 twice daily administered at days 1 to 10 and days 17 to 26. This protocol was approved by the governmental regulatory authorities and the institutional medical ethical review board. Adaptations to this protocol were implemented only after approval by these boards. Written informed consent was obtained from all patients.

In this letter, we report on the clinical experiences of the first three patients. The patients had $CAIX^+$ metastatic clear cell RCC, had undergone tumor nephrectomy, and had progressive disease after 6 to 17 months of interferon alfa (IFN-α) treatment. From all, we successfully generated functional $scFv(G250)^+$ T-cells ex vivo (Table 1). Infusions of these gene-modified T-cells were initially well-tolerated. However, after four to five infusions, liver enzyme disturbances reaching National Cancer Institute Common Toxicity Criteria grades 2 to 4 developed. These toxicities necessitated the cessation of treatment in patient 1 and patient 3, corticosteroid treatment in patient 1, and reduction of the maximal T-cell dose to 2×10^8 T-cells in patient 2 and patient 3. After treatment, patients showed progressive disease between 36 and 106 days. In order to elucidate the underlying mechanisms accounting for the liver toxicity, a liver biopsy was performed on patient 1, showing a discrete cholangitis with T-cell infiltration around the bile ducts, and CAIX expression on the bile duct epithelial cells. Although technical limitations prohibited direct identification of $scFv(G250)^+$ T-cells in these sections, these findings strongly suggest that the liver toxicity is caused by a specific attack of the $scFv(G250)^+$ T-cells against the $CAIX^+$ bile duct epithelial cells.

We transiently detected both $scFv(G250)^+$ T-cells and scFv(G250) DNA copies in the circulation of all three patients from day 3 of treatment onward, using flow cytometry and quantitative real-time polymerase chain reaction (PCR). The time period during which the transduced cells were detected in the circulation depended on the method used, that is, up to 32 days by flow cytometry and up to 53 days by PCR[4] (Table 1).

Before treatment, peripheral blood mononuclear cells did not show scFv(G250)-mediated functions, that is, specific cytolysis of $CAIX^+$ target cells and production of IFN-γ on stimulation with such cells. After infusions of scFv(G250)-transduced T cells, these activities became detectable in all three patients (Table 1).

All three patients developed low levels of anti-scFv(G250) antibodies between 37 and 100 days after the start of T-cell therapy, which were directed against the G250 idiotype (id). Remarkably, these responses were less frequent in RCC patients treated with weekly IV infusions of 50 mg chimeric G250 mAb (ie, in 6% to 30% of patients),[5,6] indicating that the expression of scFv(G250) on the cell membrane of T-cells elicits a relatively efficient immune response against the murine G250-id. Such response may hamper the effective clinical use of murine-human chimeric receptors, and may require construction of receptors from completely human mAbs.

In summary, our data show clear in vivo reactivity of autologous T-cells that have been genetically retargeted using a single-chain antibody-type receptor. The observed liver toxicity is most likely due to the reactivity of transduced T-cells against the target antigen expressed on normal tissue, that is, the epithelial cells lining the bile ducts, and thereby hinders administration of T-cells in numbers that can be expected to yield antitumor activity. We consider our observations, together with those from T-cell therapies directed against self-antigens,[7,8] relevant for other studies involving T-cell retargeting for therapeutic purposes. Ideally, the target antigens for such studies should be carefully chosen, so as to be expressed only by malignant cells and not by normal cells.

Alternatively, strategies need to be developed to attenuate activity of retargeted T-cells against normal tissues expressing target antigen to circumvent the observed adverse events.

In order to prevent liver toxicity in future patients, we have modified our clinical protocol into a conventional phase I study, and have included an infusion of 5 mg cG250 antibody 3 days before the first infusion of gene-modified T-cells. The rationale of this amended protocol is that repeated administration of cG250 has not only been shown to be clinically safe and well-tolerated by more than 200 patients,[9] but more importantly, cG250 localizes to RCC metastasis but not to the liver, after having saturated uptake by liver tissue (but not RCC metastasis) by a single low dose of cG250.[6,10,11] By pretreating patients with a single, low dose of cG250, we aim to protect the bile duct epithelium from the damaging effects exerted by $scFv(G250)^+$

Table 1. Characteristics of Preinfusion scFv(G250)-Transduced T-Cells and of Peripheral Blood Following Immunogene Therapy

Parameter	Patient 1	Patient 2*	Patient 3*
Preinfusion characteristics of scFv(G250)-transduced T-cells			
Cell and DNA copy counts			
No. of infusions	4†	5/3‡	4†
Total No. of T-cells ($\times 10^9$)	3.99	0.8/0.59	0.60
Mean % scFv(G250)$^+$ T-cells	53	52/76	63
Total No. of scFv(G250)$^+$ T-cells ($\times 10^9$)	2.13	0.43/0.42	0.38
Mean No. of scFv(G250) DNA copies per scFv(G250)$^+$ T-cell	2.3	4.5/6.8	2.8
scFv(G250)-mediated functions			
CAIX-specific cytolysis (LU_{20})§			
$LU_{20}/10^6$ scFv(G250)$^+$ T-cells	372	104/82	88
Total LU_{20}	792, 204	78, 774	33, 274
CAIX-specific IFN-γ production, ng per 10^6 scFv(G250)$^+$ T-cells per 24h	33	33/24	28
Characteristics of peripheral blood samples during immunogene therapy			
Circulating scFv(G250)$^+$ T-lymphocytes			
Peak, day	7	10/21	6
Peak level, cells/μl	5.3	2.7/1.6	0.8
Period during which cells detectable‖	3-23	3-32	3-7
Circulating scFv(G250) DNA copies			
Peak, day	tfd	17/19	8
Peak level, cells/μl	tfd	7.1/5.2	5.3
Period during which DNA detectable	tfd	tfd-53	3-32
Human anti-scFv(G250) antibodies			
Day of first appearance	37	100	79
Peak, day	57¶	100¶	79
Peak level, ng/ml	706	190	292
ScFv(G250)-mediated functions in vitro			
CAIX-specific cytolysis			
Peak, day	8	8/22	5
Peak level, $LU_{20}/10^6$ PBMC	16	29/44	26
CAIX-specific IFN-γ production			
Peak, day	8	8/22	5
Peak level, ng/ml per 10^6 PBMC per 24h	9	25/32	37

Abbreviations: scFv, single-chain antibody type; LU, lytic unit; CAIX, carboxy-anhydrase IX; IFN, interferon; h, hour; PBMC, peripheral blood mononuclear cells; tfd, too few data points to allow data assessment.
*T-cell dose reduced to a maximum of 2×10^8 T-cells.
†Treatment cycle 1 only.
‡Treatment cycle 1/treatment cycle 2.
§One LU_{20} is defined as the number of effector T-cells required to lyse 20% of 2,500 CAIX target cells in a 4 h ^{51}Cr release assay.
‖Day(s) after start of treatment (day 1 = day of first infusion).
¶Last day of observation.

T-cells. The Dutch regulatory authorities have approved this amended protocol and accrual of patients is currently ongoing.

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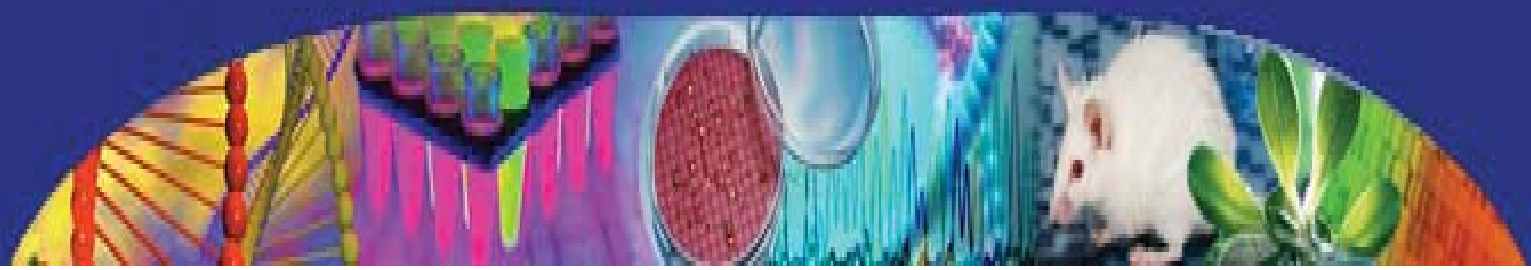
Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.



Lewis Y Protocols

Antigen Target	**Lewis Y- antigen**
Antigen Expression	**Under physiologic conditions, its expression in adults is limited on the surface of granulocytes and epithelium. Elevated expression of Lewis Y has been found in 70-90% of human carcinomas of epithelial cell origin, including breast, ovary, prostate and colon cancers. (Peinert, S., *et. al.* (2010). Gene-Modified T Cells as Immunotherapy for Multiple Myeloma and Acute Myeloid Leukemia Expressing the Lewis Y Antigen. *Gene Therapy*, 17: 678-686)**

	Chimeric Antigen Receptor Trial
Title	**Anti-Lewis-Y T cells for Hematological Malignancies (Acute Myelogenous Leukemia and Myeloma)**
PI	**Michael H. Kershaw, Ph.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Active**
Doses Proposed	**$5x10^8$ – $1x10^9$ cells**
Lymphodepletion	**Yes**
Subjects Enrolled	**2**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

Gene Therapy (2010) 17, 678–686

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SHORT COMMUNICATION

Gene-modified T cells as immunotherapy for multiple myeloma and acute myeloid leukemia expressing the Lewis Y antigen

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We have evaluated the carbohydrate antigen Lewis^Y (Le^Y) as a potential target for T-cell immunotherapy of hematological neoplasias. Analysis of 81 primary bone marrow samples revealed moderate Le^Y expression on plasma cells of myeloma patients and myeloblasts of patients with acute myeloid leukemia (AML) (52 and 46% of cases, respectively). We developed a retroviral vector construct encoding a chimeric T-cell receptor that recognizes the Le^Y antigen in a major histocompatibility complex-independent manner and delivers co-stimulatory signals to achieve T-cell activation. We have shown efficient transduction of peripheral blood-derived T cells with this construct, resulting in antigen-restricted interferon-γ secretion and cell lysis of Le^Y-expressing tumor cells. In vivo *activity of gene-modified T cells was demonstrated in the delayed growth of myeloma xenografts in NOD/SCID mice, which prolonged survival. Therefore, targeting Le^Y-positive malignant cells with T cells expressing a chimeric receptor recognizing Le^Y was effective both* in vitro *and in a myeloma mouse model. Consequently, we plan to use T cells manufactured under Good Manufacturing Practice conditions in a phase I immunotherapy study for patients with Le^Y-positive myeloma or AML.*
Gene Therapy (2010) **17,** 678–686; doi:10.1038/gt.2010.21; published online 4 March 2010

Keywords: *immunotherapy; T cells; multiple myeloma; acute myeloid leukemia*

Introduction

Hematological neoplasias have been shown to be excellent targets for immunotherapy. Both allogeneic stem cell transplantation, and donor lymphocyte infusion, as an anticancer treatment for various hematological malignancies, demonstrate the potential of the immune system.[1–7] However, allogeneic stem cell transplantation is not a suitable treatment option for the majority of patients with hematological malignancies and thus adoptive immunotherapy approaches have been explored in an attempt to harness this immune effect while reducing toxicity.

Recently, a pilot study in patients with indolent B-cell Non-Hodgkin lymphomas reported the use of gene-modified autologous T cells targeting the B-cell antigen CD20. This was the first time that chimeric T cells had been applied in humans for lymphoma treatment, and the trial demonstrated safety and clinical activity.[8] This study confirmed the clinical utility of antigen-specific T-cell therapy that has previously been demonstrated in Hodgkin lymphoma when targeting Epstein–Barr virus - related antigens.[9] Efficacy of T-cell immunotherapy with *ex vivo* expanded tumor infiltrating lymphocytes or gene-modified T cells has also been demonstrated in solid tumors, particularly against melanoma.[10,11] Compared with the process of collecting and processing tumor infiltrating lymphocytes, the technique of using transduced T cells that express a chimeric receptor recognizing a tumor antigen is potentially more easily and widely applicable.

A range of different chimeric receptor constructs have been described. The majority of these constructs consist of a single-chain antibody (scFv) as the extracellular targeting domain. This is linked to a transmembrane region and an intracellular signaling domain, usually the CD3 zeta chain. These constructs allow redirection of T-cell activity to a pre-defined target antigen regardless of the antigen specificity of the 'natural' T-cell receptor and major histocompatibility complex expression of the target cells.[12–14] In this article, we describe the use a retroviral vector encoding a chimeric T-cell receptor binding the carbohydrate antigen Lewis Y (Le^Y). The vector construct has been described before in detail by Westwood *et al*.[15] An important advantage of this particular construct is that its sequence is fully humanized and therefore less immunogenic than constructs

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containing murine elements.[16–18] Furthermore, besides CD3 zeta, it contains a co-stimulatory signaling motif from the CD28 co-receptor, which has previously been demonstrated to enhance T-cell activation compared with CD3 zeta alone.[19,20]

A further advantage of Le^Y as a target structure is that anti-Le^Y chimeric receptor expressing T cells could potentially target a broad range of malignancies that express Le^Y.[21–24] Lewis Y as a tumor-associated antigen has been shown to be correlated with an adverse prognosis in patients with lung cancer[25] and to have a pathogenetic role in colon cancer,[22] whereas expression and functional data are so far lacking for hematological diseases. Our group recently described *in vitro* and *in vivo* activity of gene-modified T cells expressing a chimeric receptor targeting Le^Y against solid tumors.[15] In this study, we have investigated the activity of T cells expressing the anti-Le^Y chimeric receptor against hematological targets. We show expression of the Le^Y antigen on a subset of multiple myeloma (MM) patient samples and cell lines as well as acute myeloid leukemia (AML) blasts. We further demonstrate specific *in vitro* and *in vivo* antitumor activity of T cells expressing the anti-LeY chimeric receptor.

Results

Lewis Y antigen expression in cell lines of MM and AML

It has been stated that Le^Y is expressed on early myeloid progenitor cells and corresponding CD34-positive leukemia cell lines.[26] On normal plasma cells, expression of carbohydrate antigens is well recognized, but Le^Y is usually not present on mature cells of the lymphoid lineage.[26] Expression of Le^Y on myeloma cells has not been described before. To explore the potential of Le^Y as a target antigen for adoptive immunotherapy in AML and MM, we tested for Le^Y expression in several commonly used cell lines representative of these diseases. Of five AML cell lines, KG-1 cells displayed very low levels of Le^Y expression, HL-60 and AMLCC1 (the latter freshly derived from primary patient sample) showed low, KG-1a moderate and K562 high expression of Le^Y (see also Figure 1a). We also assessed five MM cell lines which displayed heterogeneous Le^Y expression levels; four showed nil/very weak Le^Y expression (OPM-2, NCI-H929, U266, LP-1) and one (RPMI 8226-13) had moderately high Le^Y expression (MFI 17.9, Figure 1b).

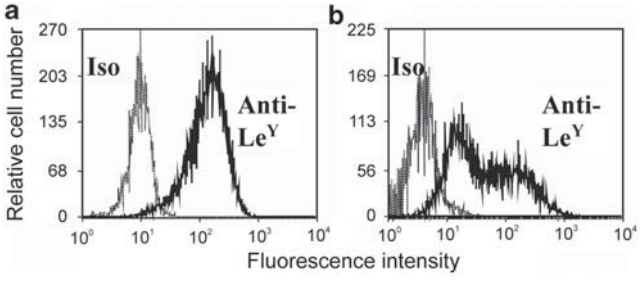

Figure 1 K562 (AML) and RPMI 8226-13 (MM) cell lines express Le^Y. Cell lines were stained with anti-Le^Y and isotype control (Iso) antibodies and analyzed using FACS. Representative overlay histograms are shown for the AML cell line K562 (**a**) and the MM cell line RPMI 8226-13 (**b**).

Lewis Y antigen expression on primary cells from patients with MM or AML

We next investigated Le^Y expression on primary cells in bone marrow (BM) samples from patients with MM or AML by fluorescence-activated cell sorting (FACS). Forty-eight fresh BM-aspirate samples from MM patients were assessed, of which 25 (52%) specimens tested positive for Le^Y expression on plasma cells with a median fluorescence intensity (MFI) of the positive samples of 9.98 compared with an MFI lymph of 0.8 (Figures 2a and b). Thirty-three BM samples from AML patients were also analyzed, 15 of which were positive for Le^Y expression (46%) with an MFI of 54.4 compared with an MFI lymph of 2.2. Thus, Le^Y expression was found in approximately half of the samples from both MM and AML patients with expression levels relatively higher in AML blasts. In contrast, tumor cells from patients with lymphoid malignancies (chronic lymphocytic leukemia, $n=9$ and Sézary Syndrome, $n=3$) were consistently negative.

Correlation of patient and disease characteristics with Le^Y expression

For 44 and 33 BM samples from patients with MM and AML, respectively, we analyzed patient and disease characteristics to explore correlations of known prognostic parameters with Le^Y expression levels. In both MM and AML, Le^Y expression was not associated with any clinical characteristics (univariate analysis, see also Table 1). Similarly, neither the number of lines of previous chemotherapy for MM or AML nor previous administration of the immune-modulatory drugs thalidomide, lenalidomide or bortezomib for MM had any impact on Le^Y expression.

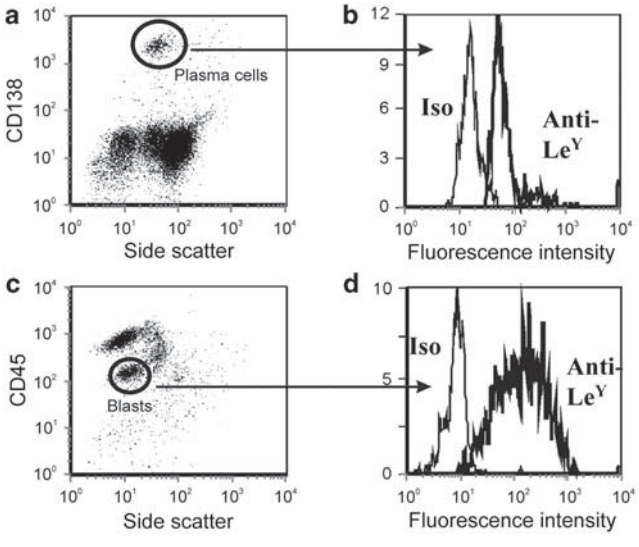

Figure 2 Lewis Y expression on primary patient samples. Identification of plasma cells by gating on CD138-positive cells (**a**) and myeloblasts according to side scatter and CD45 expression (**c**), respectively. Overlay plots of anti-Le^Y stainings and isotype controls of tumor cells from two patients with Le^Y-positive MM (**b**) and AML (**d**), respectively.

Table 1 Characteristics of patients with MM and AML whose bone marrow samples were assessed for Le^Y expression

Le^Y-expression	*Number of samples* (n)	*Clinical background available* (n)	*Median age (range)*	*Male (%)*	*Progressive/ refractory disease*	*Median previous treatments (range)*	*Median BM-infiltrate*[a] *(range)*	*Median MFI Le^Y*	*Median MFI lymph*
MM									
Positive	25	23	61 (42–77)	13 (57%)	4 (17%)	1 (0–5)	50% (10–90%)	9.9	0.7
Negative	23	21	62 (42–78)	11 (52%)	9 (42%)	2 (0–5)	50% (10–95%)	2.2	0.3
AML									
Positive	15	13	64 (40–80)	11 (85%)	3 (23%)	0 (0–3)	59% (4–97%)	54.4	2.2
Negative	18	16	64 (19–85)	11 (69%)	4 (25%)	0 (0–2)	57% (20–90%)	2.1	1.3

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; Le^Y, Lewis Y antigen; MFI, median fluorescence intensity; MM, multiple myeloma.
[a]The disease infiltration rate was determined morphologically in the biopsy for patients with MM and in the BM aspirate for patients with AML.

Transduction and expansion of T cells—phenotype of transduced cells

Transduction of primary human T cells was successfully performed with healthy donor T cells as well as with T cells derived from MM and AML patients. After optimization of the transduction protocol, the median transduction efficiency was 35.7% (range; 28.4–60%, $n = 11$) and did not differ significantly between normal donor and patient-derived cells. A subset analysis of transduced normal donor T cells revealed transduction of both CD4 and CD8 T cells (Figure 3).

In three transduction experiments, the vector copy number per cell was determined by quantitative PCR. The T cells contained 2.2, 5.4 and 9.2 vector copies per cell, which corresponded to chimeric receptor expression rates of 33.5, 30.1 and 28.4%, respectively.

Transduced anti-Le^Y T cells secrete IFN-γ in response to tumor cells

Functional analysis of transduced T cells showed specific interferon (IFN)-γ secretion in response to a 24 h co-culture with Le^Y-positive target cell lines, but not to Le^Y-negative cells. In addition, control T cells transduced with empty vector did not secrete IFN-γ in response to any cell line with the exception of the RPMI 8226-13 cells, which induced moderate IFN-γ production. Of note, transduced T cells also produced IFN-γ when cultured with AMLCC1 cells freshly derived from primary AML cells (Figure 4).

Tumor cell lysis by anti-Le^Y T cells correlates with Le^Y expression

Anti-Le^Y T cells showed specific killing of Le^Y-positive MM and AML targets in cytotoxicity assays. Specific cytotoxicity was demonstrated against the only Le^Y-positive MM cell line (RPMI8226-13) as well as the Le^Y-positive AML target cells (K562, KG1A and AMLCC1), but not Le^Y-negative cell lines (LP-1, NCI-H929, OPM-2) (Figures 5a–d). There was moderate lysis of K562 cells by non-transduced T cells in some but not all experiments.

There was a significant correlation of the amount of Le^Y expression on the target cells with the extent of tumor cell lysis ($R^2 = 0.8503$, $P = 0.026$, see also Figure 6). On the basis of these results, Le^Y-positivity was defined as an MFI ratio of the tumor cells versus control cells of five or greater.

Anti-LeY T cells are active in an MM mouse model

In three independent experiments, NOD/SCID mice were challenged with a subcutaneous deposit of RPMI 8226-13 cells, a Le^Y-positive MM cell line. One day later, half of the mice received human T cells transduced with the anti-Le^Y-scFv-CD28-ζ vector (anti-Le^Y-mice) and the other half received normal, non-transduced human T cells (control mice). The time of onset of clinically detectable subcutaneous plasmacytomas varied between experiments and often coincided with marked morbidity of the mice, mandating the killing of the mice. However, by day 60 all control mice had died from progressive tumors in all three experiments. In contrast, the incidence of plasmacytomas was delayed in the anti-Le^Y mice resulting in significantly improved overall survival of the anti-Le^Y mice compared with the control mice (Figure 7). We were unable to perform an equivalent

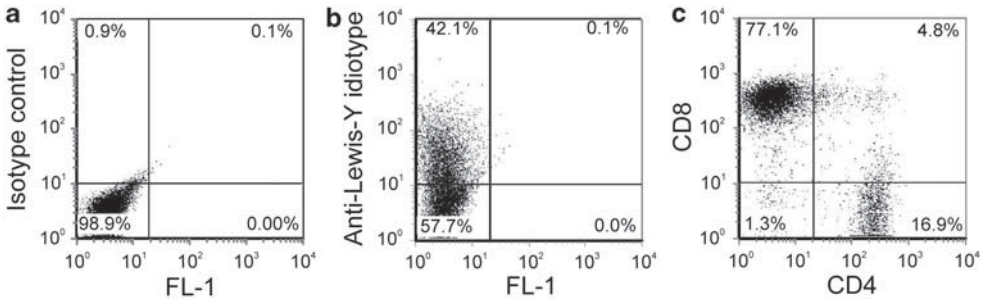

Figure 3 Transduction of peripheral blood mononuclear cell (PBMC)-derived T cells and chimeric receptor expression. FACS results of one representative experiment out of 11 with PBMC (in this case normal donor PBMC) that were activated, transduced and expanded as described, and analyzed on day 10 of the *in vitro* culture. Panel **a** demonstrates the isotype control and panel **b** shows the staining for anti-Le^{Y} chimeric receptor expression (42% positive) in the total T-cell population. Out of the anti-Le^{Y}-positive T cells, 77% were CD8 T cells and 17% were CD4 positive (**c**). Transduction with empty vector resulted in <1% of anti-Le^{Y}-positive T cells (data not shown).

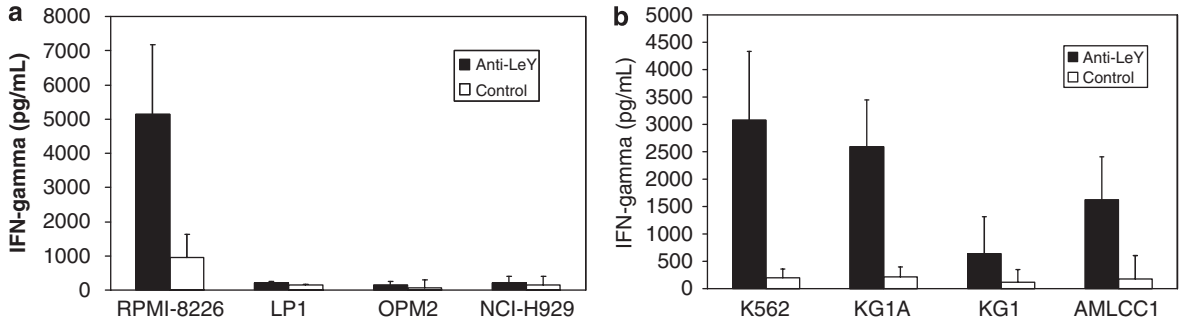

Figure 4 Anti-Le^{Y} T cells produce IFN-γ on co-culture with Le^{Y}-positive target cells. Anti-Le^{Y} cTCR-positive and mock-transduced control T cells were co-cultured with various MM and AML cell lines and with AMLCC1 cells (freshly derived from primary AML cells). The figure shows combined data from three independent experiments run as duplicates with human T cells from various healthy donors. Data are shown for MM cell lines (**a**) and AML cell lines (**b**), the error bars indicate the standard deviations. There was specific IFN-γ production by anti-Le^{Y} T cells when cultured with the Le^{Y}-positive MM cell line RPMI 8226-13, but not to Le^{Y}-negative MM cell lines LP-1, OPM-2 or NCI-H929. Of note, there was moderate IFN-γ production by control T cells in response to RPMI 8226-13 cells (**a**). There was varying IFN-γ production of anti-Le^{Y} T cells in response to different AML cell lines K562, KG1A and KG1, and to primary AML blasts-derived AMLCC1 cells. Control T cells consistently showed no significant IFN-γ production in response to the AML cell lines (**b**).

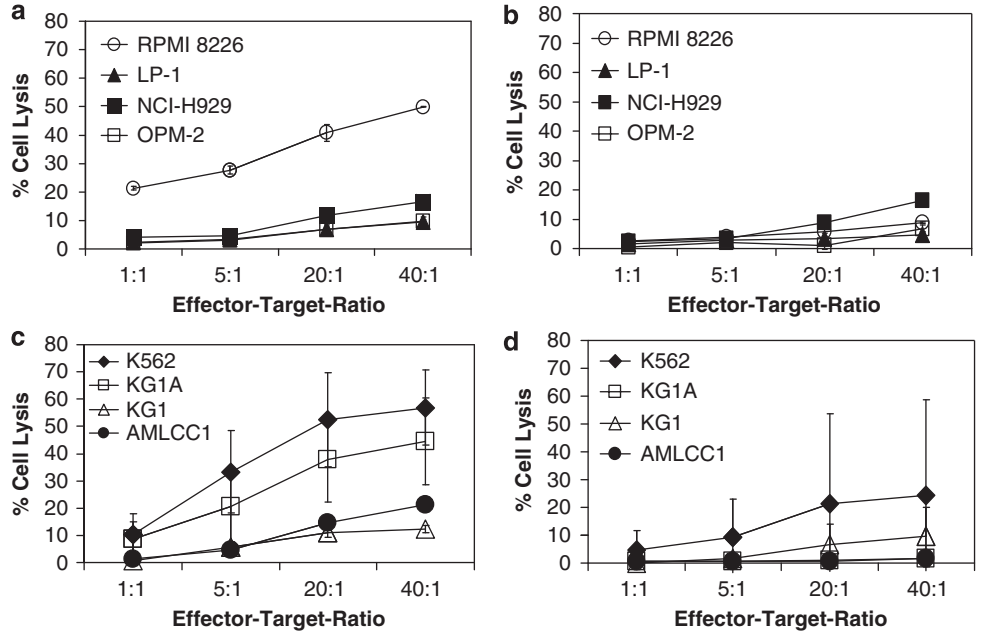

Figure 5 Le^{Y}-specific transduced human T lymphocytes are cytotoxic upon stimulation with tumor cells expressing the Le^{Y} antigen. T cells were cultured with MM (**a**+**b**) or AML (**c**+**d**) cell lines and a standard 51Cr-release assay was performed (as per methods). The cytotoxicity of anti-LeY T cells (**a**+**c**) and mock-transduced T cells (**b**+**d**) was determined as percentage of lysis (% lysis) of the different cell lines. The figure shows combined data from three independent experiments run as triplicates with human T cells from various healthy donors.

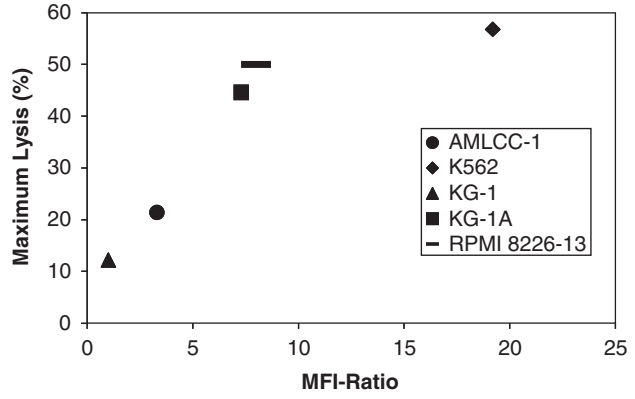

Figure 6 Correlation of Le^Y expression and specific lysis by anti-Le^Y T cells. The extent of LeY expression of different cell lines (MFI ratio) was correlated with the maximum specific lysis of those cells by normal donor anti-Le^Y T cells in the cytotoxicity assays (maximum lysis (%)).

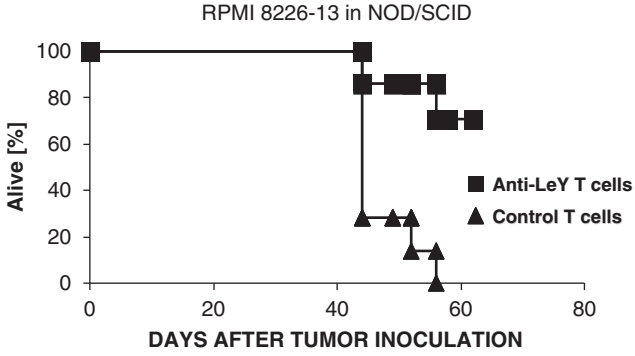

Figure 7 Adoptively transferred healthy donor-derived anti-Le^Y T cells improve survival in an MM mouse model. Shown are the results of one representative out of three experiments. On day 0, 1×10^6 cells of the Le^Y-positive RPMI 8226-13MM cell line were injected subcutaneously in NOD/SCID mice. The mice received four intravenous injections of 1×10^7 normal non-transduced T cells (control mice ($n = 7$), triangles) or the same number of T cells previously transduced with the anti-Le^Y- scFv-CD28-ζ vector (anti-Le^Y mice ($n = 7$), squares) on days 0, 1, 2 and 5. Overall survival was significantly improved for the anti-Le^Y mice compared with the control mice, $P = 0.006$, log-rank test.

experiment using an AML mouse model. Although a murine AML model with the K562 cell line has been described, concordant with reports in the literature,[26–28] we found the disease kinetics of the K562 AML mouse model far more variable and less reliable to perform comparative *in vivo* studies.

Discussion

We have previously demonstrated activity of T cells expressing a chimeric receptor directed against Le^Y *in vitro* and *in vivo* against epithelial tumors.[15] In this study, we demonstrate that the expression of Le^Y is not restricted to epithelial tumors but is also found on AML and MM. Given the previously documented expression of Le^Y on mature neutrophils,[24,29] the finding that it is also expressed on AML blasts is not entirely surprising. In contrast, the expression on plasma cells was rather unexpected, particularly given our findings of absence of Le^Y on normal circulating lymphocytes as well as on malignant B and T cells. Nonetheless, the presence of Le^Y on plasma cells is consistent with the recognition that malignant plasma cells can aberrantly express other epithelial antigens.[30–33] The biological significance of Le^Y expression on malignant plasma cells and AML blasts is unclear. We evaluated multiple clinical and laboratory parameters and found no correlation of Le^Y expression with disease subtype, stage or other known prognostic parameters in MM or AML. Similarly, amount or type of previous treatment, including immunomodulatory agents for MM, did not have any impact on cellular Le^Y expression.

Given the encouraging previous results with T cells expressing the anti-Le^Y chimeric receptor in targeting epithelial tumors, we wished to examine the suitability of Le^Y as a target antigen for the treatment of MM and AML. We established a Good Manufacturing Practice-compliant transduction protocol, which resulted in effective gene transduction into both CD4- and CD8-positive T-cell populations. The presence of antigen-specific CD4 and CD8 T cells in the adoptively transferred T-cell product is likely to be required for maximum efficacy.[34] Furthermore, we were able to demonstrate that T cells expressing the anti-Le^Y single-chain receptor were highly specific for Le^Y-positive targets, producing IFN-γ on encounter with Le^Y-positive but not Le^Y-negative plasma cells or AML blasts. With the same transduction system, but using the ovarian cancer cell line OVCAR-3 as target, we observed that both the CD8 and CD4 subsets of the anti-Le^Y T cells show a specific tumor response with production of interleukin-2 and IFN-γ (data not shown). Similarly, cytotoxicity assays revealed specific tumor cell death mediated by anti-Le^Y T cells that correlated with the extent of Le^Y expression on the target cells. The AML cell line K562 elicited a response from control T cells in some but not all experiments, which may reflect an allogeneic T-cell response to antigens such as bcr-abl or the receptor for hyaluronan acid-mediated motility.[35,36] Finally, our NOD/SCID mouse MM tumor model demonstrated superior overall survival for mice treated with anti-Le^Y T cells compared with those that received non-transduced T cells, clearly demonstrating the efficacy of this adoptive immunotherapy approach *in vivo*.

Our study reveals the previously unreported expression of the carbohydrate antigen Le^Y on AML and MM. Furthermore, we have shown *in vitro* and *in vivo* efficacy of a novel cellular immunotherapy using T cells expressing the chimeric anti-Le^Y receptor. Our results confirm that Le^Y is a suitable antigen for T-cell-mediated immunotherapy against both MM and AML. The safety of this approach is supported by the favorable toxicity profile of various 'naked' and conjugated anti-Le^Y antibodies in early phase clinical studies, as well as the lack of transforming events in T cells retrovirally transduced to express this construct.[37–40] An alternative to the use of chimeric T cells for the treatment of Le^Y expressing malignancies is the less cumbersome utilization of monoclonal antibodies.[38–40] However, chimeric T cells as used in this study effectively combine antigen specificity with direct activation of the most potent immune effector cells upon antigen binding. Therefore, in our opinion, chimeric T cells are more likely to mount

a meaningful immunologic antitumor response than antibodies alone that largely rely on secondary immune activation. Consequently, we have initiated a phase I study targeting Le^Y in patients with poor prognosis Le^Y-positive MM or AML.

Materials and methods

Cells

RPMI-8226, NCI-H929, U266, OPM-2 and LP-1 are MM cell lines, K562, KG-1 and KG-1A are AML cell lines (all from American Type Culture Collection, Manassas, VA, USA) with K562 being derived from CML in blast crisis (erythroleukemia). We established the AMLCC1 cell line from primary blasts taken from peripheral blood of an AML patient. All tumor cell lines were cultured in complete medium composed of RPMI medium 1640, 10% heat-inactivated fetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamicin and 2 mM glutamine (all from Invitrogen, Carlsbad, CA, USA). T cells were cultured in the same medium with the addition of 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol (Invitrogen) and 600 U ml^{-1} human recombinant interleukin-2 (National Cancer Institute, Frederick, MD, USA and Chiron, Emeryville, CA, USA). RPMI 8226-13 cells were generated by sorting for highly Le^Y-expressing RPMI-8226 cells per FACS and initially passaged twice subcutaneously in NOD-SCID mice to establish a cell line that reproducibly formed subcutaneous tumors on subsequent injection into mice.

Chimeric receptor construct and retroviral vector production

DNA encoding the anti-Le^Y chimeric receptor was generated by using standard molecular biology techniques using an scFv[16] generated from the humanized monoclonal antibody hu3S193.[41] The construct used in this study was derived from the one described by Westwood *et al*.[15] by excising the sequences coding for the Neomycin (Neo)-resistance gene and the IRES sequence by cutting at the *Hin*dIII/*Sph*I sites and subsequent religation. Removal of these sequences simplified the vector and eliminated translation of the *Neo*-resistance gene, thereby rendering the vector less immunogenic for potential subsequent use in humans (see also Figure 8). The construct was transfected into PA317 and GP+E86 packaging lines and resulting retroviral supernatants used to repeatedly (four times) transduce both packaging lines ('ping-pong'). The resulting GP+E86 supernatant was used to transduce the PG13 packaging line (four times). In the absence of a selectable marker, the transduced PG13 packaging line was enriched for cTCR-expressing cells by immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) targeting the chimeric receptor according to the manufacturer's instructions. The enriched cells were then subcloned and a potential high-titer producer clone was identified by flow cytometry by staining for cTCR expression on the packaging cells. From this clone, a master cell bank was generated and clinical grade retroviral supernatant produced commercially under code of Good Manufacturing Practice (cGMP) conditions (EUFETS, Idar-Oberstein, Germany).

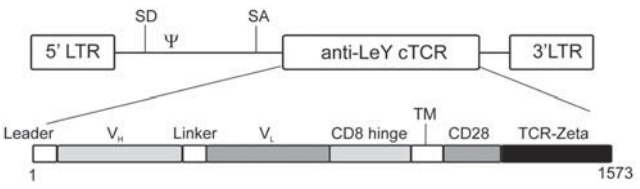

Figure 8 Schema of the scFv anti-Le^Y retroviral vector. The cartoon demonstrates the structure of the scFv anti-LeY retroviral vector. LTR, long terminal repeat; SA, splice acceptor; SD, splice donor; cTCR, chimeric T-cell receptor; VH, variable heavy chain; VL, variable light chain; TM, transmembrane region; TCR-Zeta, T-cell receptor zeta chain.

T-cell transduction and expansion

Human peripheral blood mononuclear cells were isolated from normal donor apheresis products, buffy coats or patients' peripheral blood. Briefly, peripheral blood mononuclear cells were stimulated with anti-human CD3 (OKT3 30 ng ml^{-1}, Ortho Biotech, Bridgewater, NJ, USA) and human recombinant interleukin-2 (Biological Resources Branch Preclinical Repository, NCI, Frederick, MD, USA) at 600 U ml^{-1} for 3 days. Retroviral transduction was performed by incubation with supernatant from the PG13 retroviral producer cell line on a Retronectin (Takara Bio, Otsu, Japan) matrix as per the manufacturer's instructions. Briefly, Retronectin was coated at a concentration of 6 μg cm^{-2} in non-tissue culture six-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated with 5 ml of retroviral supernatant. After 4 h, supernatant was removed and 2.5×10^6 T cells in 5 ml of complete media added for 12 h. This procedure was repeated once on the next day. Following transduction, cells were expanded for up to 21 days. During expansion, a cell density of 2×10^6/ml was maintained. Transduction efficacy was determined on day 8 or 10 after transduction by flow cytometry (FACS, see below). T-cell cultures were performed in AIMV medium (Invitrogen) supplemented with 2% human serum (BioWhittaker, Cambrex Bioscience, Walkersville, MD, USA).

In order to use Le^Y expression as a target for T-cell-based immunotherapy of MM and AML, we generated a retroviral vector as previously described.[15] In consideration of a future clinical application, the selective marker (Neo) and the preceding IRES sequence were deleted from the original construct. A cGMP-conform master cell bank was created and clinical grade supernatant was used for subsequent transduction of primary human T cells. The retroviral titer of the clinical grade supernatant was determined to be $\sim 7.5 \times 10^6$ CFU ml^{-1} based on FACS and molecular methods (quantitative PCR) by the manufacturer (EUFETS GmbH, Idar-Oberstein, Germany).

Antibodies and flow cytometry to examine T-cell transduction

Expression of the chimeric receptor was determined by staining with a mouse monoclonal antibody designated LMH-3 IgG1 specific for the idiotype of the scFv (anti-3S193 idiotype),[42] supplied by Ludwig Institute for Cancer Research (LICR), followed by phycoerythrin (PE)-conjugated anti-mouse Ig $F(ab')_2$ (Chemicon, Billerica, MA, USA). Cell surface phenotyping of

transduced cells was determined by direct staining with PE-conjugated anti-human CD8 and PE-Cy5-conjugated anti-human CD4 (Dako, Glostrup, Denmark). Cells were analyzed on a flow cytometer (FACSCalibur) using CELLQUEST software (both Becton Dickinson).

PCR for detection and quantification of the vector construct

DNA was prepared using the method published by Westwood *et al*.[37] at a concentration of 2.5×10^6 cells ml^{-1}. PCR was performed using TaqMan (Applied Biosystems, Foster City, CA, USA) chemistry according to the manufacturer's instructions on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with 10 µl DNA and primers Le^Y (forward), Le^Y (reverse) and am (reverse) at 300 nM, primer am (forward) at 100 nm and probes at 200 nm. The human melanin-concentrating hormone receptor 1 (MCHR1, am) was used as control for cell number. All samples were run in triplicate. Standard curves were prepared using serial 10-fold dilutions of pSAMEN-Le^Y plasmid or normal human peripheral blood mononuclear cells. MCHR1 was assumed to be single copy and diploid and used to calculate the Le^Y gene copy number.

Primers and probes are as follows:
Le^Y (forward): CCAAGCTGCAAATCACAGATTACA
Le^Y (reverse): GCACGAAGTGGCTGAAGTACATG
Le^Y (probe): 6FAM-ACGACGATGACAAGCTGA GCAACTCCA-TAMRA
am (forward): CACCAGCTCATGGGCAATG
am (reverse): GGTGCTGGTGAACTGACTATTGG
am (probe): VIC-CATGTGCACCCTCATCACGG CCA–TAMRA.

All primers were from Sigma (Sigma-Aldrich, St Louis, MO, USA), probes were from Applied Biosystems.

Screening for LeY expression on primary human samples of MM and AML

Fresh BM aspirate samples (100 µl) were incubated with the following antibodies: Twenty µl of 1:10 diluted murine monoclonal IgG3 anti-Le^Y antibody 3S193 (Ludwig Institute for Cancer Research, Heidelberg, Australia), directly fluorescein isothiocyanate-conjugated (Chemicon); 10 µl of undiluted CD45 PerCP (BD Biosciences); 20 µl of a 1:10 dilution in phosphate-buffered saline of the anti Le^{Y-} fluorescein isothiocyanate antibody (Chemicon). MM cells were identified by staining for CD138 (CD138APC, 5 µl, undiluted) and CD38 fluorescein isothiocyanate (10 µl, undiluted, both Becton Dickinson) versus side scatter. Myeloid blast cells were identified by gating for blasts in the CD45 versus side scatter plot. Chronic lymphocytic leukemia cells were identified by gating for lymphocytes in the CD45 versus side scatter plot and staining for CD5 (CD5 PE, clone L17F12, Becton Dickinson) and CD19 PerCP-Cy5.5 (Clone SJ25C1, Becton Dickinson). Sézary cells were identified by staining for CD3 (anti-CD3 PerCP, clone SK7, Becton Dickinson), CD4 (anti-CD4- fluorescein isothiocyanate, clone SK3, Becton Dickinson) and CD8 (anti-CD8-PE, clone SK1, Becton Dickinson). Cells were stained for 15 min at room temperature in the dark, red cells were lysed with FACS Lysis buffer (Becton Dickinson, 1 ml). Then stained cells were washed twice in phosphate-buffered saline and resuspended in 1 ml of phosphate-buffered saline for FACS analysis. The MFI above the background MFI of the isotype control was determined for plasma cells or myeloblasts, respectively, and compared with the MFI of containing lymphocytes (MFI lymph) that served as internal negative control compensating for variations in absolute fluorescence intensity levels due to use of two different FACS instruments. The definition of Le^Y positivity required a tumor cell MFI at least five times higher than the MFI of the lymphocytes (MFI lymph) and was based on functional data of anti-Le^Y T cells.

Correlation of patient and disease characteristics with Le^Y expression

The following clinical and routine laboratory parameters of both MM and AML patients were recorded on the day of the BM biopsy for investigation of Le^Y expression: age, gender, number of lines of prior treatment, hemoglobin, neutrophil and platelet count. For patients with MM, the following characteristics were also analyzed: heavy and light chain isotype of the paraprotein, presence or absence of plasmablastic or leukemic disease, extramedullary disease, adverse cytogenetics of 13q deletion and/or t(4;14), p53 mutation (by immunohistochemistry of BM biopsy), levels of β_2-microglobulin and albumin in plasma and percentage of plasma cells/plasmablasts in the BM biopsy. For patients with AML, additional characteristics analyzed were: AML subtype according to the WHO classification,[43] presence or absence of adverse cytogenetics (-7, -5, trisomy 8, abnormal 3q, t(6;9), t(9;22), 11q23 abnormalities), increased lactate dehydrogenase levels, white blood cell count $>$or $\leqslant 30 \times 10^9/l$, and percentage of myeloblasts in the nucleated cell fraction of the BM aspirate.

For all patients included in the analysis, the medical history was reviewed independently by two investigators (HMP and SP) in order to determine the clinical status of the patients' disease at the time of the BM biopsy. On the basis of this information, each patient's disease was classified as being clinically high risk (rapidly progressive or treatment refractory) or not high risk. Each of the above listed characteristics was analyzed for correlation with Le^Y antigen expression in a univariate analysis (unpaired *t*-test; GraphPad Software, La Jolla, CA, USA).

Correlation of Le^Y expression and tumor cell lysis

A Pearson correlation was performed of the MFI ratio of tumor cells to Le^Y-negative control cells with the extent of tumor cell lysis by anti-Le^Y T cells, and the Pearson r^2 and two-tailed *P*-values were determined (GraphPad Software).

Cytokine secretion and cytotoxicity assays

T-cell antitumor response assays were performed as described.[44] Briefly, IFN-γ secretion was determined by enzyme-linked immunosorbent assay on culture supernatants following overnight co-culture of transduced T cells (1×10^6/ml) and tumor cells (5×10^5/ml). The MM cell lines RPMI-8226-13, NCI-H929, OPM-2 and LP-1 and the AML cell lines K562, KG-1 and KG-1A (all from American Type Culture Collection) as well as the AML cell line AMLCC1, established from peripheral blood-derived myeloblasts, were used as target cells.

Cytotoxicity was assessed by using a 4-h ^{51}Cr-release assay as described previously.[15] Briefly, the target cells were loaded with ^{51}Cr and incubated with transduced T cells in an increasing effector to target ratio for 4.5 h with specific killing measured by ^{51}Cr release. Empty vector-transduced T cells served as control.

Tumor treatment in vivo

On day 0, NOD-SCID mice ($n = 6$–7 per group, Walter and Eliza Hall Institute, Melbourne, Australia) were irradiated at 2.5 Gy and injected subcutaneously with 1×10^6 RPMI 8226-13 cells in 200 µl of phosphate-buffered saline. In three experiments, mice received 1×10^7 adoptively transferred anti-Le^Y T cells intravenously on days 0, 1, 2 and 5 following tumor inoculation. Controls included mice receiving no T cells and mice receiving control T cells transduced with empty vector (in equal numbers as those mice receiving anti-Le^Y T cells and on the same days). All mice were assessed second daily for the first 4 weeks and then daily thereafter. When the mice developed a tumor mass of >1 cm in diameter, the mice were killed. Overall survival was estimated by the Kaplan–Meier method and calculated from the day of tumor cell injection to the day a mouse was found dead or had to be killed. Group comparisons were performed using the log-rank test. Experimental procedures were approved by the Animal Ethics Committee of the Peter MacCallum Cancer Centre and conducted in accordance with institutional guidelines.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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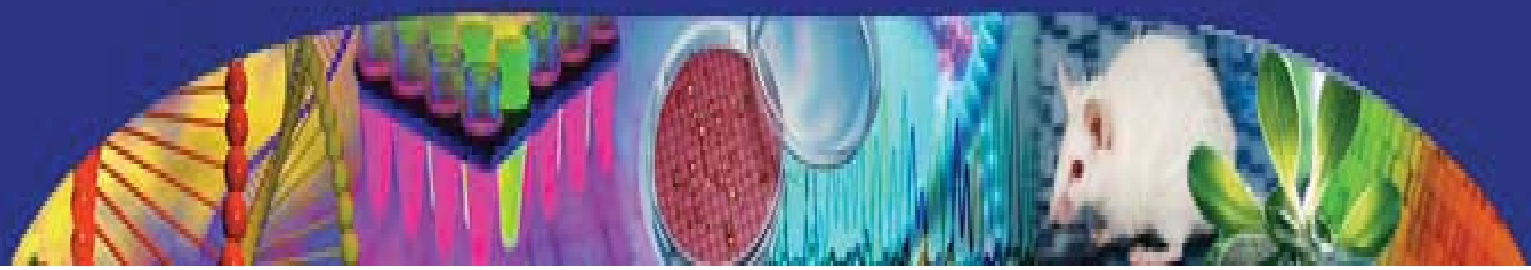


TAG-72 Protocols

Antigen Target	**Tag-72**
Antigen Expression	**Oncofetal tumor-associated glycoprotein found on the surface of many cancer cells, including breast, colon, and pancreatic cells.**

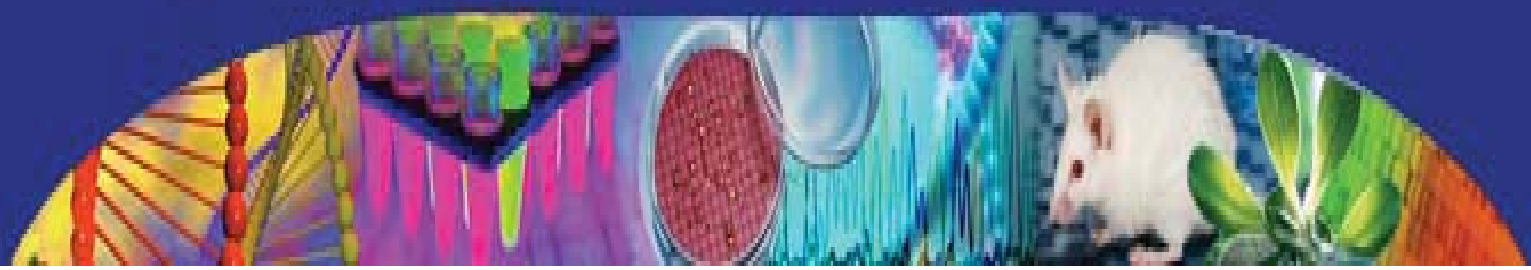
	Chimeric Antigen Receptors Trials Registered with OBA
Title	**Protocol # 198: A Phase I/II Study of Autologous CC49-Zeta Gene-Modified T Cells and alpha-Interferon in Patients with Advanced Colorectal Carcinomas Expressing the Tumor-Associated Antigen, TAG-72**
PI	**Robert S. Warren, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD3 zeta**
Status	**Closed**
Doses Proposed	**$1x10^8$ – $1x10^{10}$ cells administered IV every 2 weeks for a total of 3 – 5 doses**
Lymphodepletion	**No**
Subjects Enrolled	**Dosed: 10**
Summary of Unexpected and Related Events	**One subject was removed due to development of a serious adverse event (left central retinal artery thrombosis, loss of vision, and confusion) 3 days following the 2nd infusion. ESR was > 100 and temporal artery biopsy was negative for arteritis. This subject had a history of prior spontaneous deep venous thrombosis of the lower extremity.**
Summary of Results	**There were no tumor responses. Although CC49-zeta T cells were detected by PCR in the blood, they were cleared in some subjects following repeat infusions. An induced anti-idiotype humoral immune response to the CC49-zeta receptor was detected in at least 7/10 patients.** **Warren, R. S., *et. al.*, Studies of Regional and Systemic Gene Therapy with Autologous CC49-Zeta Modified T cells in Colorectal Cancer Metastatic to the Liver, 1998: *Abstract Presented at the 7th International Conference on Gene Therapy of Cancer*, San Diego CA**

Title	**Protocol # 239: A Phase I/II Study of Hepatic Infusion of Autologous CC49-Zeta Gene-Modified T Cells in Patients with Hepatic Metastasis from Colorectal Cancer**
PI	**Emily K. Bergsland, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD3 zeta**
Status	**Closed**
Doses Proposed	**$1x10^9$ – $1x10^{10}$ cells administered via hepatic artery infusion every 2 weeks for a total of 3 – 4 doses**
Lymphodepletion	**No**
Subjects Enrolled	**Treated: 6**
Summary of Unexpected and Related Events	**SAEs were reported in three patients:** **1) The day after the second infusion, one subject was admitted with a Grade 2 fever, Grade 3 increase in bilirubin and anemia. The events resolved and the subject received further infusions. Following the fourth infusion, the subject had an elective liver biopsy and developed a post-biopsy hematoma and anemia. One month after this subject completed all infusions, the subject was admitted for fever, jaundice and anemia.** **2) Two hours after the fourth infusion, one subject developed fever, atrial fibrillation and hypotension. CT showed evidence of pulmonary edema and a pleural effusion. This subject had a history of chronic atrial fibrillation.** **3) Twenty-five minutes after the fourth infusion, one subject was noted to have a decrease in oxygen saturation. The subject was observed and discharged the next day.**
Summary of Results	**There were no tumor responses. Gene-modified T cells were detected in the blood of some subjects. Three of 3 subjects analyzed had evidence of a CC49 anti-idiotype humoral immune response.**

STUDIES OF REGIONAL AND SYSTEMIC GENE THERAPY WITH AUTOLOGOUS CC49-ZETA MODIFIED T CELLS IN COLORECTAL CANCER METASTATIC TO THE LIVER. *RS Warren, GA Fisher, EK Bergsland, R Pennathur-Das, J Nemunaitis, AP Venook, KM Hege. University of California, San Francisco, CA; Stanford University, Palo Alto, CA; PRN Research, Dallas, TX; Cell Genesys, Foster City, CA.*

Two Phase I/II trials have been initiated in which patients with metastatic colorectal cancer to the liver are treated with gene-modified autologous CD4 and CD8 T cells administered systemically (IV) or via the hepatic artery (IA). The T cells are genetically altered to express a novel receptor, CC49-zeta, which conveys specificity for tumor targets. CC49 is a humanized monoclonal antibody directed against TAG-72 (an oncofetal mucin expressed on many adenocarcinomas) which is linked to the zeta chain of the T cell receptor (involved in T cell activation). In the IV trial, 14 patients were enrolled and underwent lymphapheresis, T cell activation, expansion, and retroviral gene transduction. The target cell dose of 4 $x10^{10}$ T cells was achieved in a mean of 14 days (range 12-17). Mean transduction efficiency was 39% (range 12-59%). The final products contained a mean of 56% CD4 T cells (range 34-82%) and 41% CD8 T cells (range 13-73%). 10 patients have received at least one infusion of CC49-zeta gene-modified T cells. 6 patients were treated in the dose escalation cohort (range 10^8 to 10^{10} T cells every two weeks), and 4 in the Phase II cohort (10^{10} T cells every two weeks x 3). All patients received α-interferon with each T cell infusion to upregulate tumor expression of TAG-72. No dose limiting toxicity was observed. One serious adverse event (SAE) was reported: a central retinal artery thrombosis in a patient with a prior history of venous thrombosis. No replication competent retrovirus was detected in 7/7 patients tested. Gene-modified T cells were detected by PCR in the peripheral blood of 6/6 patients for up to 10 weeks. Over the same period, serum TAG-72 levels decreased by >80% in 6 of 8 patients consistent with TAG-72-specific anti-tumor activity. Continued TAG-72 suppression below the pre-infusion level was evident in the first patient evaluable at 6 months. There were no radiographic responses; 1 patient had stable disease. In the IA trial, 3 patients have initiated CC49-zeta gene-modified T cell infusions via the hepatic artery (range 10^9 to 10^{10} cells every 2 weeks). 2 SAEs have been reported. 1 patient was hospitalized with transient fevers, abdominal pain, hyperbilirubinemia, anemia, and increased LDH. Another patient developed hyperbilirubinemia. Results of serum TAG-72 and follow-up radiographic imaging are pending. $Indium^{111}$ – labeled T cell trafficking studies suggest increased tumor localization with IA delivery compared to IV administration. We conclude that intravenous infusion of CC49-zeta gene-modified T cells is safe and shows evidence suggestive of *in vivo* anti-tumor activity as measured by changes in serum TAG-72. Tumor localization of these cells may be enhanced by regional delivery and accrual to the IA study continues.

Presented at the 7th International Conference on Gene Therapy of Cancer, San Diego, CA, Nov 19-21, 1998



HER2 Protocols

Antigen Target	**HER2/neu**
Antigen Expression	**Primarily expressed in tumor specific tissues but has also been noted in normal tissues (e.g. lung, liver, and breast).**

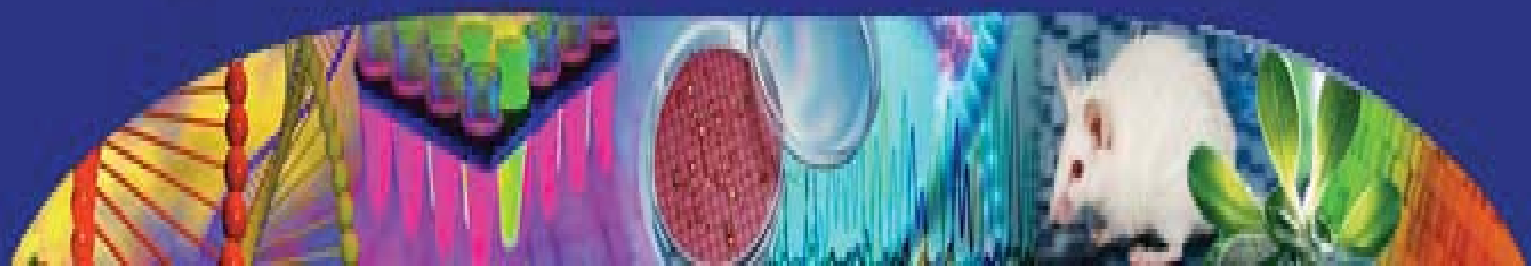
	Chimeric Antigen Receptors Trials Registered with OBA
Title	**Protocol # 920: Phase I/II Study of Metastatic Cancer that Expresses Her-2 Using Lymphodepleting Conditioning Followed by Infusion of Anti-Her-2 Gene Engineered Lymphocytes**
PI	**Steven Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/CD3 zeta, CD28, and 4-1BB**
Status	**Not Currently Enrolling**
Doses Proposed	**$1x10^9$ – $1x10^{11}$ mg/kg**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 250 mg/m^2**
Subject Enrolled	**1**
Summary of Unexpected and Related Events	**One patient suffered a cytokine storm with eventual death** **Morgan, RA, *et. al*. (2010). Case Report of a Serious Adverse Event Following the Administration of T Cells Transduced with a Chimeric Antigen Receptor Recognizing ERBB2. *Molecular Therapy* 18: 843-851.**
Summary of Results	**None**

Title	**Protocol # 926: Patient Specific Protocol for Administration of EBV Specific CTLs Expressing HER2/neu Chimeric Receptors for Lung Cancer (single subject protocol)**
PI	**Helen E. Heslop, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Subject treated in 2008**
Doses Proposed	**$2x10^7$ cells/m^2**
Lymphodepletion	**No**
Subjects Enrolled	**1**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 969: Administration of HER2 Chimeric Antigen Receptor Expressing T Cells for Subjects with Advanced Osteosarcoma (HEROS)**
PI	**Nabil Ahmed, M.D., M.Sc.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Active**
Doses Proposed	**$1x10^4$ – $1x10^8$ cells/m^2**
Lymphodepletion	**No**

Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 970: Administration of HER2 Chimeric Antigen Receptor Expressing CMV-Specific Cytotoxic T Cells in Patients with Glioblastoma Multiforme (HERT-GBM)**
PI	**Nabil Ahmed, M.D., M.Sc.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**IND submitted 6/30/10**
Doses Proposed	**$1x10^4$ – $1x10^8$ cells/m^2**
Lymphodepletion	**None**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 971: Administration of HER2 Chimeric Receptor and TGFβ Dominant Negative Receptor (DNR) Expressing EBV Specific Lymphocytes for Subjects with Advanced HER2 Positive Lung Malignancy (HERCREEM)**
PI	**Stephen Gottschalk, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**

Status	**Active**
Doses Proposed	**$1x10^4$ – $1x10^8$ cells/m^2**
Lymphodepletion	**No**
Subjects Enrolled	**1**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 973: Phase I/II Study in Subjects with Recurrent and Resistant Glioblastoma Multiforme (GBM) of Intracavitary CMV-specific Cytotoxic T Lymphoctyes (CTL) Expressing Chimeric Antigen Receptors Targeting HER2 (GLITCHER)**
PI	**Stephen Gottschalk, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Not initiated**
Doses Proposed	**$1.5x10^7$ – $1.2x10^8$ cells/m^2**
Lymphodepletion	**No**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

Title	**Protocol # 1020: Administration of EBV-specific Cytotoxic T Cells Expressing HER2 Chimeric Antigen Receptor to Subjects with Advanced Osteosarcoma (ECHO)**
PI	**Nabil Ahmed, M.D., M.Sc.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Not initiated**
Doses Proposed	**$1x10^4$ – $1x10^8$ cells/m^2**
Lymphodepletion	**None**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**



GD2 Protocols

Antigen Target	**GD2**
Antigen Expression	**Expressed on neuroblastoma cells at high levels but at very low levels on normal tissues.**

	Chimeric Antigen Receptors Trials Registered with OBA
Title	**OBA Protocol # 563: Administration of Peripheral Blood T-Cells and EBV Specific CTLs Transduced to Express GD-2 Specific Chimeric T Cell Receptors to Patients with Neuroblastoma**
PI	**Chrystal U. Louis, M.D., M.P.H.**
Vector and Signaling Moieties	**Retrovirus/EBV specific T cells or activated T cells with CAR for GD2**
Status	**Active**
Doses Proposed	**$1x10^8 – 2x10^8$ cells/m^2**
Lymphodepletion	**After 1st 2 cohorts, lymphodepleting antibody CD45 was added for 6 subjects**
Subjects Enrolled	**19 (each received two products – CTLs or activated T cells transduced with distinguishable GD2 CARs)**
Summary of Unexpected and Related Events	**One subject developed fever about 2 weeks after the T cell infusion along with abdominal pain in the region of the liver metastases. Imaging showed tumor necrosis. A biopsy confirmed the presence of necrotic tumor, with areas of residual viable tumor, and infiltration with $CD4^+$ and $CD8^+$ T cells. The subject's fever was, therefore, possibly due to an infection but also possibly due to infused T cells mediating necrosis at the tumor sites.**
Summary of Results	**Eight high risk subjects with no evaluable disease at the time of infusion remain in remission. In 11 subjects with active disease who were infused, the best clinical response was complete remission in 3 subjects, partial response in 1 subject, tumor necrosis in 2 subjects, stable disease in 2 subjects and no response in 3 subjects.** **Pule, M., *et. al.* (2008). Virus-specific T cell Engineered to Coexpress Tumor-Specific Receptors: Persistence and Antitumor Activity in Individuals with Neuroblastoma. *Nature Medicine* 14:1264-1270.**

nature medicine

Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma

Martin A Pule[1], Barbara Savoldo[1], G Doug Myers[1,2], Claudia Rossig[1], Heidi V Russell[1,2], Gianpietro Dotti[1,3], M Helen Huls[1], Enli Liu[1], Adrian P Gee[1–3], Zhuyong Mei[1], Eric Yvon[1], Heidi L Weiss[4], Hao Liu[4], Cliona M Rooney[1,2,5], Helen E Heslop[1–3] & Malcolm K Brenner[1–3]

Cytotoxic T lymphocytes (CTLs) directed to nonviral tumor–associated antigens do not survive long term and have limited antitumor activity *in vivo*, in part because such tumor cells typically lack the appropriate costimulatory molecules. We therefore engineered Epstein-Barr virus (EBV)-specific CTLs to express a chimeric antigen receptor directed to the diasialoganglioside GD2, a nonviral tumor–associated antigen expressed by human neuroblastoma cells. We reasoned that these genetically engineered lymphocytes would receive optimal costimulation after engagement of their native receptors, enhancing survival and antitumor activity mediated through their chimeric receptors. Here we show in individuals with neuroblastoma that EBV-specific CTLs expressing a chimeric GD2-specific receptor indeed survive longer than T cells activated by the CD3-specific antibody OKT3 and expressing the same chimeric receptor but lacking virus specificity. Infusion of these genetically modified cells seemed safe and was associated with tumor regression or necrosis in half of the subjects tested. Hence, virus-specific CTLs can be modified to function as tumor-directed effector cells.

The promise of tumor antigen–specific T lymphocytes for the treatment of melanoma and EBV-associated malignancies[1–7] has led to efforts to retarget effector T cells and thereby extend the range of tumors that they can treat. A common strategy has been to introduce a synthetic receptor with an antigen-binding domain from an antibody coupled to a signal-transducing endodomain derived from the native T cell receptor into activated T cells (ATCs)[8]. These chimeric antigen receptors (CARs) thus have the specificity of an antibody coupled to the cytotoxic effector mechanisms of the T cell. To date, however, this strategy has had only limited success, owing in part to the lack of essential costimulatory signals to the T cell during engagement of its CAR and perhaps also to the introduction of the CAR into regulatory T (T_{reg}) cells, as well as into conventional T effector cells[9]. Consequently, even when the infusion of large numbers of CAR-bearing T cells is supplemented with exogenous growth factors, such as interleukin-2 (IL-2), survival *in vivo* is poor and antitumor activity minimal[10,11]. By contrast, small numbers of CTLs with native receptor specificity directed to persistent human viruses such as EBV can survive long term after infusion and eradicate even bulky EBV-associated malignancies, such as Hodgkin's disease and nasopharyngeal cancer[2,12–14]. A contributing factor to the superior survival and function of EBV-specific CTLs is that engagement of their native receptors by EBV-infected B cells produces extensive co-stimulation during their preparation *ex vivo* and by encounters with (latent) viral antigens on antigen-presenting cells *in vivo*[15].

This knowledge has given rise to the concept of engineering antigen-specific CTLs to provide them with a second specificity for tumor antigens[16–18]. However, many tumors generate their own immunosuppressive environment[19,20], and it is unknown whether the desirable functional characteristics of CTLs would be retained in individuals with cancer once these cells co-express a CAR that retargets them to a tumor-associated antigen. We therefore administered EBV-specific CTLs expressing such a CAR to individuals with neuroblastoma to determine whether the survival times of these CAR-CTLs are indeed longer than those of ATCs expressing the same tumor-directed CAR. We made this comparison in the same individuals by expressing functionally identical but molecularly distinguishable tumor-specific CARs in the two discrete T cell populations. Thus, each subject acted as a 'self control', avoiding the confounding variables otherwise inherent in a comparison of CTL versus ATC behavior in a heterogeneous group of humans with cancer. Here we show the superior persistence and retained cytotoxicity of CAR-CTLs compared to CAR-ATCs, as well as measurable tumor responses after infusion of these genetically engineered cells into subjects with neuroblastoma. With additional

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Table 1 Subject characteristics

Subject number	Age, sex	Disease stage[a] and status at treatment	Sites of evaluable tumor[b]	Dose of each cell product[c] (cells per m^2)	Response at 6 weeks after treatment
1	10, M	4 (*MYCN*$^-$); relapsed (bulk)	Bone marrow, bone, pleura	2×10^7	PD
2	11, M	4 (*MYCN*$^-$); refractory (bulk)	Liver, bone, diaphragm, lung	2×10^7	PD
3	4, F	4 (*MYCN*$^-$); refractory (bulk)	Extradural/calvarial mass	2×10^7	PR by MIBG scan; CR by 16 weeks
4	10, F	4 (*MYCN*$^+$); relapsed (NED)	NED	1×10^8	NED
5	9, F	4 (*MYCN*$^+$); relapsed (bulk)	Bone, brain, dura	1×10^8	SD
6	15, F	4; relapsed (bone marrow)	Bone marrow	1×10^8	NED (marrow clear)
7	3, M	4 (*MYCN*$^+$); refractory (bulk)	Abdominal mass, bone, bone marrow, lung	1×10^8	SD
8	5, F	3 (*MYCN*$^+$); relapsed (bulk)	Left scapular lesion	1×10^8	Tumor necrosis
9	6, F	4 (*MYCN*$^+$); relapsed (NED)	NED	1×10^8	NED
10	6, F	4 (*MYCN*$^+$); relapsed (bulk)	Liver	2×10^8	Tumor necrosis
11	7, M	4 (*MYCN*$^-$); relapsed (NED)	NED	2×10^8	NED

[a]Disease stages (3 and 4) are based on criteria of the Evans system. The *MYCN* oncogene status is given in parentheses. *MYCN*$^+$ indicates amplified *MYCN*; *MYCN*$^-$ indicates nonamplified *MYCN*. NED, no evidence of disease; PD, progressive disease; PR, partial response; CR, complete response; SD, stable disease; bulk, extensive disease. [b]Disease was evaluated before treatment and 6 weeks after treatment. [c]Dose of each transduced T cell population given.

refinements and further clinical testing, infusion of CAR-CTLs may provide a general approach to the cell therapy of cancer.

RESULTS

Modification, phenotype and *ex vivo* activity of CTLs and ATCs

Both CTLs and ATCs were transduced with a CAR directed to the GD2 antigen, which is present on the tumor cells of most individuals with neuroblastoma, a pediatric malignancy derived from neural crest cells[21]. The GD2-specific CAR vectors were made from the same 14G2a antibody[16], but each incorporated a distinguishable noncoding 3′ oligonucleotide. Through PCR analysis, these distinct signals allowed us to determine the proportion of GD2 signal coming from each vector source (**Supplementary Fig. 1** and **Supplementary Methods** online). These distinguishable CARs were introduced into autologous ATCs and EBV-specific CTLs from each individual. We rotated the transducing vector between ATCs and CTLs in consecutive subjects to ensure that apparent differences in persistence and function between the transduced cells would not be due to differences in vector transduction efficiency or function. Eleven individuals with neuroblastoma who had EBV-specific IgGs (indicating persistent virus infection) received a single injection of an equal number of CAR-CTLs and CAR-ATCs, for a total dose of 2×10^7 to 2×10^8 cells (**Table 1**).

Before infusing the modified T cells, we characterized their phenotype and *ex vivo* function. The transduction efficiency for both cell types was consistently above 35% (**Fig. 1a**,**b**), with a good correlation between the percentages of positive cells found by PCR and by immunofluorescence, with the mean (± s.d.) efficiency for ATCs exceeding that for CTLs ($P = 0.02$ for each comparison). CAR-ATCs and CAR-CTLs both consisted of a polyclonal mixture of CD4$^+$ and CD8$^+$ T cells with few CD56$^+$ natural killer (NK) cells present, but the ratio of CD4$^+$ to CD8$^+$ cells was consistently higher in the CTL population than in the ATC population ($P = 0.05$) and covered a wider range of values (**Fig. 2a**). At the time of freezing, 2.2–4.4% of cells in the ATC population had a central memory (CD45RO$^+$CCR7$^+$CD62L$^+$) phenotype, whereas 42–76% had an effector memory phenotype (CD45RO$^+$CCR7$^-$CD62L$^-$; **Fig. 2b**). Between 23% and 44% were CD45RO$^-$ and CD45RA$^+$ (**Fig. 2b**), showing the expected mixture of naive and memory T cells[22]. CAR-CTLs at freezing were uniformly CD45RO$^+$ and CD45RA$^-$ and uniformly CCR7$^-$ and CD62L$^-$, so their phenotype was consistent with the presence of effector memory cells (**Fig. 2b**). CXCR4, (the receptor for SDF1, a chemokine associated with marrow homing) was more highly expressed on ATCs ($P = 0.02$) than on CTLs, but all other chemokine receptors and adhesion molecules tested were comparably expressed by the two populations (**Fig. 2c**), suggesting that the ATCs and CTLs would have similar trafficking properties *in vivo*.

To ensure that the transduced CTLs retained their EBV specificity (mediated through their native receptor) and that both CAR-CTLs and CAR-ATCs gained specificity against GD2$^+$ neuroblasts, we measured cytotoxic effector function against EBV$^+$ B cells and GD2$^+$ neuroblastoma cells. The transduced CTLs killed autologous but not allogeneic EBV$^+$ B cells (lymphoblastoid cell lines, LCLs), whereas the transduced ATCs failed to kill either of these targets (**Fig. 2d**). Conversely, an allogeneic GD2$^+$ neuroblast line (LAN-1), which expresses few major histocompatibility complex (MHC) class I molecules, was effectively killed by both CAR-CTLs and CAR-ATCs (**Fig. 2d**). As expected, therefore, CAR-CTLs recognize and kill EBV$^+$ target cells through their native receptor (in an MHC-restricted manner), whereas both CAR-CTLs and CAR-ATCs kill GD2$^+$ neuroblasts through their MHC-unrestricted chimeric receptor.

In vivo persistence of modified CTLs and ATCs

We anticipated that the CAR-CTLs would persist at an initially higher level than the CAR-ATCs because of the additional co-stimulation they

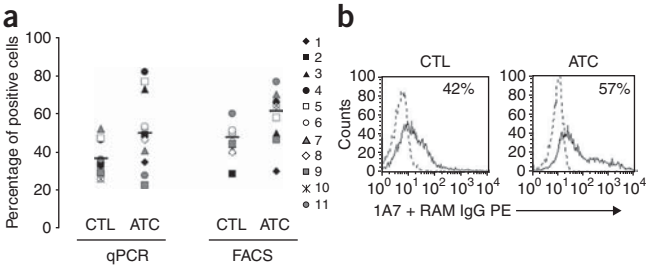

Figure 1 Transduction of CTLs and ATCs with GD2-specific CARs. (**a**) Transduction efficiency of subject-derived CTLs and ATCs by the GD2 vectors, as evaluated by quantitative PCR (qPCR, left) and FACS (right). Each symbol represents 1 of the 11 individual subjects, and the horizontal lines indicate the mean group value. Surface expression by FACS and qPCR amplification are described in Methods. (**b**) FACS analyses of receptor expression shown in more detail for subject 5. Percentages represent the proportion of transduced cells.

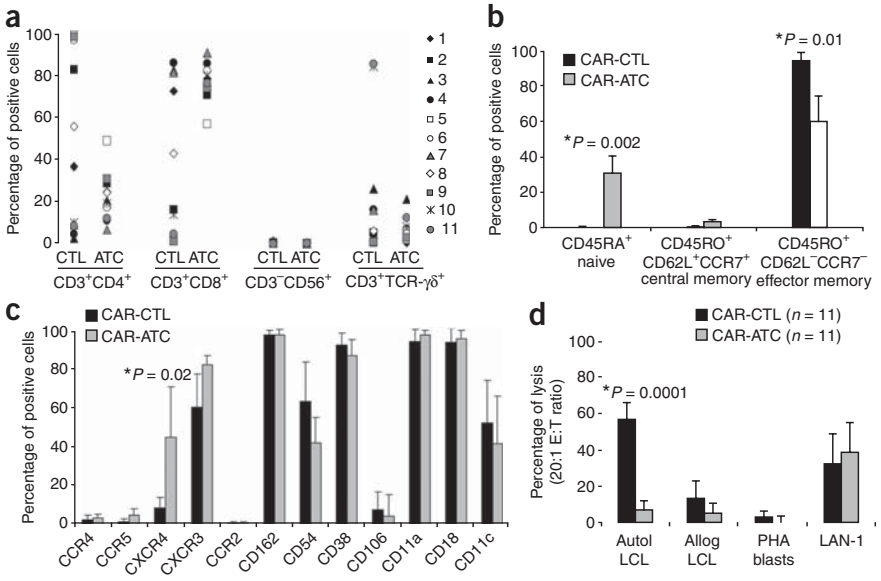

Figure 2 Immunophenotypes of CAR-transduced CTLs and ATCs. (**a**) Phenotypic composition of CTL and ATC population after transduction with the GD2-specific CARs. Percentages of $CD4^+$ and $CD8^+$ T cells, natural killer cells ($CD3^-CD56^+$) and T cells expressing TCR-γδ are shown. Each symbol represents a transduced cell line infused into a single subject. A significant difference between CTLs and ATCs was observed only for the percentage of $CD8^+$ cells ($P = 0.05$). (**b**) Expression of naive, central memory and effector memory surface markers on GD2-specific CAR-CTLs and CAR-ATCs. The data are means ± s.d. (**c**) Expression of chemokine receptors and adhesion molecules on GD2-specific CAR-CTLs and CAR-ATCs. The data are means ± s.d. (**d**) Results of standard ^{51}Cr release assay at an effector:tumor cell (E:T) ratio of 20:1. Data represent the mean ± s.d. percentage of specific chromium released from the CAR-CTLs and CAR-ATCs generated from each of the 11 subjects. Targets were autologous LCLs, allogeneic LCLs, autologous PHA blasts and LAN-1 cells.

received *ex vivo* from the EBV antigens expressed by autologous lymphoblastoid cell lines[23] and subsequently persist longer because they had the advantage of activation through their native receptors by endogenous antigen and concomitant co-stimulation from EBV-infected B cells[24]. Thus, in each subject, we measured the PCR signal from the vector associated with CAR-CTLs and from the vector associated with CAR-ATCs. As expected, within 24 h of infusion, the PCR signal from the CAR-CTLs consistently reached higher levels than that from the CAR-ATCs and was detectable beyond 6 weeks, compared to only 3 weeks for the modified ATCs (**Fig. 3**). These higher initial and subsequent CAR-CTL numbers in the circulation seem to be the consequence of *in vitro* and then *in vivo* native antigen receptor engagement rather than the result of any intrinsic differences in antigen-independent survival between CAR-ATCs and CAR-CTLs (**Supplementary Table 1**, **Supplementary Figs. 2** and **3**, and **Supplementary Results** online). Overall, the mean ± s.e.m. area under the curve (positive cells × duration of signal) was more than tenfold higher for CAR-CTLs than for CAR-ATCs (0.189 ± 0.082 vs. 0.014 ± 0.004, $P = 0.05$; **Fig. 3**).

It is unlikely that the number of effector cells infused had a substantial impact on the outcome of the experiments. Indeed, as we have previously observed[21], the percentages of gene-modified ATCs and CTLs in peripheral blood do not change if larger numbers of cells are infused[15]. Nor were these percentages increased in the present study after partial lymphodepletion mediated by monoclonal antibodies to CD45 (ref. 25) (**Supplementary Results**). It is also possible that the observed differences in the numbers and persistence of CAR-ATCs versus CAR-CTLs in peripheral blood resulted from increased trafficking to marrow by CAR-ATCs, which typically express the CXCR4 receptor, or by immediate trafficking to tumor sites. However, less than half of the ATC population (mean 43.8%) expressed CXCR4 (**Fig. 2c**), and marrow aspirates and tumor biopsies lacked evidence of increased CAR-ATC infiltration (data not shown).

CAR-CTLs show sustained proliferation and cytotoxicity *in vivo*

CAR-expressing CTLs and ATCs introduced into individuals with advanced cancer can become anergic[20]. This functional loss has multiple causes, including increased numbers and activity of T_{reg} cells and secretion of inhibitory cytokines such as transforming growth factor-β by the tumor itself[19,26]. We therefore asked whether the CAR-CTLs in peripheral blood at 4–24 weeks after infusion had become unresponsive. After culturing peripheral blood mononuclear cells (PBMCs) from six individuals with their autologous EBV-expressing B cells, we measured the intensity of the CAR-derived signal by PCR amplification (**Fig. 4**). Even after 24 weeks in the treated subjects, the CAR-CTLs consistently expanded in response to native receptor stimulation by EBV^+ target cells (**Fig. 4b**, **Supplementary Fig. 4** and **Supplementary Results** online), resulting in a 2–20-fold enrichment of PCR signal. We found no evidence for such expansion in the CAR-ATC population (**Fig. 4a**), although highly specific methods for selective expansion of CAR-ATCs in *ex vivo* cultures are lacking. Enrichment of CAR DNA was accompanied by a corresponding enrichment of CAR expression on the cell surface (**Fig. 4c**). To show that these CAR-enriched cells (from a 2-week sample) also retained cytotoxic activity against cells targeted through both native and chimeric receptors, we cultured them with autologous EBV^+ targets and allogeneic $GD2^+$ neuroblasts and confirmed the

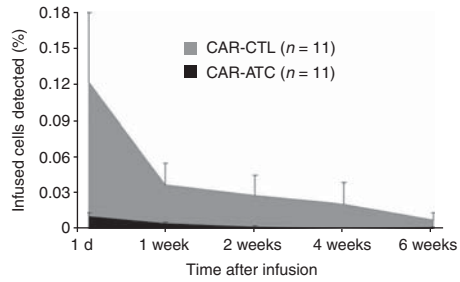

Figure 3 *In vivo* persistence of infused CAR-CTLs versus CAR-ATCs in peripheral blood as determined by real-time quantitative PCR. A comparison of mean ± s.e.m. areas under the curve of the qPCR signal for ATCs and CTLs detected in PBMCs of treated subjects at the indicated times after infusion is shown. Five of the subjects (7 –11) received monoclonal antibodies to the common leukocyte antigen (CD45)[25] at 2–3 d before ATC and CTL infusion and had 44–91% depletion of endogenous circulating lymphocytes[25]. There were no measurable differences between the areas under the curve for either ATCs or CTLs between subjects 1–6 (no CD45) and 7–11 (CD45-treated).

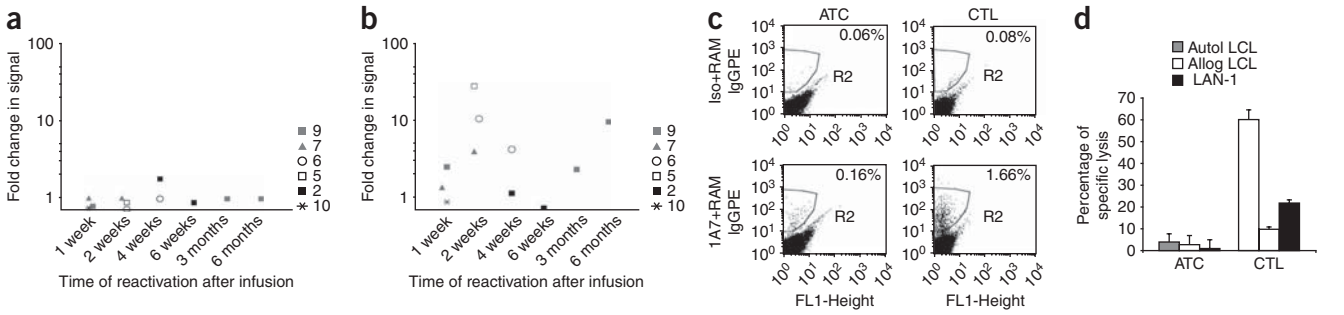

Figure 4 Reactivation of CAR-CTLs *ex vivo*. (**a**,**b**) Fold change in the level of GD2-receptor-transgene positivity in the cultures before and after exposure to EBV antigen in ATCs (**a**) and CTLs (**b**). PBMCs were collected at increasing times after infusion (indicated on the *x* axis) and re-expanded *ex vivo* in the presence of EBV$^+$ targets (total of three or four stimulations). Each symbol represents a single subject. (**c**) Change in percentage of cells expressing the GD2 chimeric receptor on *ex vivo*-reactivated CTLs. The *y* axis for the top panels indicates the isotype control, whereas for the bottom panels it indicates antibody 1A7. Percentages represent the proportions of cells expressing the CAR. (**d**) Cytotoxicity of *ex vivo*-reactivated CTLs against autologous LCLs, allogeneic LCLs or LAN-1 cells. The data are means ± s.d. of triplicate experiments.

persistence of recognition and killing through both native and chimeric receptors (**Fig. 4d**). Hence, even after prolonged *in vivo* survival, the CAR-expressing CTLs can remain responsive to signals through their native receptors and retain tumor-directed cytotoxic effector function through the chimeric receptors. By contrast, CAR-ATCs could not be detected among mononuclear cells in peripheral blood (even after mitogen stimulation) and thus were not available for assessment.

Tumor responses after infusion of modified T cells

Eleven individuals with neuroblastoma were treated with CAR-CTLs and CAR-ATCs. Four of the eight with evaluable tumors had evidence of tumor necrosis or regressions (summarized below), including a sustained complete remission (**Table 1**). None developed detectable antibodies to CAR-CTLs, and there were no adverse events attributable to the genetically modified T cells in the 11 subjects followed for up to 24 months.

Subject 3, a 4-year-old female whose disease was refractory to high-dose chemotherapy and radiation, had a persistent lesion (4 cm) in the left parietal region of the skull. This mass arose from the bone, extended into the extradural space (**Fig. 5a**) and accumulated meta-iodobenzylguanidine (MIBG). The extradural component resolved within 6 weeks after treatment, and the MIBG scan became negative 4 months later. This subject has remained in complete remission for more than 12 months without further therapy.

Subject 6, a 15-year-old female with recurrent disease after chemotherapy, irradiation and double autologous stem cell transplantation, had extensive marrow disease repeatedly documented on bilateral bone biopsies (**Fig. 5b**). Re-examination 4 weeks after treatment revealed no residual tumor in either biopsy site, but 4 weeks later extramedullary disease became evident (data not shown).

Subject 8, a 5-year-old female with recurrent disease after chemotherapy, irradiation, and autologous stem cell transplantation, had a slowly progressing lesion (10 cm) in the left scapula. Two weeks after cell infusion, she developed fever, local (axillary) lymphadenopathy and then pain at the site of the tumor. Computed tomography imaging showed necrosis in the scapular lesion and axillary lymph nodes (data not shown); necrosis was confirmed by biopsy (**Supplementary Fig. 5** online). A residual tumor persisted, but its size remained stable at 12 months after treatment (data not shown).

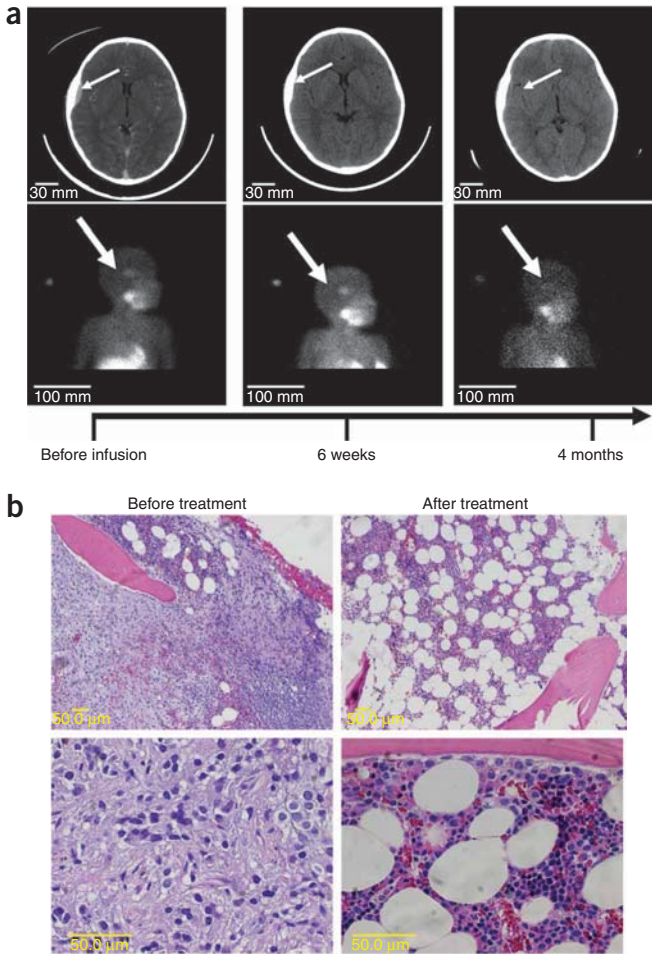

Figure 5 Resolution of neuroblastoma in subjects 3 and 6 after infusion of genetically engineered T cells. (**a**) Sequential anatomic (MRI) and functional (MIBG) imaging of the head and neck of subject 3, a 4-year-old girl with relapsed metastatic neuroblastoma, with an extradural mass and overlying calvarial bone involvement. Progressive resolution of the extradural mass and loss of MIBG uptake in the lesion by 4 months after infusion are indicated by arrows. The signal in the salivary glands remains due to normal uptake. (**b**) H&E stain showing postinfusion normalization of bone marrow in subject 6 after extensive infiltration by neuroblasts.

Subject 10, a 6-year-old female with recurrent disease after chemotherapy, radiation and autologous stem cell transplantation, had multiple metastatic lesions (2–6 cm) in the liver. Twelve days after receiving the modified T cells, she developed fever and hepatalgia. Computed tomography imaging showed hyperlucency of the multiple liver lesions (data not shown), a finding indicative of necrosis that was subsequently confirmed on biopsy (**Supplementary Fig. 6** online). The liver lesions were rapidly resurgent (data not shown), leading to the subject's death at 4 months after treatment.

We were unable to relate these responses to the clinical characteristics of the individual tumors (such as size, extent or genetic markers) or to the infused dose of the genetically engineered T cells. Needle biopsy specimens of necrotic tumor sites from subjects 6, 8 and 10, although revealing T cell infiltrates, lacked PCR signal for CAR-CTLs or CAR-ATCs, suggesting that the observed tumor responses may have resulted from indirect mechanisms of cytotoxicity[27].

DISCUSSION

Our results show that human virus-specific CTLs expressing a synthetic chimeric antigen receptor directed to a nonviral tumor–associated antigen persist in higher numbers and for longer times after administration to individuals with cancer than do activated T cells expressing the same receptor but lacking viral specificity. Moreover, these engineered CTLs retain the ability to recognize both virus-infected and tumor targets through their native and chimeric receptors, respectively, and their infusion may be associated with subsequent tumor necrosis or sustained complete remission. Hence, virus-specific CTLs seem to offer distinct advantages as tumor-directed effector cells.

The chimeric antigen receptor we describe was derived from a combination of an antibody and a T cell receptor, but retargeting may also be achieved by cloning the MHC-peptide–binding α and β chains of the native T cell receptor (TCR-αβ) itself and transferring them to polyclonal T cells[1,28]. Although encouraging results have been obtained[1], the αβ receptors are MHC restricted, so that multiple receptors and vectors must be made to cover common MHC polymorphisms for every peptide. More problematically, many tumors, including neuroblastoma, evade T cell immune responses by down-regulating their MHC expression or by degrading their antigen-processing mechanisms[19,29,30]. Thus, T cells expressing αβ receptors may be of value for only a restricted number of tumors.

A limitation of CAR-mediated T cell therapy has been the brief persistence of the engineered cells, even when they are infused in large numbers. We suggest that CAR-CTLs were able to overcome this limitation in the present study (**Fig. 3**) because of the *ex vivo* and subsequent *in vivo* antigen stimulation and co-stimulation they received after engagement of their native receptor. The almost immediate difference in survival seen between CAR-CTLs and CAR-ATCs at less than 24 h after infusion is unlikely to have resulted from differences in *in vivo* proliferation and more probably reflects the greater ability of CAR-CTLs to recirculate in the peripheral blood, perhaps by avoiding trapping in capillary beds[31]. The subsequent survival advantage of CAR-CTLs over CAR-ATCs does not seem to be an artifact resulting from differences in the intrinsic, antigen-independent survival ability of the two populations (**Supplementary Figs. 2** and **3**), but rather a consequence of continuing native receptor stimulation by latent EBV antigen on endogenous antigen-presenting cells. Both *in vitro* and *in vivo* preclinical studies have shown[16,18] that CAR-CTL survival requires continued native receptor stimulation; otherwise, these cells cease to divide and undergo apoptosis within 2–3 weeks (**Supplementary Fig. 4**). Moreover, in an earlier clinical study[15], we adoptively transferred an autologous CTL monoculture containing EBV-, cytomegalovirus (CMV)- and adenovirus-specific CTLs to stem cell transplant recipients and observed persistence of the autologous CMV-specific and EBV-specific CTLs in the latently infected CMV- and EBV-seropositive recipients. By contrast, adenovirus-specific CTLs persisted only when there was concomitant adenovirus infection of the recipient (and thus antigen stimulation). Because the CMV-, EBV- and adenovirus-specific CTLs used in the study had all received the same *in vitro* stimulation and co-stimulation from the same antigen-presenting cells, we concluded that native receptor stimulation *in vitro* is insufficient to produce subsequent long-term persistence of CTLs in peripheral blood and that *in vivo* engagement of the native receptor is required as well.

Our CTLs and ATCs each expressed a chimeric receptor containing only the ζ-chain of the T cell receptor. An alternative means of preparing CAR^+ T cells is to incorporate additional costimulatory endodomains such as CD28 into the CAR[32] or to express transgene-encoded costimulatory ligands from the effector T cell itself[33]. These strategies initiate costimulatory signaling to the nucleus after CAR engagement and thereby help to compensate for the lack of physiological co-stimulation when chimeric receptors engage most tumor cells[32]. Despite promising results in preclinical testing[34], such constitutively expressed 'compound' CARs cannot mimic the complicated temporal or spatial pattern of costimulatory signals required for the full and sustained activation and function of effector T cells, suggesting that they may undergo suboptimal activation unless the targeted tumor itself expresses a range of additional costimulatory receptors or ligands[34]. By contrast, when virus-infected B cells engage EBV-CTLs through their native receptor, a panoply of physiological co-stimulation follows[35], increasing the likelihood of an effective T cell response. Similarly, in our study, the T cells were activated *ex vivo* by a CD3 mitogenic antibody (OKT3) alone before transduction. Other investigators have used a mixture of OKT3 and the costimulatory molecule CD28 to prepare ATCs. Although this combination may facilitate *ex vivo* expansion of ATCs to the larger numbers infused in those studies, the transience of the activation response to CD28 and its incompleteness as a costimulatory signal[26–29] means that such cells may not have *in vivo* survival superior to that of the OKT3-stimulated ATCs used here[36].

We found no correlation between the dose of the genetically modified cells infused and their subsequent numbers in peripheral blood, and neither of these measures correlated with tumor response, a result similar to previous observations[13,14]. Indeed, with few exceptions, the precise mechanisms by which T lymphocytes damage or destroy solid human tumors *in vivo* are far from defined[7,14]. Although it is possible that earlier tumor biopsies would have enabled detection of gene-modified cells, an alternative explanation is that effective T cell–based cancer therapies stimulate an immune cascade involving multiple types of antitumor effector cells, including T lymphocytes with different origins and different antigen specificities from those actually infused[27,28]. Nonetheless, given that functional CAR-CTLs persist after infusion, we suggest that the EBV-specific CAR–expressing CTLs, rather than the modified ATCs, initiated the tumor responses and necrosis observed in this study. It is noteworthy that even large numbers of CAR-ATCs ($>1 \times 10^9$ cells) have failed to influence tumor survival or growth in previous studies[10], supporting a clinically important antitumor contribution from the EBV-specific CTLs expressing the chimeric antigen receptor. Thus, we have shown that a CAR introduced into virus-specific CTLs has biological advantages over an identical CAR introduced into OKT3-activated primary T cells. This general model will probably be of value in studies of other

combinations of CARs and native receptors[17,37] and may yield improved clinical results as we implement refinements made on the basis of a better understanding of the antitumor mechanisms involved[1,31].

METHODS

Subjects. This study was open to individuals who had recurrent or refractory advanced-stage neuroblastoma or were unable to receive or complete standard therapy (**Table 1**). All were seropositive for IgG specific for the viral capsid antigen of EBV, had appropriate organ function and performance scores and lacked human antibodies to mouse antigen or to rat antigen. We obtained 30–60 ml of peripheral blood for production of EBV-transformed LCLs, phytohemagglutinin blasts, and gene-modified ATCs and EBV-CTLs under current 'good tissue practice' conditions. The investigation was approved by the US Food and Drug Administration, the Recombinant DNA Advisory Committee and the Institutional Review Board of Baylor College of Medicine. All participants or their guardians gave informed consent on enrollment. Assent given by the participants was as is appropriate for their age and level of development.

We treated subjects with single infusions of CAR-ATCs and CAR-CTLs at the doses given in **Table 1** and evaluated them for tumor responses 4–6 weeks after infusion[38]. We assessed toxicity on the basis of physical examinations, performance tests and laboratory tests of organ function conducted at 1, 2, 4 and 6 weeks after infusion and again at 3, 6, 9 and 12 months after infusion. We tested the retroviruses for their replication competence before the study and at 3, 6, and 12 months after infusion.

Generation of retroviral constructs. The retroviral vector encoding the scFv domain targeting the GD2a antigen has been previously described[16]. To generate two PCR-distinguishable retroviral constructs (Zeta-5 and Zeta-6), we opened SFG.lacZ (a splicing Maloney murine leukemia virus–based vector generously provided by R.C. Mulligan)[39] with *Nco*I and *Bam*HI, removing the *lacZ* gene. We generated a pair of double-stranded DNA cassettes with *Xho*I- and *Bam*HI-compatible ends by annealing oligonucleotides with sequences 5′-TCGAACGCGTCATCATC-3′ with 5′-CTAGCTACTACT GCGCA-3′ and oligonucleotides with sequences 5′-TCGATGCATGCAAC CTC-3′ with 5′-CTAGCTCCAACGTACGT-3′, respectively. We cloned the chimeric receptor with either of these cassettes into the opened SFG vector to generate the Zeta-5 and Zeta-6 vectors, which on confirmatory sequencing differed only in a 12–base pair stretch between the receptor stop codon and the 3′ long terminal repeat. This 12–base pair stretch includes an *Mlu*I site in Zeta-5 and an *Sph*I site in Zeta-6.

Generation and validation of retrovirus packaging cell lines. We transfected the Phoenix Eco cell line (American Type Culture Collection SD3444) with vectors Zeta-5 or Zeta-6 to generate a pseudotyped transient supernatant, which we used repeatedly to transduce PG13 cells (gibbon ape leukemia virus pseudotyping packaging cell line; American Type Culture Collection CRL-10686). We detected the GD2-specific CAR by FACS analysis on the transduced PG13 cells with the idiotypic antibody 1A7 (TriGem, Titan Pharmaceutical)[16]. After single-cell cloning, we used the highest-titer clone for each vector to establish a master cell bank. We released the clones for clinical use only after safety testing and vector sequencing; none produced replication-competent retrovirus. We stored the final viral supernatant at –80 °C and tested it before clinical release. Virus titers ranged from 6×10^5 to 1.6×10^6 virus particles per ml.

Generation and transduction of activated T cells and cytotoxic T cells. To generate CAR-ATCs, we transduced PBMCs, activated them with OKT3 (Ortho Biotech) antibody and recombinant human IL-2 (rhIL-2, 100 U ml^{-1}, Proleukin Chiron) in 24-well plates precoated with a recombinant fibronectin fragment (FN CH-296, Retronectin Takara). At 48 h after transduction, we expanded T cells with rhIL-2 (50 U ml^{-1}) added every 3 d. We generated EBV-CTLs as previously described[23] with PBMCs stimulated with gamma-irradiated (40 Gy) autologous LCLs. For transduction, we plated EBV-CTLs (obtained after at least three stimulations) in retronectin-coated 24-well plates with the retroviral supernatant. We transduced CAR-ATCs on day 3 after culture initiation and froze them on day 15 (± 3 d), and we transduced CAR-CTLs on day 23 (± 5 d) after culture initiation and froze them on day 45 (± 9 d).

Immunophenotyping. We stained ATC and CTL lines with monoclonal antibodies to CD3, CD4, CD8, CD56, CD19, TCR-αβ, TCR-γδ, CD62L, CD27, CD28, CD45RA, CD45RO, CCR2, CCR4, CCR5, CCR7, CXCR3, CXCR4, CD162, CD54, CD38, CD106, CD11a, CD11c and CD18 (Becton-Dickinson). We included control samples labeled with appropriate isotype-matched antibodies in each experiment. We detected the 14g2a CAR with the idiotypic antibody 1A7 (ref. 16). We analyzed the cells by FACScan (Becton-Dickinson) equipped with a filter set for four fluorescence signals.

Chromium release assay. We evaluated the cytotoxic specificity of ATCs and EBV-CTLs with a standard 4-h ^{51}Cr release assay, as previously described[8,17].

Real-time quantitative PCR. We used quantitative PCR to quantify the retrovirus integrants for both the Zeta-5 and the Zeta-6 vectors in PBMCs. After DNA extraction with the QIAamp DNA Blood Mini Kit (Qiagen), we amplified the DNA in duplicate with primer and probe sequences (Applied Biosystems) for the Zeta-5 and Zeta-6 vectors in the ABI Prism 7700 Sequence Detector (Perkin-Elmer). The baseline range was set at cycles 6–15 with the threshold at ten standard deviations above the baseline fluorescence. To generate DNA standards, we established single-cell clones from the Jurkat cell line and transduced them with either Zeta-5 or Zeta-6. We serially diluted DNA obtained from these clones with DNA extracted from nontransduced control Jurkat cells (**Supplementary Fig. 1** and **Supplementary Methods**).

Before and at different times afterinfusion, we extracted DNA was extracted from the PBMCs of subjects. We amplified both marker sequences as described above and plotted the results as the mean percentage of positive cells for all 11 subjects, with a known positive cloned line used as a reference standard. Comparison of mean ± s.e.m. areas under the curve for ATCs and CTLs was based on data from the primary PCR analysis above.

Statistical analyses. We relied on descriptive statistics (means, ranges and standard deviations or standard errors) to summarize most datasets. We compared the data by the two-tailed *t*-test or by the nonparametric Wilcoxon signed-rank test (area-under-the-curve analysis). Differences with a P value of <0.05 were considered statistically significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

This study was developed and designed by M.A.P., C.M.R., H.E.H. and M.K.B. The principal investigators on the clinical trial were G.D.M. and H.V.R. M.A.P. and C.R. performed the preclinical studies. M.A.P. and Z.M. manufactured the clinical vectors, and M.A.P. and G.D. designed the vectors and developed the gene transfer monitoring studies. B.S., G.D., E.L. and E.Y. performed the PCR and immune reconstitution studies. H.L.W. and H.L. provided statistical support. C.M.R., M.H.H. and B.S. supervised CTL and ATC preparation, and A.P.G. supervised quality assurance. B.S., G.D. C.M.R., H.E.H. and M.K.B. contributed to the writing of the paper.

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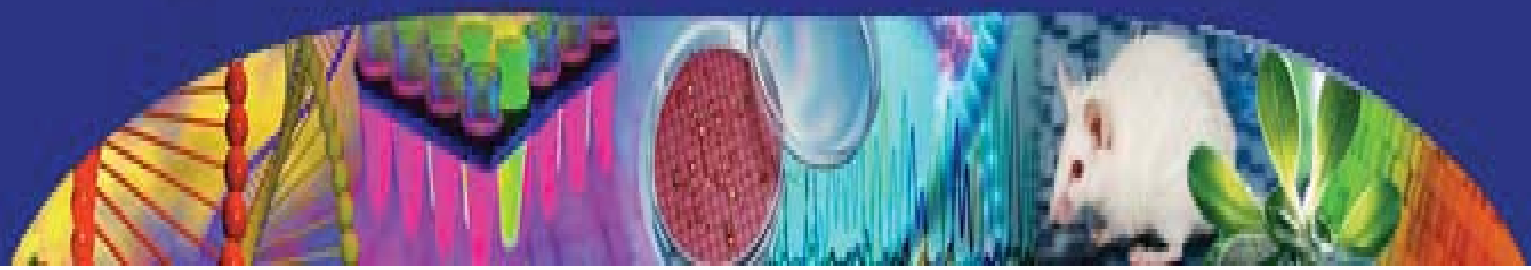

CD19 Protocols

Antigen Target	**CD19**
Antigen Expression	**Expressed on mature and immature B cells but not hematopoietic stem cells**

	Chimeric Antigen Receptor Trials Registered with OBA
Title	**Protocol # 543: Phase I Study to Evaluate the Safety of Cellular Immunotherapy for CD19+ Follicular Lymphoma Using Autologous T Cell Cytolytic Clones Genetically Modified to be CD19-Specific and Express HyTK (1st generation)**
PI	**Laurence J. Cooper, M.D., Ph.D.**
Vector and Signaling Moieties	**Plasmid/CD3 Zeta**
Status	**Closed**
Doses Proposed	**$1x10^8$ cells/m^2 – $4x10^9$ cells/m^2**
Lymphodepletion	**Rituximab at 375 mg/m^2 x 1 to reduce the number of circulating normal and malignant B cells prior to adoptive immunotherapy. Fludarabine 25 mg/m^2/day for 5 days to help eradicate and prevent a host immune response directed against the genetically modified T cells.**
Subjects Enrolled	**2**
Summary of Unexpected and Related Events	**1 hospitalization due to fever and chills 7 hours after infusion.**
Summary of Results	**Jensen, M.C., *et. al*. (2010). Antitransgene Rejection Responses Contribute to Attenuated Persistence of Adoptively Transferred CD20/CD19-Specific Chimeric Antigen Receptor Redirected T Cells in Humans. *Biol of Blood and Marrow Transplantation* 1-12.**

Title	**Protocol # 721: A Phase I Trial for the Treatment of Purine Analog-Refractory Chronic Lymphocytic Leukemia Using Autologous T Cells Genetically Targeted to the B Cell Specific Antigen CD19**
PI	**Renier Brentjens, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Active**
Doses Proposed	**$1x10^8$, $3x10^8$, $1x10^9$ cells**
Lymphodepletion	**Cyclophosphamide chemotherapy at escalating dose levels of 1.5 gm/m^2, 2.2 gm/m^2 and 3.0 gm/m^2 prior to the infusion of the tumor targeted T cells**
Subjects Enrolled	**4**
Summary of Unexpected and Related Events	**Subject became hypotensive, required intubation, subsequently developed bradycardia, and expired when the healthcare proxy requested withdrawal of support.** **Brentjens, R., *et. al*. (2010). Treatment of Chronic Lymphocytic Leukemia with Genetically Targeted Autologous T Cells: Case Report of an Unforeseen Adverse Event in a Phase I Clinical Trial. *Molecular Therapy* 18: 666-668.**
Summary of Results	**None**

Title	**Protocol # 776: Phase I Study of CD19 Chimeric Receptor Expressing T Lymphocytes in B-Cell Non-Hodgkin's Lymphoma and Chronic Lymphocytic Leukemia**
PI	**Helen E. Heslop, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Active**
Doses Proposed	**$2x10^7$, $1x10^8$, $2x10^8$ cells/m^2**
Lymphodepletion	**No**
Subjects Enrolled	**Lines manufactured for 6 subjects with 4 infused thus far.**
Summary of Unexpected and Related Events	**None**
Summary of Results	**So far, it has been shown that CAR-CD19-28zeta+ cells survive better than CAR-CD19zeta+ cells.**
Title	**Protocol # 793: Pilot Study of Redirected Autologous T Cells Engineered to Contain Anti-CD19 Attached to TCRζ and 4-1BB Signaling Domains in Patients with Chemotherapy Resistant Or Refractory CD19+ Leukemia and Lymphoma**
PI	**David L. Porter, M.D.**
Vector and Signaling Moieties	**Lentivirus/ 4-1 BB:CD3 zeta**
Status	**Active**

Doses Proposed	**$5x10^9$ – $1x10^{10}$ cells**
Lymphodepletion	**Yes, CARs infused two days post chemotherapy**
Subjects Enrolled	**2 enrolled, none dosed**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 813: Pilot Study of Genetically Modified Haploidentical Natural Killer Cell Infusions for B-lineage Acute Lymphoblastic Leukemia**
PI	**Dario Campana, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/4-1 BB:CD3 zeta**
Status	**Active**
Doses Proposed	**$1x10^6$ – $5x10^7$ cells/kg**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m^2/day x 5 days**
Subjects Enrolled	**1 (infusion planned for 6/8/2010)**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

Title	**Protocol # 892: Phase I Study to Evaluate Cellular Immunotherapy Using Genetically-Modified Autologous CMV x CD19 Bispecific T Cells Following T-Cell Depleted Autologous Peripheral Blood Stem Cell Transplantation for Patients with Recurrent/Refractory Intermediate Grade B-Lineage Non-Hodgkin Lymphoma**
PI	**Leslie L. Popplewell, M.D.**
Vector and Signaling Moieties	**Lentivirus/CD3 zeta**
Status	**Not initiated**
Doses Proposed	**$5x10^7$ – $5x10^8$ cells/m^2**
Lymphodepletion	**Rituximab at 375 mg/m2 to lower the B cell numbers to reduce the recipient's inflammatory response upon receiving the genetically modified CTL and also increase the availability of infused T cells to immediately target lymphoma cells**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 915: Phase I Study of the Administration of Peripheral Blood T Cells or EBV Specific CTLs Expressing CD19 Chimeric Chronic Lymphocytic Leukemia Receptors for Advanced B-Cell Non Hodgkin's Lymphoma and Chronic Lymphocytic Leukemia**
PI	**Helen E. Heslop, M.D.**

Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Active**
Doses Proposed	**$2x10^7$, $1x10^8$, $2x10^8$ cells**
Lymphodepletion	**No**
Subjects Enrolled	**2 dosed (1 as an emergency after FDA approval)**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 922: Adoptive Immunotherapy for CD19+ B-Lymphoid Malignancies using Sleeping Beauty Transposition to Express a CD19-specific Chimeric Antigen Receptor in Autologous Ex Vivo expanded T cells**
PI	**Laurence J. Cooper, M.D., Ph.D.**
Vector and Signaling Moieties	**Plasmid containing 2 transgenes 1) CD19 CAR with CD 28, CD3 zeta and 2) Sleeping Beauty transposase**
Status	**Active**
Doses Proposed	**$5x10^7$ - $5x10^9$ cells/m^2**
Lymphodepletion	**Yes; BEAM radiation conditioning regimen for autologous hematopoietic stem cell transplantation**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

Title	**Protocol # 940: Treatment of B Cell Malignancies with T Cells Expressing an Anti-CD19 Chimeric Receptor: Assessment of the Impact of Lymphocyte Depletion Prior to T Cell Transfer**
PI	**Steven A. Rosenberg, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Active**
Doses Proposed	**$1x10^9$ - $1x10^{11}$ cells/m^2**
Lymphodepletion	**Cyclophosphamide 60 mg/kg, Fludarabine 25 mg/m^2**
Subjects Enrolled	**At least 2**
Summary of Unexpected and Related Events	**Adverse events that have been reported are likely due to expected side effects of IL-2 and one death due to infection with 2009 H1N1.**
Summary of Results	**None**
Title	**Protocol # 945: Phase I/II Study of the Administration of Multi-virus-specific Cytotoxic T Lymphocytes (CTLs) Expressing CD19 Chimeric Receptors for Prophylaxis or Therapy of Relapse of Acute Lymphoblastic Leukemia Post Hematopoietic Stem Cell Transplantation (MultiPRAT)**
PI	**Catherine Bollard, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**

Status	**Active**
Doses Proposed	**1.5x 10^7 – 1.2x10^8 cells/m^2**
Lymphodepletion	**Conditioning regimen for allogeneic hematopoietic stem cell transplantation**
Subjects Enrolled	**Lines manufactured for 7 subjects; None infused as FDA requires evidence of relapse and all are still in remission after transplant**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 985: A Phase I Trial of Precursor B Cell Acute Lymphoblastic Leukemia (B-ALL) Treated with Autologous T Cells Genetically Targeted to the B Cell Specific Antigen CD19**
PI	**Renier Brentjens, M.D., Ph.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Active**
Doses Proposed	**1x10^7 – 1x10^8 cells**
Lymphodepletion	**Cyclophosphamide 3 gm/m^2 cells/kg**
Subjects Enrolled	**1**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

Title	**Protocol # 998: Administration of anti-CD19-Chimeric-antigen-receptor-transduced T cells from the original transplant donor to patients with recurrent or persistent B-cell malignancies after allogeneic stem cell transplantation**
PI	**Michael R. Bishop, M.D.**
Vector and Signaling Moieties	**Retrovirus/CD28, CD3 zeta**
Status	**Not initiated**
Doses Proposed	**$5x10^5$ - $3x10^7$ cells/kg**
Lymphodepletion	**Conditioning regimen for allogeneic hematopoietic stem cell transplantation**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 1003: CD19-specific T Cell Infusion in Patients with B-Lineage Lymphoid Malignancies after Allogeneic Hematopoietic Stem-Cell Transplantation**
PI	**Laurence J. Cooper, M.D., Ph.D.**
Vector and Signaling Moieties	**Plasmid containing 2 transgenes 1) CD19 CAR with CD 28, CD3 zeta and 2) Sleeping Beauty transposase**
Status	**Not initiated**
Doses Proposed	**$1x10^6$ - $1x10^9$ cells/m^2**

Lymphodepletion	**Conditioning regimen for allogeneic hematopoietic stem cell transplantation**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**
Title	**Protocol # 1022: Adoptive Immunotherapy for CD19+ B-cell Malignancies using Sleeping Beauty Transposition to Express a CD19-specific Chimeric Antigen Receptor in Allogeneic Neonatal Ex Vivo expanded T cells**
PI	**Laurence J. Cooper, M.D., Ph.D.**
Vector and Signaling Moieties	**Plasmid containing 2 transgenes 1) CD19 CAR with CD 28, CD3 zeta and 2) Sleeping Beauty transposase**
Status	**Not initiated**
Doses Proposed	**$1x10^6$ – $1x10^9$ cells/m^2**
Lymphodepletion	**Conditioning regimen for allogeneic hematopoietic stem cell transplantation**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

Title	**Protocol # 1025: Pilot Study of Donor Lymphocyte Infusions Using Donor T Cells Engineered to Contain Anti-CD19 Attached to TCR-ζ and 4-1 BB Signaling Domains in Patients with Relapsed CD19+ ALL After Allogeneic Stem Cell Transplantation**
PI	**David L. Porter, M.D.**
Vector and Signaling Moieties	**Lentivirus/ 4-1 BB: CD 3 zeta**
Status	**Not initiated**
Doses Proposed	**$1x10^8$ cells/kg**
Lymphodepletion	**Conditioning regimen for allogeneic hematopoietic stem cell transplantation**
Subjects Enrolled	**0**
Summary of Unexpected and Related Events	**None**
Summary of Results	**None**

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Antitransgene Rejection Responses Contribute to Attenuated Persistence of Adoptively Transferred CD20/CD19-Specific Chimeric Antigen Receptor Redirected T Cells in Humans

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Immunotherapeutic ablation of lymphoma is a conceptually attractive treatment strategy that is the subject of intense translational research. Cytotoxic T lymphocytes (CTLs) that are genetically modified to express CD19- or CD20-specific, single-chain antibody–derived chimeric antigen receptors (CARs) display HLA-independent antigen-specific recognition/killing of lymphoma targets. Here, we describe our initial experience in applying CAR-redirected autologous CTL adoptive therapy to patients with recurrent lymphoma. Using plasmid vector electrotransfer/drug selection systems, cloned and polyclonal CAR^+ CTLs were generated from autologous peripheral blood mononuclear cells and expanded in vitro to cell numbers sufficient for clinical use. In 2 FDA-authorized trials, patients with recurrent diffuse large cell lymphoma were treated with cloned $CD8^+$ CTLs expressing a CD20-specific CAR (along with NeoR) after autologous hematopoietic stem cell transplantation, and patients with refractory follicular lymphoma were treated with polyclonal T cell preparations expressing a CD19-specific CAR (along with HyTK, a fusion of hygromycin resistance and HSV-1 thymidine kinase suicide genes) and low-dose s.c. recombinant human interleukin-2. A total of 15 infusions were administered (5 at 10^8cells/m^2, 7 at 10^9cells/m^2, and 3 at 2×10^9cells/m^2) to 4 patients. Overt toxicities attributable to CTL administration were not observed; however, detection of transferred CTLs in the circulation, as measured by quantitative polymerase chain reaction, was short (24 hours to 7 days), and cellular antitransgene immune rejection responses were noted in 2 patients. These studies reveal the primary barrier to therapeutic efficacy is limited persistence, and provide the rationale to prospectively define T cell populations intrinsically programmed for survival after adoptive transfer and to modulate the immune status of recipients to prevent/delay antitransgene rejection responses.

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KEY WORDS: Cellular immunotherapy, Adoptive therapy, T lymphocyte, Clinical trial

INTRODUCTION

Although conventional chemotherapy, radiation therapy, and antibody therapy can be efficacious in treating lymphoma, relapse and progressive disease are the major sources of patient morbidity and mortality [1,2]. Experimental evidence that the cellular immune system can eradicate lymphoma provides a basis for the development of therapies aimed at amplifying antitumor immune responses [3,4]. The adoptive transfer of lymphoma-specific T cells is one strategy to augment antilymphoma immunity. A significant challenge to executing this strategy is the isolation of T cells specifically reactive to lymphoma. Alternately, the ex vivo derivation of tumor-specific T lymphocytes by genetic modification to express tumor-targeting chimeric antigen receptors (CARs) is a rapidly evolving focus of translational cancer

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immunotherapy [5,6]. Antibody-based CARs are HLA-unrestricted and thus can be used in patient populations with target-antigen–positive tumors.

We have constructed 2 CARs specific for the B cell lineage antigens CD20 and CD19 for the purpose of targeting lymphomas and leukemias [7,8]. When expressed in cytotoxic T lymphocytes (CTLs), these CARs redirect effector cells to lyse B-lineage lymphoma targets [7,8]. Here we report our initial clinical experience in manufacturing and infusing autologous T cells expressing CD20R or CD19R in patients with relapsed B cell lymphoma under City of Hope–held FDA-authorized trials BB-IND-8513/IRB 98142 and BB-IND-11411/IRB 01160, respectively.

MATERIALS AND METHODS

Patients

City of Hope Internal Review Board (IRB) protocols 98142 and 01160 were activated for patient accrual following IRB and Institutional Biological Safety Committee approval, FDA authorization (BB-IND-8513 and BB-IND-11411, respectively), and National Institutes of Health Office of Biotechnology Activities registration (9907-330 and 0207-543, respectively). In brief, for IRB 98142, patients were eligible if they had immunohistopathologically documented $CD20^+$ diffuse large cell lymphoma (DLCL) with a history of recurrent or refractory disease and did not have central nervous system metastases. After leukapheresis, patients began salvage/mobilization chemotherapy, then underwent hematopoietic stem cell transplantation (HSCT). The first of 3 escalating-dose T cell infusions was given at 28 days post-HSCT. For IRB protocol 01160, patients were eligible if they had pathologically documented follicular lymphoma (FL) with evidence of progression after previous rituximab therapy and did not have central nervous system metastases or a history of allogeneic HSCT. These patients were enrolled no sooner than 3 weeks after their most recent cytotoxic chemotherapy.

Plasmid Vectors

The plasmid expression vectors encoding the CD20R chimeric immunoreceptor and the neomycin phosphotransferase cDNAs and the CD19R chimeric immunoreceptor and the selection-suicide HyTK (a fusion of hygromycin resistance and HSV-1 thymidine kinase suicide genes) cDNAs have been described previously [7,8] (Figure S1A). In brief, the chimeric construct consists of V_H and V_L gene segments of the CD20-specific Leu-16 or CD19-specific FMC63 monoclonal antibodies (mAbs), an IgG hinge-C_{H2}-C_{H3} region, a CD4 transmembrane region, and the cytoplasmic domain of the CD3ζ chain (Figure S1B).

Isolation, Transfection, Selection, Cloning, and Expansion of T Cells

The methods for OKT3 stimulation of peripheral blood mononuclear cells (PBMCs), and for PBMC electroporation, selection, cloning (IRB 98142 only), and subsequent growth using the rapid expansion method (REM), consisting of recursive 14-day cycles of activation with OKT3, recombinant human interleukin (rHuIL)-2, and PBMC/lymphoblastoid cell line (LCL)-irradiated feeders, have been described previously [9]. The overall T cell product manufacturing schemas for each trial are depicted in Figure S1C.

Cell Product Quality Control Tests

The cell product quality control tests (QCTs) performed and the requisite test results for product release are summarized in Table S1.

Confirmation of Plasmid Vector Integration (IRB 98142 Only)

A single site of plasmid vector chromosomal integration was confirmed by Southern blot analysis of *XbaI*/*Hind*III-digested T cell genomic DNA using a 420-bp NeoR-specific probe generated using the pcDNA3.1(-) plasmid as a template [9]. The pass criterion of this test was defined as detection of a single band.

Confirmation of CAR Expression

Western blot analysis for CAR expression has been described previously [10]. In brief, reduced whole-cell lysates are subjected to Western blot analysis with an anti-human CD3-ζ (cytoplasmic tail)-specific mAb 8D3 (BD Pharmingen, San Diego, CA). This probe detects both the 16-kDa endogenous ζ and the 66-kDa CAR ζ. Pass criteria were defined as visualization of both the 16-kDa and 66-kDa bands. Flow cytometry analysis for surface CAR expression was determined using a fluorescein isothiocyanate (FITC)-conjugated Fc-specific antibody (Jackson ImmunoResearch, West Grove, PA). Pass criteria were defined as unimodal positive staining for Fc compared with the FITC-conjugated isotype control (BD Biosciences, San Jose, CA).

Surface Phenotype Determination

T cell products were evaluated for cell-surface phenotype using standard staining and flow cytometric procedures with FITC-conjugated mAbs (BD Biosciences), followed by analysis on a FACScaliber analyzer (BD Biosciences). The pass criterion was ≥90% positive staining for TCR-αβ and CD8 (IRB 98142) or CD3 (IRB 01160) compared with the isotype control. Independent of the QCT guidelines, other correlative surface markers included CD4 for IRB 98142 and both CD4 and CD8 for IRB 01160.

Assay for Antilymphoma Cytolytic Activity

Cytolytic activity of CAR^+ CTLs against ^{51}Cr-labeled human lymphoma Daudi cells was assessed as described previously using a 4-hour chromium release assay [8]. The pass criterion was ≥50% specific lysis at an effector-to-target ratio of 25:1.

Viability

Viability was determined by standard trypan blue dye exclusion. The pass criterion was >90% viability.

Sensitivity to Ganciclovir Ablation (IRB 01160 Only)

To test for acquired cytocidal sensitivity to ganciclovir (GCV), aliquots of cells were harvested from 5-day REM cultures, then maintained for 14 days in 37.5 U/mL rHuIL-2 with or without 1 μM GCV. Then the cells were harvested and subjected to viability testing. The pass criterion was ≤25% viability in the GCV-treated cultures.

Assay for Antigen/IL-2–Independent Growth

First, 5×10^6 cells were washed and plated in antigen- and IL-2–free culture media at the end of a 14-day REM cycle. Parallel cultures of Jurkat T cells (American Type Culture Collection, Manassas, VA) (IRB 98142) or T cells cultured in the presence of 37.5 U/mL rHuIL-2 (IRB 01160) served as controls for expansion and viability. For IRB 98142, following an 11-day incubation, cultures were harvested, counted using trypan blue, plated into 96-well plates at 6000 viable cells per well, and pulsed with 1 μCi of ^3H-TdR. DNA was harvested following a 4-hour incubation at 37°C. For IRB 01160, viable cell numbers of 14-day cultures were determined by flow cytometry as described previously [11]. Release criteria specified that cells must exhibit <10% of the Jurkat cpm (IRB 98142) or <10% of the $IL\text{-}2^+$ control cell number (IRB 01160).

Sterility

Sterility tests were performed according to an FDA Center for Biologics and Evaluation of Research–mandated schedule. Aliquots of media from the T cell cultures were plated onto bacterial and fungal growth media. Mycoplasma detection was conducted on media aliquots using the Gen-Probe Mycoplasma Tissue Culture-NI Rapid Detection System (Gen-Probe, San Diego, CA), and endotoxin levels were determined by enzyme-linked immunosorbent assay. Pass criteria were negative bacterial, fungal, and mycoplasma results, along with an endotoxin level <5 EU/kg recipient weight.

Adoptive Transfer of T Cells

Processed and cryopreserved cell banks were thawed and expanded in culture to the desired cell numbers before being resuspended in 0.9% NaCl with 2% human serum albumin in a clinical reinfusion bag. T cells were reinfused i.v. over 30 minutes through either a central line or an age-appropriate sized i.v. catheter inserted into a peripheral vein. The infusion bag was mixed gently every 5 minutes during the infusion. The intrapatient dose escalation plan is shown schematically in Figure S2. In IRB 01160, fludarabine (Flu) was administered after the first T cell infusion as a potential nonmyeloablative (NMA) immunosuppressive regimen for attenuating possible rejection responses against the transferred T cells. The guidelines provided in the National Cancer Institute's (NCI) Common Toxicity Criteria, version 2.0 (https://ctep.ifo.nih.gov/l) were followed for the monitoring of toxicity and adverse event reporting. Rules for dose escalation, de-escalation, and cancellation were strictly enforced and resulted in 3 of the 4 treated patients deviating from the planned infusion cell dose escalation at least once.

In Vivo Persistence of Transferred T Cells

For IRB 98142, PBMCs from heparinized peripheral blood samples were isolated and analyzed for percentage of transfected cells by quantitative polymerase chain reaction (qPCR) as described previously [12] using primers and probes to quantify CD20R copy number (details available on request).

For IRB 01160, samples were received, processed, stored, and analyzed in accordance with current good laboratory practice guidelines. A validated qPCR-based assay to quantify CD19R plasmid vector DNA in samples was developed and performed using an MJ Research DNA engine with a Chromo 4 continuous fluorescence detector qPCR module (Bio-Rad, Hercules, CA). Real-time qPCR was performed in a 20-μL reaction mixture volume containing 50 ng DNA, 10 μL of IQ SYBR Green Supermix (Bio-Rad; catalog no. 170-8880), and 0.5 pmol of each primer. Quantification of the CD19R transgene sequence in DNA isolated from patient PBMCs was evaluated using qPCR to amplify a 182-nucleotide fragment that spanned the CD4 transmembrane–zeta junction within the transgene coding sequence, and a standard curve derived by dilution of DNA isolated from a clone with a single integration of the CD19R transgene (primers, probes, and amplification conditions available on request). The qualification studies for this amplification reaction demonstrated no amplification from healthy donor-derived PBMCs (n = 5), whereas the transgene sequence could be quantified in a PBMC sample if the transgene containing DNA composed as little as 0.1% of the total DNA sample.

Analysis of Antitransgene Rejection Responses

Pretreatment and post-treatment PBMCs (on days +75, +77, and +50 from UPN006, UPN009, and

UPN035, respectively) were first stimulated with irradiated therapeutic T cells (3000 rads) or LCLs with and without pcDNA3.1(-) plasmid (8000 rads) plus irradiated pretreatment PBMCs as feeder cells at a 10:1 responder-to-stimulator ratio in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM L-glutamine (Irvine Scientific), 25 mM Hepes (Irvine Scientific), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Irvine Scientific), and 10% heat-inactivated human serum. One week later, the same irradiated stimulator, as well as irradiated pretreatment PBMC feeders (3500 rads), were added at a 1:1:1 responder-to-stimulator-to-feeder ratio. This stimulation schema was repeated up to 2 more times (once weekly) until sufficient numbers were obtained for chromium-release assays. Cytolytic activity of these stimulated PBMC against ^{51}Cr-labeled targets was analyzed as described previously using a 4-hour chromium-release assay [8].

For IRB 01160, cellular antitransgene immune responses were evaluated directly ex vivo using a combination of T cell receptor (TCR) Vβ spectratyping and CD107 degranulation assays. For the TCR Vβ spectratyping analysis, RNA was isolated from PBMC collected before and after infusion using the RNAqueous-4 PCR Kit for Isolation of DNA-free RNA (Applied Biosystems/Ambion, Austin, TX), and cDNA was then synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). TCR Vβ spectratyping analysis was performed on cDNA samples essentially as described previously [13] using pools of Vβ-specific primers. A parallel series of amplifications using cDNA generated from pooled healthy donor PBMCs was performed as a quality control for amplification of each Vβ family. Aliquots of the amplification mixes were run on sequencing gels, followed by analyses using Genemapper v3.7 software (Applied Biosystems). The CD107 degranulation/mobilization assay was performed essentially as described previously [14], using patient PBMCs collected before and after infusion as effectors and infused T cell product or OKT3-expanded preinfusion PBMCs (i.e., autologous T cells) as targets. To detect spontaneous degranulation, a control sample without target cells was included in every experiment. FITC-conjugated anti-CD107a and anti-CD107b (BD Biosciences) were added directly to the tubes before incubation. After 5 hours of coincubation, cells were washed twice and stained with PE-Cy5-conjugated anti-CD8β and PE-conjugated anti-TCR Vβ23 (Beckman Coulter, Fullerton, CA) for 30 minutes at room temperature in the dark, rewashed, and analyzed on a FC500 flow cytometer using FCS Express v3.0 software (Beckman Coulter), with gating on CD8β$^+$ and Vβ23$^+$ lymphocytes.

Serologic Anti-CAR Immune Response Analysis

Serum was isolated from patient blood samples collected in red-top (no additive) tubes using an established laboratory standard operating procedure and a qualified assay to detect CAR-specific serologic responses in samples. Samples were allowed to clot for 2-1/2 hours at room temperature, then centrifuged at 1000 × g for 15 minutes at 4°C. Serum was collected, aliquoted, and frozen immediately at −80°C. Flow cytometry detection of potential serum antibody responses against the anti-CD19R transgene was performed using parental versus CD19R-expressing Jurkat cell lines as indicator cell lines. The presence of antibodies in patient serum that specifically bound to CD19R$^+$ Jurkat cells was evaluated by a subsequent incubation with FITC-conjugated AffiniPure F(ab')$_2$ fragment goat anti-human IgG (Fcγ; Jackson ImmunoResearch). The cutoff for a negative response was established by defining the 95% one-sided prediction interval using a pool of non–CAR-reactive serum samples from healthy volunteers.

RESULTS

Patient Characteristics

The patients in these studies either had DLCL (BB-IND 8513/IRB 98142) or follicular non-Hodgkin lymphoma (NHL; BB-IND 11411/IRB 01160) (Table S2). One of the 4 research participants had bulky disease, including sites at the neck, chest, lymph nodes, and pelvis, at the time of enrollment. Three of the 4 participants had received rituximab (chimeric mAb specific for CD20) therapy before the first infusion of therapeutic T cells. The average duration of time from leukapheresis to first infusion was 106 days, and was affected by the time required to manufacture the T cell product and/or the timing of the patient's recovery from salvage therapy.

Generation of Genetically Modified T Cells

Cell products meeting all quality control release tests (Table S1) were successfully generated for 2 of the 5 patients enrolled on IRB 98142, and for both patients enrolled on IRB 01160. The failure to release products for 3 of the patients enrolled on IRB 98142 stemmed from an inability to isolate T cell clones that expressed CD8, expressed endogenous TCR, or expanded adequately in vitro. The results of Southern blot analysis indicating the desired single-site insertions of the CD20R transgene within the released clones of IRB 98142 are depicted in Figure 1. Western blot and cell-surface expression profiles of T cell products for both trials are also depicted, confirming expression of the CAR protein. These cells were further subjected to flow cytometry analysis for confirmation of the T cell subset markers CD4, CD8, and either TCR-αβ or CD3. All of the cell products used in therapy also exhibited redirected killing of CD19$^+$ and CD20$^+$ human Daudi lymphoma targets in 4-hour

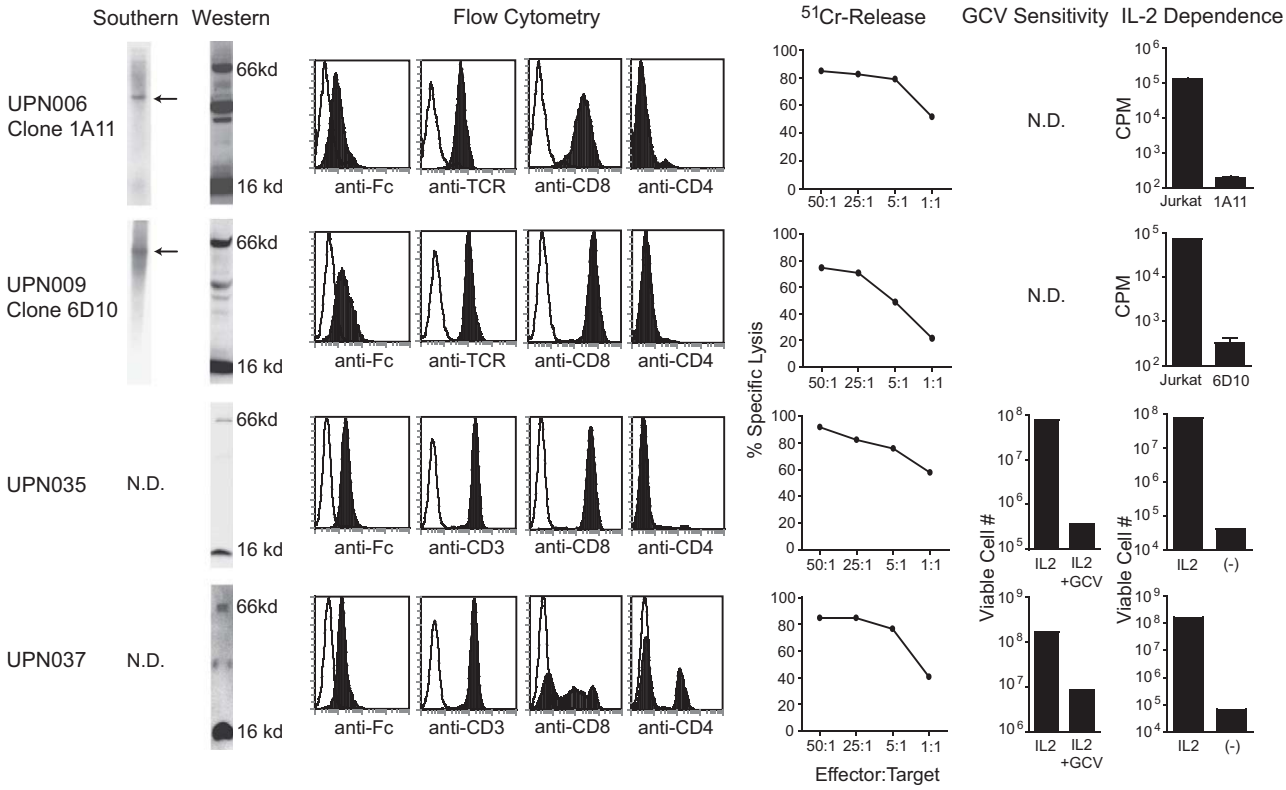

Figure 1. T cell products meet release requirements. Depicted from left to right: Southern blots of T cell genomic DNA using an HyTK-specific probe showing existence of single bands as indicated by arrows; Western blots revealing both the 16-kDA endogenous CD3ζ and the 66-kDA CE7R chimeric ζ bands detected with anti-human CD3ζ cytoplasmic tail specific antibody; flow cytometry analysis for surface expression of the chimeric receptor using anti-Fc antibody, or for the T cell markers CD8, CD4, and TCR or CD3, where isotype control staining is indicated with the open histogram; ability of CTL clones to lyse $CD19^+$ $CD20^+$ Daudi targets was determined in a 4-hour ^{51}Cr release assay; ganciclovir (GCV) sensitivity using a flow cytometry–based assay for viable cell numbers after 14 days of culture with either rHuIL-2 or rHuIL-2 + GCV; assays for IL-2 dependence were performed using ^3H-thymidine incorporation measurements (cpm) of Jurkat T cells versus the indicated T cell clones after 11 days of culture in the absence of rHuIL-2 (UPN006, UPN009), or using a flow cytometry–based assay for viable cell numbers after the T cell products were cultured in the presence versus the absence of rHuIL-2 for 14 days (UPN035 and UPN037). N.D., not done.

chromium release assays. Furthermore, all of the cell lines retained their dependence on exogenous rHuIL-2 for survival and proliferation, and the HyTK-expressing lines of IRB 01160 tested positive for sensitivity to GCV-mediated ablation.

Treatment Experience

As depicted in Figure 2, the intrapatient dose escalations were carried out as planned (compare with Figure S2), with the exception that the $10^{10}/m^2$ cell dose was never given in IRB 98142 because of protocol toxicity dose modification rules (UPN006 and UPN009). Indeed, because of grade 2 hepatic toxicities that were noticed with the first infusion dose of $10^8/m^2$ in UPN006, the second infusion was repeated at $10^8/m^2$, followed by an escalation to $10^9/m^2$ for the third infusion. Patient UPN009 exhibited a drop in hemoglobin after the second infusion that, although clinically insignificant (from 10.6 to 8.6), represented a Common Toxicity Criteria grade change from grade 1 to grade 2 anemia status, requiring repetition of the $10^9/m^2$ dose for the third infusion based on the protocol's defined rules for dose escalation. In UPN006, the second infusion was cancelled and rescheduled because of a puncture in the bag, which compromised the integrity of the T cell product. In UPN037, the last infusion was cancelled because of the detection of contaminated T cell product. Neither myeloablation (MA) followed by HSCT nor Flu resulted in a drop in absolute lymphocyte count (ALC) below the normal range (Table S3).

There were no grade 3 or higher adverse events with a possible correlation to administration of 10^8 T cells per m^2. However, examination of the adverse events at 10^9 T cells per m^2 revealed one case of grade 3 self-limited lymphopenia in both IRB 98142 and IRB 01160, possibly attributed to cell administration (Table 1). At 2×10^9 T cells per m^2, grade 3 lymphopenia and grade 3 eosinophilia each occurred once in IRB 01160; both resolved spontaneously with no adverse sequelae to the patients. Overall, the safety profile of this adoptive transfer therapy was acceptable.

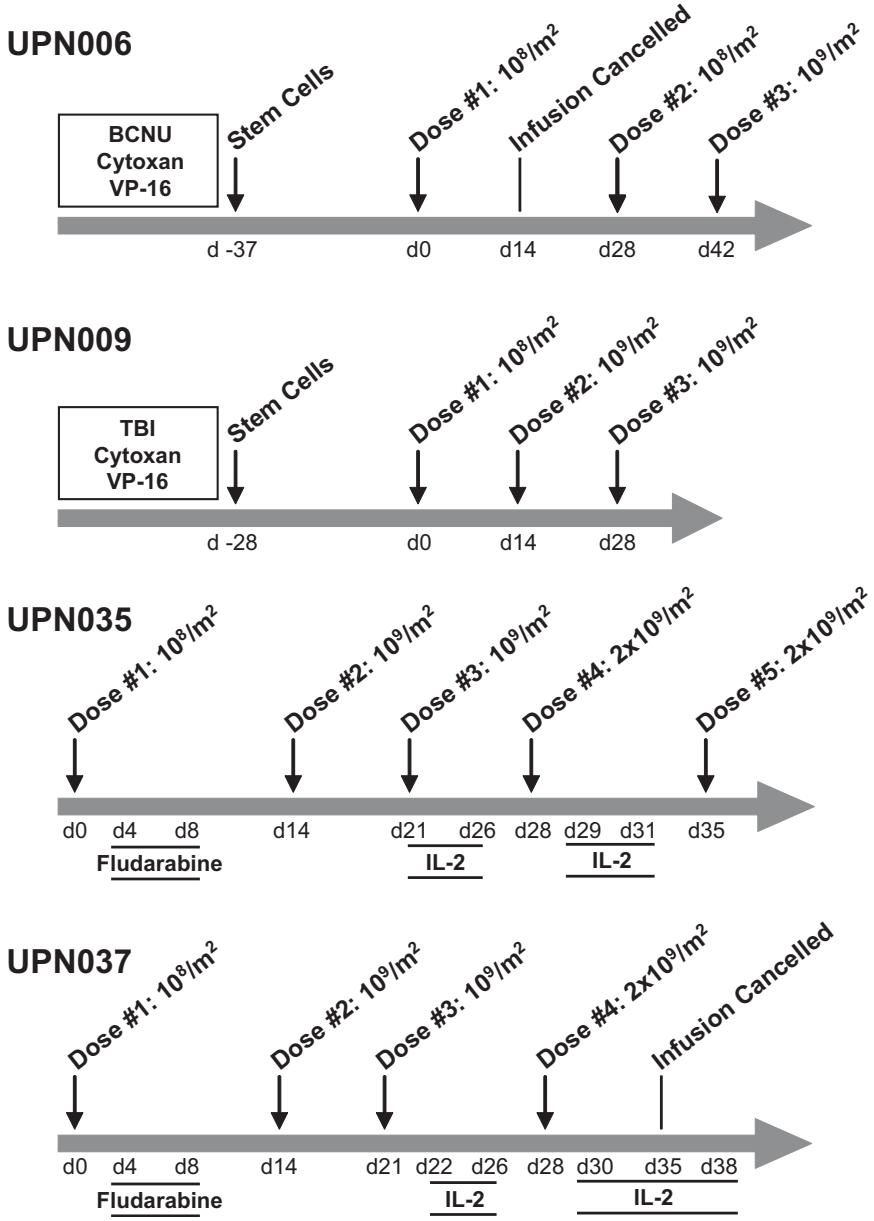

Figure 2. Treatment regimens for each patient. First i.v. infusions of T cells were administered on day 0 for each patient. For UPN006 and UPN009, fractionated total body irradiation (TBI) and/or myeloablative chemotherapies administered to UPN006 and UPN009 are indicated just before administration of $CD34^+$ autologous stem cells. BCNU, bis-chloronitrosourea; cytoxan, cyclophosphamide; VP-16, etoposide. For UPN035 and UPN037, administration of fludarabine (i.v. at 25 mg/m^2) occurred between days 4 and 8 after the first T cell infusion, and rHuIL-2 administration (5×10^5 IU/ m^2 BID) was initiated after the third T cell infusion.

Follow-Up Clinical Status of Patients

Although this was a phase I clinical trial with a primary purpose of determining safety, we also monitored the disease and survival status of each patient. For IRB 98142 (CD20R; DLCL), UPN006 (last infusion on February 24, 2000) relapsed in September 2001, whereas UPN009 (last infusion on November 22, 2000) continues to be in remission after autologous HSCT. At the time of the writing of this report, both UPN006 (after additional treatment) and UPN009 are alive and in remission. For IRB 01160 (CD19R; FL), UPN035 (last infusion on May 4, 2006) presented with a new diagnosis of $CD19^+/CD20^+$ DLCL in June 2006, and died in June 2007. UPN037 (last infusion on February 15, 2007) displayed progression on computed tomography scan in September 2007, and is currently alive and undergoing additional treatment.

In Vivo Persistence of Transferred T Cells

Quantitative PCR performed to detect CD20R and CD19R plasmid copy numbers in PBMCs as a surrogate marker of the presence of adoptively transferred T cells showed varying T cell persistence among patients (Figure 3). Only 1 of the 4 patients (UPN006)

Table 1. Adverse Event Summary

IRB Trial	T Cell Dose, Cells/m^2	Event*	Occurrences
98142	10^9	Lymphopenia	1
01160	10^9	Lymphopenia	1
	2×10^9	Lymphopenia	1
		Eosinophilia	1

IRB indicates internal review board; NCI, National Cancer Institute.
*Only events of grade 3 or higher, according to the NCI Common Toxicity Criteria, with possible attribution to T cell administration are reported.

had a detectable level of transferred T cells at 1 week after the first infusion of 10^8 cells/m^2. A detectable level of transferred T cells at 1 week after infusion of 10^9 cells/m^2 was found in only 2 of the 7 higher doses (UPN006 infusion 3 and UPN009 infusion 2), and at no time were transferred T cells detected at 1 week after infusion of 2×10^9 cells/m^2. Thus, adoptively transferred T cell persistence did not appear to correlate with cell dose. Compared with the persistence after the initial infusion, UPN009, UPN035, and UPN037 also displayed significantly reduced levels of transferred T cells 24 hours after each additional infusion, suggesting the possibility of an antitransgene immune response mounted against the administered T cells (Figure 3).

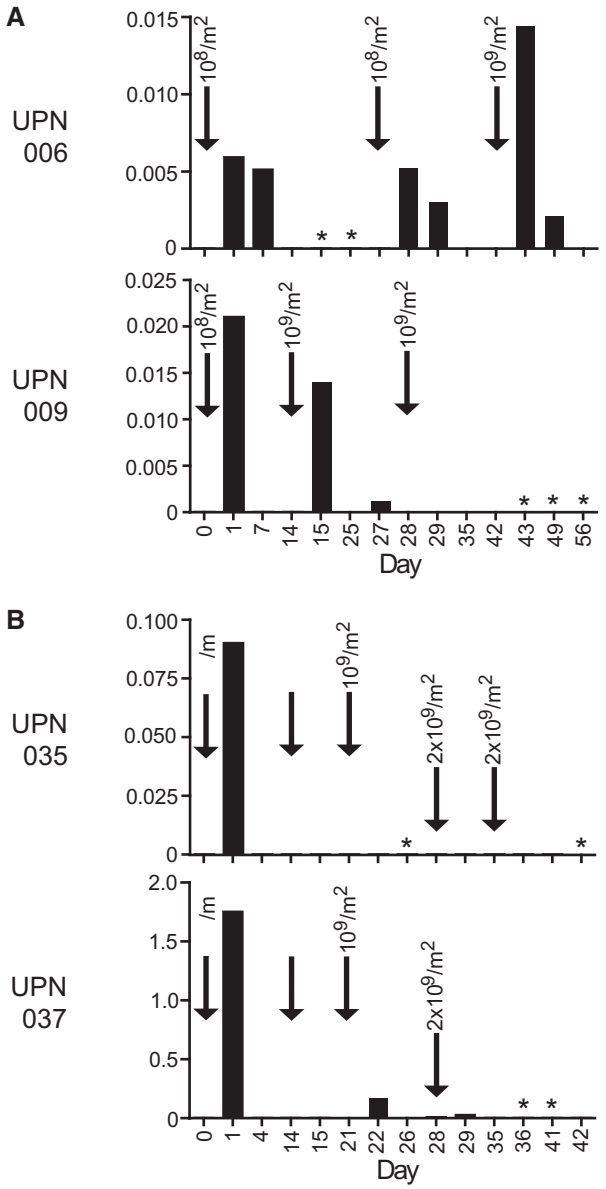

Figure 3. Transferred T cells do not persist long term in vivo. Using real time quantitative PCR, the percent of cells in the PBMC that were positive for the CD20R (A) or CD19R (B) genes were determined as an indicator of the relative amount of chimeric receptor expressing T cells in the PBMC samples collected at the indicated days during the treatment schedule. Escalating infusion doses are indicated by arrows. *Cells not harvested.

Detection of Transgene-Specific Immune Responses

For IRB 98142 (CD20R; DLCL), the development of cellular immune responses against the infused T cell products was evaluated. For these analyses, PBMCs were collected from UPN006 and UPN009 before and after T cell administration, and, after in vitro stimulation, were compared for cytotoxic activity using chromium-release assays (Figure 4). The use of irradiated lymphoblastoid cells (xLCLs) to stimulate the PBMCs resulted in successful lysis of ^{51}Cr-labeled LCLs, indicating that functional effector cells could be derived from each patient's PBMC samples. Interestingly, when the irradiated autologous T cell clones were used to stimulate the PBMCs, cytotoxic responses were seen against the ^{51}Cr-labeled T cell clone only in the posttreatment sample collected from UPN009 (Figure 4A). This immunoreactivity against the T cell clone 6D10 used in therapy appeared to be specific for neomycin phosphotransferase but not the CAR, because cytotoxic responses could be observed against ^{51}Cr-labeled LCLs that had been transduced with the pcDNA3.1(-) vector, which directs the expression of neomycin phosphotransferase, but lacks the CD20R transgene (Figure 4B). To better analyze the specificity of the rejection response, UPN009's posttreatment PBMCs that had been stimulated with irradiated 6D10 cells were cloned in limiting dilution; all clones were similarly specific for NeoR (Figure 4C).

For IRB 01160 (CD19R; FL), the development of both antibody and cellular immune responses against the infused T cell products was evaluated. For the antibody analyses, serum collected from UPN035 and UPN037 at enrollment and after T cell administration were both negative for antibody reactivity against the surface-expressed CD19R transgene using a flow cytometry–based assay (Figure 5A); however, evidence was found for cell-mediated immunoreactivity against the infused T cells. Examination of the TCR Vβ gene

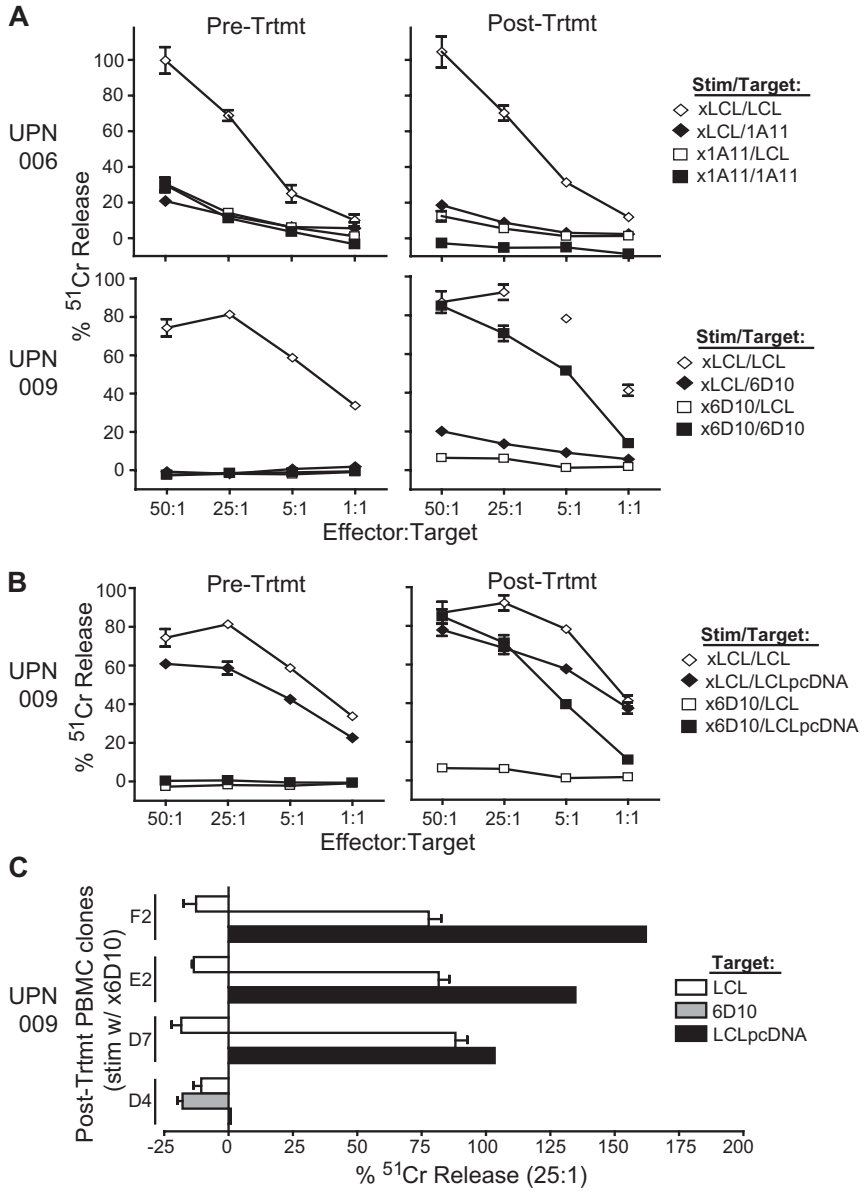

Figure 4. Transgene rejection response detected when T cells administered after HSCT. In the trial targeting CD20$^+$ diffuse large-cell lymphoma, PBMCs collected before treatment and at day 75 (UPN006) or day 77 (UPN009) after initiation of treatment were stimulated in vitro with irradiated LCL as a control (xLCL) or the corresponding irradiated CTL clone that had been administered (i.e., x1A11 or x6D10). Effectors were then used in a 4-hour ^{51}Cr-release assay using either LCL or the corresponding CTL as targets (A) or, in the case of UPN009, using LCL that had been transfected with the pcDNA3.1(-) vector lacking the CD20R transgene as targets (B). (C) Clones derived from UPN009 day 98 PBMC were also stimulated in vitro with irradiated 6D10 CTL and then analyzed for cytolytic activity against ^{51}Cr-labeled LCL, 6D10, or pcDNA3.1(-) vector transfected LCL to determine specificity of transgene-specific response. Percent ^{51}Cr-release at an E:T of 25:1 in each case is depicted for four representative clones.

repertoire through spectratyping sequence analysis of PBMCs collected from UPN035 and UPN037 both before and after T cell administration revealed alterations in the Vβ profiles of the posttreatment PBMCs, with the appearance of unique clonotypes in the post-infusion samples indicative of a new immunoreactive response (Figure 5B). Furthermore, flow cytometry analysis of the TCR Vβ23$^+$ and Vβ14$^+$ subpopulations in UPN035's posttreatment PBMCs collected 2 weeks after the first infusion showed significant surface CD107 expression, an indicator of lysis-associated degranulation, on coculture with the infused T cell product (Figure 5C and data not shown). This specific degranulation was not observed with the pretreatment PBMCs or when the posttreatment PBMCs were co-cultured with control T cells. Similar flow cytometric assays could not be carried out with UPN037's PBMC because of the lack of commercially available antibodies specific for the TCR V*β* genes (Vβ15 and Vβ21) that arose in this patient. The pretreatment and posttreatment PBMCs from UPN035 also were stimulated in vitro with irradiated LCLs or infused T cell product and compared for their cytotoxic activity using chromium-release assays (Figure 5D). As seen

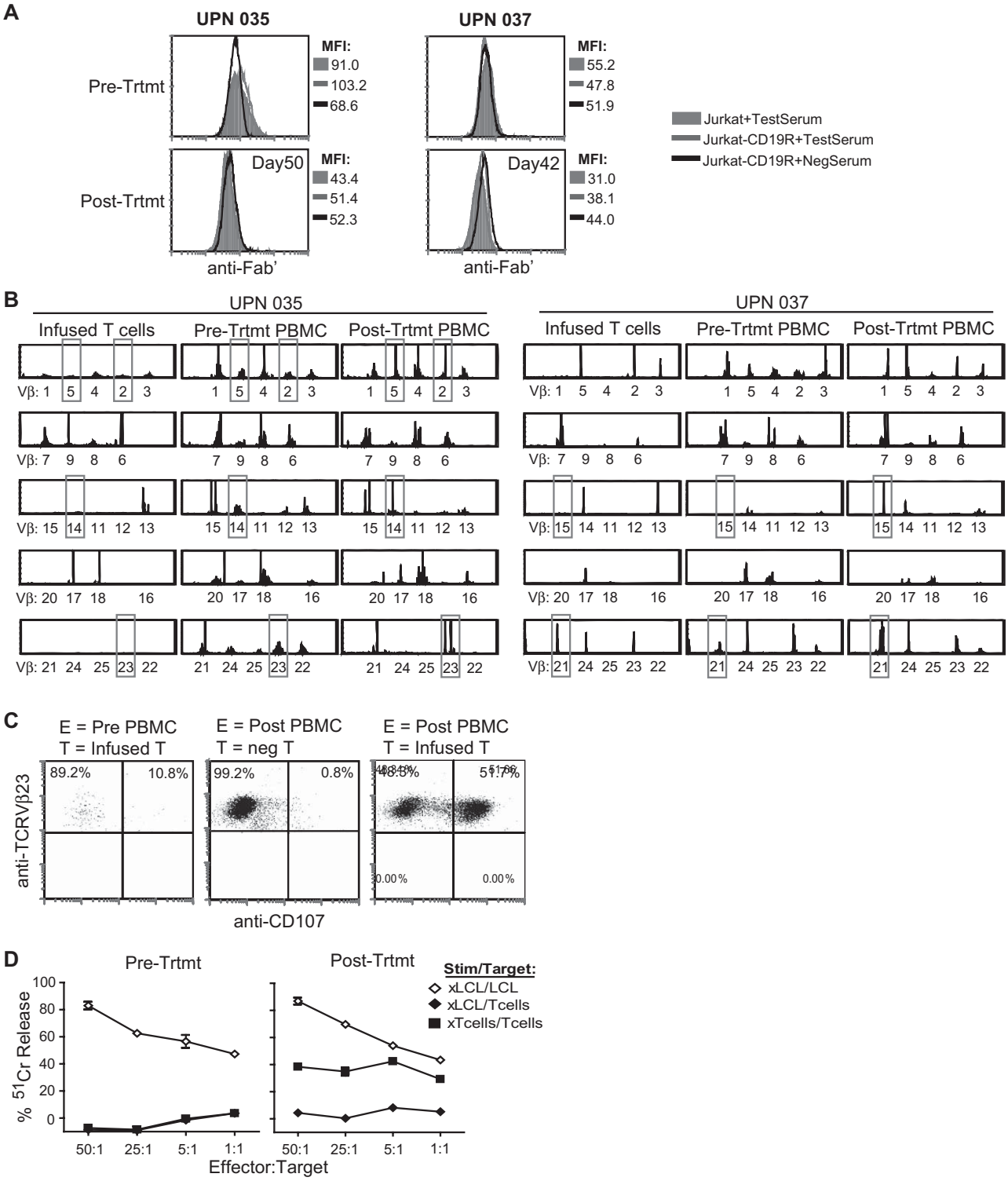

Figure 5. Rejection response detected when T cells were administered following fludarabine administration. (A) In the trial targeting CD19$^+$ FL, serum collected at the time of patient enrollment (Pre-Trtmt) and at day 50 (UPN035) or day 42 (UPN037) after initiation of treatment was examined for immunoreactivity against Jurkat cells expressing the CD19R (red line) in a flow cytometry based assay. Parental Jurkat cells (grey histogram), and a known nonreactive serum (black line) were used as negative controls. (B) TCR Vβ profiles of the infused T cell product, day 0 PBMCs (collected just before first T cell infusion; Pre-Trtmt), and day 14 PBMC (collected before the second T cell infusion, Post-Trtmt) were determined by spectratyping analysis. Alterations in Vβ usage that were observed pretreatment versus posttreatment are highlighted by red boxes. (C) The TCR Vβ23$^+$ population of pretreatment and day 14 PBMC from UPN035 was further analyzed by flow cytometry for surface CD107 expression as a marker of degranulation on coculture with the infused T cell product (infused T), or nonmodified autologous T cells (neg T). (D) Pretreatment and day 50 PBMC from UPN035 were stimulated in vitro with irradiated LCL as a control (xLCL) or with the irradiated T cell product (xTcells). Effectors were then used in a 4-hour ^{51}Cr-release assay using either LCL or T cells as targets.

in UPN009 in IRB 98142, functional effector cells were derived from both of UPN035's PBMC samples, but cytolytic activity against the ^{51}Cr-labeled T cells was seen only in the posttreatment sample. Taken together, these data suggest that, at least in some cases, the lack of T cell persistence observed in these 2 trials was because of immune rejection responses mounted by the patient's endogenous T cells.

DISCUSSION

More than 55,000 new cases of NHL are diagnosed each year in the United States, and the incidence of this disease is increasing [15,16]. Intermediate-grade B cell lymphomas (ie, diffuse large cell, mantle cell, marginal zone) and low-grade FLs are the most common subtypes of NHL, accounting for approximately 80% of cases. Most patients have widespread disease at the time of diagnosis and are treated with some combination of chemotherapy, radiation therapy, and rituximab. Unfortunately, more than two-thirds will relapse with their disease, and only 10% of these patients can be salvaged [17]. Efforts to improve survival in recurrent NHL focus primarily on the use of MA conditioning and autologous HSCT [18-21], a strategy that is curative in approximately 46% of selected patients. However, the selected group of salvageable patients (aged <60 years, complete remission after primary treatment, and no known marrow or central nervous system disease) represents less than one-third of those with relapsed intermediate grade lymphomas. Patients with chemotherapy-resistant recurrent disease have a <15% 5-year event-free survival after HSCT, and those with refractory disease at the time of transplantation are rarely cured. Similarly, patients with mantle cell lymphoma and low-grade FL, whose disease becomes refractory to chemotherapy and radiation, have a poor prognosis despite high-intensity salvage therapy [22-25]. These findings have prompted the evaluation of additional strategies to eradicate lymphoma minimal residual disease after cytoreductive chemotherapy/radiation/rituximab, including the immunotherapeutic targeting of malignant B cells with adoptively transferred antigen-specific T cells.

Here, we have described our initial experience testing the feasibility and safety of lymphoma adoptive therapy with CTLs genetically modified to express redirecting CD20- and CD19-specific CARs. Our T cell production platform relied on plasmid vector electrotransfer into patient PBMC preparations. Although this approach facilitated regulatory approval and diminished expenses relative to the use of a viral vector platform, the low efficiency of chromosomal integration and sustained transgene expression encumbered the production platform to multiple rounds of activation/propagation in selection drugs (G418 and hygromycin B). Nevertheless, drug-resistant CAR$^+$ T cells were isolated in each of the 7 enrolled patients. The reason why 3 of the enrolled subjects on protocol IRB 98142 did not have clones released was that only CD4$^+$CD8$^-$ clones were isolated, when the release criteria specified CD4$^-$CD8$^+$ clones. This skewed result in the production runs was specific to lymphoma patients in this trial and likely reflects the repertoire changes in these patients because of disease and/or previous therapy at the time of apheresis for T cell production. Another observed limitation in the production platform during generation of polyclonal lines in IRB 01160 was the discordance between CAR expression and hygromycin resistance. The plasmid vector used in the trial drives the CAR and HyTK from 2 separate promoters, allowing chromosomal integration events that result in deletion of the CAR-encoding portion of the vector or transcriptional repression of the CAR promoter. As a result, we found demonstrable CAR expression in only a subset of polyclonal cell preparations. This problem potentially can be resolved in plasmid vectors using single promoter systems in which the two transgene open reading frames are separated by an internal ribosome entry site element or directly integrated into a single polypeptide with a cleavable linker element. Our group has now redesigned our platform to use self-inactivating lentiviral vectors and shortened ex vivo culture duration (~28 days), improving the percentage of CAR-expressing T cells in polyclonal cell preparations and eliminating the need for bacterial drug-resistance gene coexpression.

The primary focus of these studies was to establish the safety of this approach. In this regard, T cell infusions were well tolerated up to 2×10^9 cells/m^2. The most common event that could be attributed to T cell infusion was transient self-limited lymphopenia lasting less than 7 days. We suspect that this phenomenon is related to redistribution of the endogenous circulating repertoire as a consequence of infused cell product; whether it is based on cytokine/chemokine elaboration on activation or other mechanisms remains to be delineated. In the 2 patients who demonstrated immunologic rejection of the infused cell products, the third and subsequent cell doses in these 2 patients elicited a self-limited (<24 hours) febrile response with rigors. Despite the dramatic systemic febrile response, the patients did not exhibit cardiovascular instability or other overt toxicities associated with a "cytokine storm" syndrome. A toxic death proximal to cell infusion has been recently reported [26] in a patient with bulky chronic lymphocytic leukemia who received cyclophosphamide before administration of redirected T cells expressing a CD19-specific CAR with both CD28-costimulatory and CD3-ζ activation–signaling domains. The lack of serious toxicities in our patients might result from the limited numbers of circulating B cells at the time of T cell

infusion; the IRB 98142 patients were 28 days from MA autologous HSCT, whereas the IRB 01160 patients experienced B cell reduction as a consequence of rituxan. However, analysis of peripheral blood samples after the last T cell infusion, as well as the follow-up clinical status of these patients, indicate that this strategy did not result in sustained B cell lymphopenia, as would be expected based on the transient engraftment of infused effector cells. Another possible explanation for the observed lack of toxicity is an attenuated cytokine response to activation based on the CAR having only a CD3-ζ–activation domain. Carefully designed future trials should test the effects of these parameters as they relate to the tolerability of cell infusions.

The NCI's Surgical Branch has demonstrated that the frequency and magnitude of melanoma-reactive tumor-infiltrating lymphocyte (TIL) engraftment can be enhanced by rendering patients lymphopenic before adoptive transfer and administering high-dose rHuIL-2 after transfer. TIL products were previously difficult to detect in very high numbers after adoptive therapy engraftment in about 50% of patients treated with the most intensive lymphodepleting regimens consisting of MA chemotherapy/TBI with CD34-selected stem cell rescue. Despite having received MA HSCT, the patients on IRB 98142 were not lymphopenic on day +28 when infusion of CD20-specific $CD8^+$ clones commenced. Similarly, the patients on IRB 01160 were given a 5-day course of Flu at 25 mg/m^2/dose without achieving lymphopenia before T cell transfer. Given the compelling data from the NCI melanoma trials, we plan to administer T cell products to lymphoma patients in conjunction with their autologous HSCT procedure on day +2, when lymphopenia is profound.

Our experience clearly identifies the issue of transgene immunogenicity as a mechanism that limits persistence. The plasmid electrotransfer platform required that we select drugs for stably integrated clones/lines. The rejection response observed was cellular and focused on NeoR in UPN009, whereas the transgene specificity of the rejection responses in UPN035 and UPN037 was not characterized further. The ability of transgenes expressed in T cells to elicit immune responses after adoptive transfer was clearly established by the findings of Berger et al. [27], in which the limited persistence of adoptively transferred $HyTK^+$ T cells correlated with anti-HyTK transgene-specific immune responses. Furthermore, although studies by that group suggest that an NMA immunosuppressive regimen could prolong the in vivo persistence of T cells by attenuating possible rejection responses [28], Flu applied after the first dose of T cells in IRB 01160 apparently failed as an immunopreparative strategy to delete antitransgene-specific responses. Thus, more effective immunosuppressive/ lymphodepleting regimens might be advantageous for future clinical application. The use of more efficient vector transduction systems (eg, lentiviral vectors or the Sleeping Beauty system [29]) that would negate the requirement for ex vivo selection also might allow the omission of NeoR and HyTK. Even without the selection markers, the CARs themselves are expected to be immunogenic based on mouse single-chain variable fragments and fusion sites in the chimera. We expect that providing a window for cells to evade immunologic rejection by patient lymphodepletion will be the most practical strategy for limiting early rejection responses, whereas late rejection responses have the advantage of eliminating the gene-modified cells, which, if timed appropriately, could be exploited as a safety feature.

Poor in vivo persistence is the major problem in the cancer adoptive therapy field in general, likely related primarily to the intrinsic programming of T cells to survive after adoptive transfer. Effector T cells are inherently short-lived [30], and it has been suggested that acquisition of an effector phenotype during in vitro culture and expansion is a major reason for the poor survival of transferred T cells [31]. The adoptive transfer of virus-specific effector T cells from memory cell precursors can result in long-term repopulation in humans, however. Thus, the use of memory T cells, which are known to self-renew [32], and/or virus specific T cells, which would receive optimal costimulation after engagement of their native receptors, as populations for genetic redirection has become of increasing interest. Indeed, it has recently been reported that in neuroblastoma patients, Epstein-Barr virus-specific T cells engineered to coexpress tumor-specific receptors survived longer than those lacking virus specificity and were associated with tumor regression or necrosis in half of the subjects tested [33]. It also was recently reported that, in a macaque model of adoptive transfer, antigen-specific $CD8^+$ T cell clones derived from central memory T (T_{CM}) cells persisted long term in vivo, reacquiring phenotypic and functional properties of memory T cells and occupying T_{CM} cell niches [34]. Accordingly, we have begun to develop a clinically compatible immunomagnetic selection system to isolate T_{CM} cells from human PBMCs for subsequent processing to generate CAR redirected effector cells. We propose viral-specific, T_{CM}-derived, anti–tumor effector cell infusion in lymphoma patients during profound lymphopenia shortly after autologous HSCT as the next logical iteration of our translational research in lymphoma immunotherapy.

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Financial disclosure: Michael C. Jensen has a major ownership interest ($10,000 or more) as a patent holder. Laurence J. Cooper has minor ownership interest (<$10,000) as founder and majority owner of InCellerate Inc, a company that commercializes genetically modified T cells. None of the other authors has any conflicts of interest to disclose.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbmt.2010.03.014

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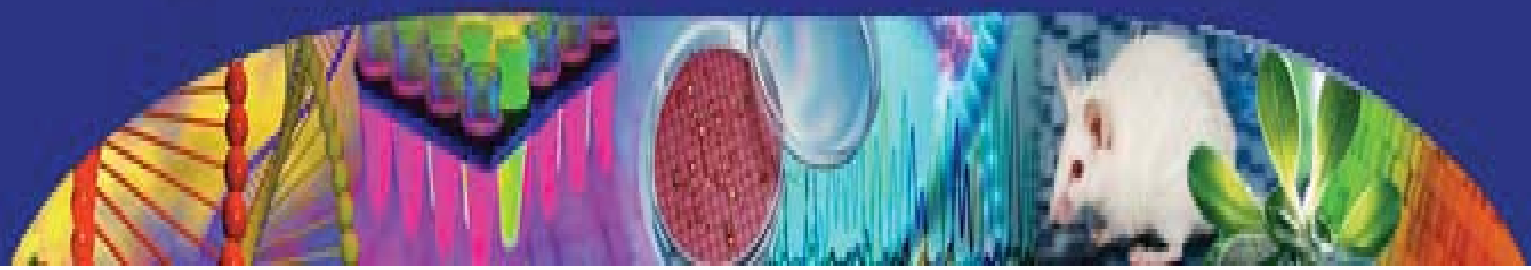

CD20 Protocols

Antigen Target	**CD20**
Antigen Expression	**Expressed on mature B cells and is involved in cell cycle regulation, activation, proliferation and differentiation of B cells. Minimal expression on early pre-B cells and no expression on plasma cells. Expressed to varying degrees on B cell malignancies.**

	Chimeric Antigen Receptors Trials Registered with OBA
Title	**Protocol # 330: Pilot Phase I Study to Evaluate the Safety of Cellular Immunotherapy Using Genetically Modified Autologous CD20-Specific CD8+ T Cell Clones for Patients with Recurrent/Refractory CD20+ Lymphoma Undergoing Autologous Peripheral Blood Stem Cell Transplantation**
PI	**Michael C.V. Jensen, M.D.**
Vector and Signaling Moieties	**Plasmid/CD3 Zeta**
Status	**Protocol was closed in 2002. Long term follow-up continues.**
Doses Proposed	**$1x10^8 – 1x10^{10}$ cells/m^2**
Lymphodepletion	**Undergoing Autologous Stem Cell Transplantation. Infusion 28 days after stem cell infusion.**
Subjects Enrolled	**3/5 individuals enrolled did not receive the gene modified cells; 2 because of failure to isolate gene-modified clones that met all quality control.**
Summary of Unexpected and Related Events	**None**
Summary of Results	**As of 2009, one subject is still in long term follow-up. The other subject who received cells declined to continue follow-up.**

Title	**Protocol # 491: A Phase I Study to Evaluate the Safety of Cellular Immunotherapy Using Genetically-Modified Autologous CD20-Specific T Cell Clones for Patients with Relapsed CD20+ Mantle Cell or Indolent Lymphomas**
PI	**Oliver W. Press, M.D., Ph.D.**
Vector and Signaling Moieties	**Plasmid/CD3 zeta**
Status	**Closed**
Doses Proposed	**$1x10^8$ cells/m^2 for infusion #1 and then 3.3 X 10^9 cells/m^2 for infusion #3**
Lymphodepletion	**Cytoreductive Chemotherapy if bulky disease or actively growing lymphoma or if lymph nodes > 5 cm or who have > 5000 circulating B cells/mm^3 blood**
Subjects Enrolled	**10; 7 completed as of 3/2010**
Summary of Unexpected and Related Events	**There were no significant safety issues noted on this protocol. All participants who received post-infusion IL2 injections had mild to moderate, expected adverse effects associated with IL2. With use of IL2, modified T cells were detected in blood samples taken up to 13 weeks post infusion.**
Summary of Results	**Of the 7 subjects treated, 2 maintained a previous complete remission (CR), 1 achieved a partial response (PR) and 4 had stable disease.** **Till, B. G., *et. al.* (2008). Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 112: 2261-271.**
Title	**Protocol # 863: A Pilot Study To Evaluate the Safety and Feasibility of Cellular Immunotherapy Using Genetically Modified Autologous CD20-Specific T cells for Patients with Relapsed or Refractory Mantle Cell and Indolent B Cell Lymphomas**
PI	**Oliver W. Press, M.D., Ph.D.**
Vector and Signaling Moieties	**Plasmid/CD3 zeta, 4-1BB:CD28**

Status	**Open**
Doses Proposed	**$1x10^8$ cells/m^2 for T cell infusion #1 followed by $1x10^9$ cells/m^2 and then 3.3 x 10^9 cells/m^2 for infusion #3**
Lymphodepletion	**Cyclophosphamide 1000mg/m^2**
Subjects Enrolled	**4 subjects enrolled and 3 treated to date out of proposed enrollment of 12**
Summary of Unexpected and Related Events	**Protocol consent amended due to a serious adverse event involving orthostatic hypotension, fever and hypoxia in a subject following infusion requiring overnight hospitalization. The subject above was 75 years of age and received the first infusion without complications and then was admitted due to orthostatic hypotension with second infusion. DSMB was consulted and the 3rd infusion was given with premedications and a 500 ml bolus. Infusion # 3 (4 x 10^9 cells/m^2) was given but fresh cells were used in lieu of frozen. About 5 hours post infusion, the subject developed a fever and a drop in O2 sats (98% - 92%). Supportive care was provided and symptoms resolved by morning allowing IL-2 administration. The subject's CXR showed no signs of pulmonary edema.**

Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells

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Adoptive immunotherapy with T cells expressing a tumor-specific chimeric T-cell receptor is a promising approach to cancer therapy that has not previously been explored for the treatment of lymphoma in human subjects. We report the results of a proof-of-concept clinical trial in which patients with relapsed or refractory indolent B-cell lymphoma or mantle cell lymphoma were treated with autologous T cells genetically modified by electroporation with a vector plasmid encoding a CD20-specific chimeric T-cell receptor and neomycin resistance gene. Transfected cells were immunophenotypically similar to CD8$^+$ effector cells and showed CD20-specific cytotoxicity in vitro. Seven patients received a total of 20 T-cell infusions, with minimal toxicities. Modified T cells persisted in vivo 1 to 3 weeks in the first 3 patients, who received T cells produced by limiting dilution methods, but persisted 5 to 9 weeks in the next 4 patients who received T cells produced in bulk cultures followed by 14 days of low-dose subcutaneous interleukin-2 (IL-2) injections. Of the 7 treated patients, 2 maintained a previous complete response, 1 achieved a partial response, and 4 had stable disease. These results show the safety, feasibility, and potential antitumor activity of adoptive T-cell therapy using this approach. This trial was registered at www.clinicaltrials.gov as #NCT00012207. (Blood. 2008;112: 2261-2271)

Introduction

Several lymphoma subtypes are incurable with standard chemotherapy and radiation, but immune-based therapies have emerged as effective treatment and offer a potential for cure. Monoclonal antibodies (Abs) against the B-cell lymphoma marker CD20 have activity alone,[1,2] in combination with chemotherapy,[3-5] or conjugated with radiation-emitting nuclides.[6-8] Adoptive cellular therapy with nonmyeloablative allogeneic stem cell transplantation (SCT) or donor lymphocyte infusion (DLI) can eradicate tumors, resulting in long-term survival, even in highly chemotherapy-refractory lymphomas.[9-11] Both of these immunotherapy approaches have limitations, however, because antibodies fail to cure many types of lymphoma, and SCT and DLI, although potentially curative, cannot be used in many patients because of significant toxicity and transplantation-related mortality.

Because the graft-versus-tumor effect of SCT and DLI appears to be mediated by alloreactive donor T lymphocytes,[12,13] generating T cells specific for tumor antigens minimally expressed in normal tissues is an attractive strategy for harnessing this antitumor effector activity. One technique involves genetically modifying autologous T cells to express a chimeric T-cell receptor (cTCR) that targets a tumor antigen and induces antigen-specific T-cell activation, proliferation, and killing. Because this antigen-induced activation of the T cell occurs in an MHC-independent fashion, a single vector can be used universally to confer recognition of a selected target antigen. By introducing the cTCR into autologous T cells, the risk of graft-versus-host disease is eliminated. Such genetically modified T cells have been designed to target antigens associated with a variety of tumors, with success in animal models[14-16] and some early evidence of clinical efficacy in human subjects.[17]

Our group has developed a technique to manufacture CD20-specific T cells by transfecting peripheral blood mononuclear cells (PBMCs) with a linearized naked DNA plasmid encoding a cTCR derived from a murine anti–human CD20 Ab.[18-20] The cell-surface antigen CD20 is an attractive target for immune-based therapies because it is present in more than 90% of B-cell lymphomas, is expressed at a high copy number, is stable on the cell surface, and does not internalize on binding Abs.[21] These modified T cells secrete interleukin-2 (IL-2) in an antigen-dependent manner,[19] selectively kill CD20$^+$ target cells in vitro,[20] and eradicate human xenograft tumors in mice.[22] Application of this approach to the treatment of lymphoma in human subjects has not yet been described. We report here the results of a proof-of-concept clinical trial in which ex vivo–expanded, genetically modified autologous CD20-specific T cells were used as adoptive cellular therapy for patients with relapsed or refractory indolent B-cell non-Hodgkin lymphoma (NHL) and mantle cell lymphoma (MCL). We show that these T cells can be reproducibly generated and expanded to therapeutic numbers, exhibit in vitro antitumor cytotoxicity, persist in vivo for up to 9 weeks, and appear to be safe, well tolerated, and potentially capable of mediating in vivo antitumor activity.

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An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

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Methods

Clinical protocol

This clinical protocol was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board, the University of Washington Institutional Biosafety Committee, the US Food and Drug Administration, and the Recombinant DNA Advisory Committee of the National Institutes of Health. Informed consent was obtained in accordance with the Declaration of Helsinki. Patients were eligible if they had a pathologically confirmed diagnosis of $CD20^+$ MCL or indolent B-cell lymphoma, had relapsed or refractory disease after at least one prior chemotherapy, were deemed not to be candidates for (or refused) stem cell transplantation, and had serologic evidence of prior Epstein-Barr virus (EBV) exposure (because the TM-LCL cell line used in T-cell culture is EBV-transformed). Patients were excluded if they received fludarabine or cladribine within 2 years before apheresis (but could receive these drugs as cytoreductive therapy after apheresis), anti-CD20 Ab within 4 months of T-cell infusions, or chemotherapy within 4 weeks of T-cell infusions; had lymph nodes more than 5 cm or more than 5000 circulating lymphoma cells in the peripheral blood at the time of T-cell infusions, a previous allogeneic stem cell transplantation, or human anti–mouse Ab (HAMA) seropositivity; required corticosteroids during the study period; had pulmonary or central nervous system involvement with lymphoma; were HIV-seropositive; or were pregnant.

Patients underwent leukapheresis after signing informed consent, and then they were allowed to receive cytoreductive chemotherapy for disease control or debulking during the 2- to 4-month period of T-cell generation, at the discretion of their referring physician. For patients A to E, PBMCs were activated, transfected, and plated at limiting dilution with the intention of isolating and subsequently expanding T-cell clones. This approach proved to be laborious and inefficient, however, and the protocol was modified for patients F to I to allow expansion of modified cells in bulk culture. Patients subsequently received 3 infusions of autologous CD20-specific T cells 2 to 5 days apart in escalating doses (10^8 cells/m^2, 10^9 cells/m^2, and 3.3×10^9 cells/m^2) followed by 14 days of subcutaneous low-dose (500 000 IU/m^2) interleukin-2 (IL-2) injections twice daily (patients F-I only). Patients then underwent clinical follow-up to evaluate toxicities related to therapy, which were assessed according to National Institutes of Health Common Terminology Criteria for Adverse Events, version 3.0 (http://ctep.cancer.gov/). A Data and Safety Monitoring Board was assembled that performed reviews of the safety data every 6 months. Clinical responses were assessed according to International Working Group criteria.[23]

T-cell transfection, selection, and expansion

All cell culture for therapeutic use was performed in the Cell and Gene Therapy Core Laboratory at the University of Washington General Clinical Research Center, under current good manufacturing practice standards. PBMCs collected by apheresis were diluted 1:2 with PBS containing 200 mg/L EDTA, isolated by density gradient centrifugation over Ficoll-Paque (GE Healthcare, Little Chalfont, United Kingdom), washed, and resuspended in RPMI 1640 medium containing 2 mmol of L-glutamine, 25 mmol HEPES, and 10% fetal calf serum. Cells were activated with 30 ng/mL OKT3, and after overnight incubation recombinant human IL-2 was added (50 U/mL).

Electroporation and selection. On day 4 of culture, cells were harvested and resuspended in chilled hypo-osmolar electroporation buffer (Eppendorf North America, New York, NY) at 20×10^6 cells/mL. Cell suspensions were mixed with linearized plasmids (25 μg/mL) encoding a CD20-specific scFvFc:ζ cTCR,[18-20,24] and divided into aliquots into chilled 0.2-cm electroporation cuvettes. Cells were electroporated with an Eppendorf Multiporator at 250 V for 40 microseconds (μsec) as previously described.[20] Approximately 3 days after electroporation, G418 was added to flasks (at 0.8 mg/mL). Cells were selected by G418 for 8 days before generating cells by limiting dilution.

Generation and expansion of genetically modified T cells. Transfected cells from patients A through E were selected in G418, and attempts were made to generate T-cell clones by limiting dilution as previously described.[24,25] Although the intention was to isolate clonal populations derived from a single progenitor cell, the plating density required to yield reliable growth of T cells resulted in the presence of 1 to 3 clones per well, as subsequently determined by Vβ TCR spectratyping. For patients F through I, G418-resistant transfected cells were grown in bulk cultures as previously described.[25] As cell numbers increased, T cells were transferred to 1-L or 3-L tissue culture bags (Lifecell, Branchburg, NJ). During the expansion, 5 to 8 stimulation cycles were performed. Fresh T cells were infused in patients A and B. For logistic reasons, T cells from patients D, F, G, H, and I were cryopreserved between days 70 and 132 after apheresis in Plasmalyte-A containing 5% HSA and 10% DMSO and thawed 3 to 4 hours before infusion (48 hours before infusion for patient D). Release criteria included detectable cTCR expression by flow cytometry, negative bacterial, fungal, and *Mycoplasma* cultures, endotoxin level no more than 5 EU/kg per hour, Gram stain–negative on day of infusion, greater than 80% cell viability, $TCR\alpha/\beta^+$ and $CD3^+$ phenotype by flow cytometry, IL-2 growth dependence, and CD20-specific cytotoxicity.

T-cell clonality assays

T-cell clonality was determined by polymerase chain reaction (PCR) amplification of rearrangements at the T-cell receptor gamma (TCRγ) locus as previously described,[26] except that $V_\gamma I$-$J_\gamma 1/2$, $V_\gamma II$-$J_\gamma 1/2$, $V_\gamma I$-$J_\gamma P1/2$, and $V_\gamma II$-$J_\gamma P1/2$ rearrangements were amplified in a single multiplex PCR reaction and analyzed by capillary electrophoresis on an Applied Biosystems Model 3130 (Foster City, CA). See Document S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) for detailed methods.

Vβ spectratyping was also performed by flow cytometry. Cells were labeled with monoclonal antibodies CD8 ECD and IOTest Beta Mark Kit (Beckman Coulter, Fullerton, CA). The expression of each of the 24 T-cell receptor isoforms present in the Beta Mark Kit (approximately 70% coverage of the normal human TCR Vβ repertoire) were determined independently on the $CD8^+$ T-cell populations, and a threshold of 85% positivity for a single isoform or an absence of expression of all 24 isoforms outside the reference range was considered to represent a clonal expansion. Samples showing 2 or more isoforms outside the reference range were considered oligoclonal.

Western blot assay

Whole cell lysates of modified T cells were probed with a mouse anti–human CD3ζ monoclonal Ab (BD PharMingen, San Diego, CA) as previously described.[20]

Cytotoxicity assays

T-cell cytotoxicity was analyzed 2 to 7 weeks before planned T-cell infusions to permit selection of optimal "clones" of T cells for expansion. CD20-specific cytotoxicity was assessed with the use of standard chromium-release assays with the following target cell lines: EL4-CD20 (a murine T-cell lymphoma line transfected to express the human CD20 molecule), the parental CD20–nontransfected EL4 cell line, or the Daudi Burkitt lymphoma cell line, as previously described.[25] Cytotoxicity assays were repeated in some patients just before T-cell infusions and showed levels of cytotoxicity comparable to assays performed 2 to 7 weeks before infusion.

Flow cytometry for immunophenotypic characterization of T cells and lymphocyte subset analysis

Flow cytometry was performed with the use of standard methods. Briefly, cells cryopreserved within 1 day of the first T-cell infusion were thawed, washed, and labeled with the indicated monoclonal Ab for 15 minutes at room temperature in the dark. The samples were then washed once, resuspended in a dilute DNA binding dye (DAPI), incubated for 10 minutes, and approximately 20 000 events acquired on an LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using software

developed in our laboratory (WoodList). Positivity for DAPI was used to exclude nonviable cells, and thresholds for positivity were determined with unstained cells and isotype control Ab, as appropriate. Antibodies were used at the manufacturer's recommended concentrations. A complete list of Abs used is included in Document S1. Flow cytometry to detect cTCR expression was performed using a FITC-labeled polyclonal goat anti–mouse IgG Fab-specific Ab (Sigma-Aldrich, St Louis, MO) as previously described.[25]

Detection of modified T cells in vivo

PBMCs collected serially after T-cell infusions were isolated by Ficoll density-gradient centrifugation, and genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). The standard consisted of 10-fold serial dilutions of purified scFvFc:ζ plasmid DNA starting at 10^6 copies/μL, with each sample containing 1 μg of preinfusion PBMC DNA to control for background signal. The negative control was preinfusion PBMC genomic DNA. A 72-bp (base pair) fragment containing portions of the CD3ζ chain and adjacent CD4 transmembrane domain sequences was amplified using forward primer 5′-TCGCCGGCCTCCTGCTTT-3′ and reverse primer 5′-CGTCTGCGCTCCT-GCTGA-3′. The probe used was 5′-FAM-TGGGCTAGGCATCTTCTTCA-GAGTGAA-TAMRA-3′. Primers that amplify a fragment of the β-actin gene (TaqMan B-actin Detection Reagent Kit; Applied Biosystems) were used as an internal control and for normalization of DNA quantities. Quantitative real-time PCR was performed in triplicate with 1 μg DNA in each reaction, using TaqMan Universal PCR Master Mix in a 7900HT Sequence Detection System (all Applied Biosystems).

Immune response assays

Two assays were performed to test for humoral immune responses to the cTCR. In the first assay, 96-well enzyme-linked immunoabsorbent assay (ELISA) plates were coated with 0.5 μg Leu-16 murine anti–human CD20 Ab (BD Biosciences, San Diego, CA) in pH 9.6 carbonate buffer and blocked with 5% milk before adding samples of goat anti–mouse IgG Fab-specific Ab (standard curve; Jackson ImmunoResearch Laboratories, West Grove, PA), serially diluted 2% BSA/PBS (negative control), baseline patient serum (negative control), $HAMA^+$ patient serum (positive control), or study subject serum from serial postinfusion time points. Biotinylated Leu-16 murine anti–human CD20 Ab (BD Biosciences; 10 μg/mL) was added to each well as the primary Ab, followed by 1:1000 horseradish peroxidase-Avidin D (BD Biosciences). Samples were incubated for 30 minutes at room temperature and washed 3 times with 0.01 M PBS/0.3% Tween between each step. Color reagent (2,2,-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt; Sigma-Aldrich) at 0.42 mg/mL in citrate buffer (citrate 10.5 mg/mL, pH 4.0) plus hydrogen peroxide (100 μL/12 mL buffer) was added to each well; absorbency was read with a Bio-Tek XS ELISA reader (Bio-Tek Instruments, Winooski, VT). Optical density measurements were converted to concentration values as calculated from the standard curve. In the second assay, flow cytometry was used to assess the presence of anti-cTCR Ab in posttreatment patient serum samples (see Document S1 for detailed methods).

Cellular immune response assays were performed by coincubating patient-derived PBMCs (10^6 cells/mL) serially collected after T-cell infusions with irradiated anti-CD20 cTCR-expressing T cells (10^6 cells/mL) from infused batches, at a 2:1 ratio. After 2 rounds of stimulation 1 week apart, the PBMCs were tested in ^{51}Cr release cytotoxicity assays using either autologous T cells transfected with the cTCR-encoding plasmid or nontransfected autologous PBMC as target cells at a 25:1 E/T ratio. In the first 2 patients treated we also assessed the responsiveness of recovered T cells to histocompatibility locus antigen–disparate cells as a positive control.

Results

Study design and patient characteristics

The primary objective of this study was to assess the feasibility, safety, and toxicity of adoptive therapy using patient-derived T cells bearing a CD20-specific cTCR to treat indolent and mantle cell lymphomas. Autologous PBMCs were collected by apheresis, genetically modified, and expanded ex vivo, a process that typically required 2 to 4 months. During this interval patients underwent cytoreductive chemotherapy if necessary for tumor debulking or to maintain disease control. Subjects were then treated with 3 infusions of modified CD20-specific T cells, 2 to 5 days apart, at incremental doses (10^8 cells/m^2, 10^9 cells/m^2, and 3.3×10^9 cells/m^2) similar to those used in previous adoptive T-cell therapy trials,[27] but with a shorter interval between infusions to limit the potential for development of an immune response against the transfected cells. The last 4 patients received low-dose subcutaneous injections of IL-2 twice daily for 14 days after the final T-cell infusion to enhance in vivo T-cell survival and proliferation. Patients then underwent follow-up for clinical and research end points, and long-term monitoring for adverse events for 2 years. The study design is outlined in Figure 1.

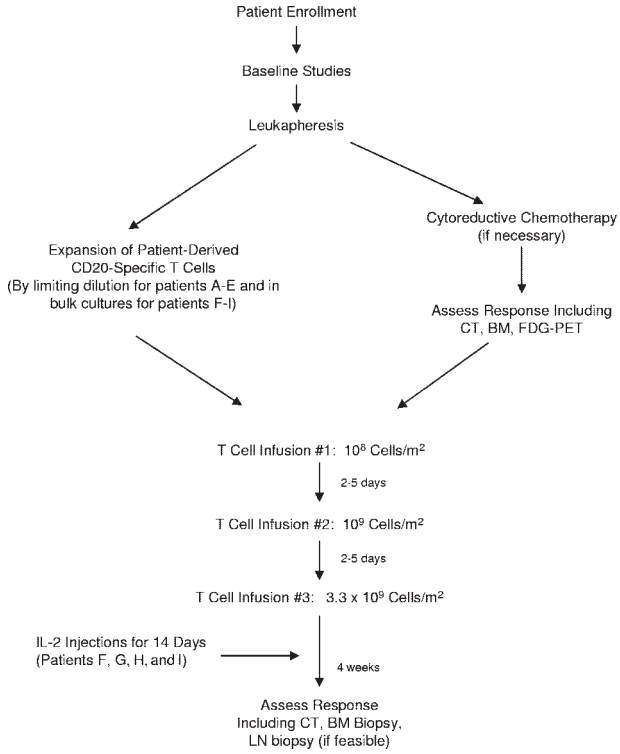

Figure 1. Schema of clinical protocol.

Nine patients with relapsed or refractory indolent B-cell NHL or MCL were enrolled: 8 men and 1 woman between the ages of 43 and 77 years; 8 had relapsed follicular lymphoma, and 1 had relapsed MCL. Patients had been treated with a median of 2 prior therapies (range, 1-7 therapies; Table 1).

Generation and expansion of autologous CD20-specific T cells

PBMCs collected by apheresis were stimulated with anti-CD3 Ab (OKT3) and IL-2 and transfected by electroporation with a naked DNA plasmid encoding a cTCR consisting of a murine kappa leader sequence, CD20-specific scFv derived from the Leu16 murine Ab, human IgG1 C_H2C_H3 hinge, human CD4 transmembrane, and human CD3ζ intracellular signaling domain, as well as a neomycin resistance gene (neoR) under a separate promoter (Figure 2A).[20,25] Anti-CD20 cTCR surface expression was confirmed by Western blot (Figure 2B) and flow cytometry (Figure S3).

Table 1. Patient characteristics

Patient	Age, y	Sex	Diagnosis	Stage	Prior therapies	Cytoreductive therapy before T-cell infusions
A	44	F	FL	IV-B	R-CHOP	CVP
B	70	M	FL	II-A	CHOP, rituximab, ^{131}I-tositumomab	CVP
C	47	M	FL	IV-B	ProMACE/MOPP, ASCT, fludarabine (10 cycles)	CVP
D	60	M	FL	IV-A	Rituximab	CVP
E	63	M	MCL	IV-A	R-HyperCVAD, GCD-R	None
F	46	M	FL	IV-A	R-CVP	FND
G	43	M	FL	IV-A	CHOP, IFN, CY + VP16, R-CY, CY + DEX, GCD-R, ASCT	None
H	46	M	FL	IV-B	R-CHOP, fenretinide	FND
I	77	M	FL	III-A	R-CVP, R-CHOP, GCD-R	^{131}I-tositumomab

F indicates female; M, male; FL, follicular lymphoma; MCL, mantle cell lymphoma; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; R, rituximab; CVP, cyclophosphamide, vincristine, and prednisone; ProMACE/MOPP, procarbazine, methotrexate with leucovorin, doxorubicin, cyclophosphamide, etoposide, mechlorethamine, vincristine, and prednisone; ASCT, high-dose therapy followed by autologous stem cell transplantation; HyperCVAD, cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with cycles of high-dose cytarabine and methotrexate; GCD, gemcitabine, carboplatin, and dexamethasone; FND, fludarabine, mitoxantrone, and dexamethasone; IFN, interferon-α; CY, cyclophosphamide; VP16, etoposide; and DEX, dexamethasone.

Modified T cells were generated for the first 5 patients by limiting dilution and selected for CD20 cytotoxicity by chromium release assay and cTCR expression by flow cytometry. At the plating density required to reproducibly generate modified T cells, the resulting T-cell populations consisted of cells derived from 1 to 3 clones of T cells as assessed by Vβ TCR spectratyping and TCRγ clonality testing by PCR (Figure 3A; Table S1). This expansion and selection process proved to be laborious and inefficient, requiring approximately 4 months to achieve the target cell dose. Moreover, T cells generated by limiting dilution could not be expanded adequately for infusions in 2 of these initial 5 patients, and in 2 of the other 3 patients the target cell doses could not be reached (Table 2).

We subsequently elected to modify the protocol to include expansion of T-cell transfectants in bulk culture to circumvent the difficulties of expanding T cells after limiting dilution. Successful expansion of modified T cells was achieved for the subsequent 4 patients using this approach, and the time required to reach the target cell dose was reduced by approximately 50% (Figure 4). Vβ TCR spectratyping and TCRγ clonality testing by PCR showed more heterogeneous T-cell populations in these bulk cultures, although several of the cultures contained prominent T-cell clones (Figure 3B; Table S1). Three of these 4 patients received all planned doses of T cells. The target cell number was reached for the fourth patient as well, but the third infusion consisted of only 2×10^9 cells/m^2 because of a loss of cells during a quality control assay.

Immunophenotype of modified T cells

The phenotype of ex vivo–expanded cTCR-bearing T cells has not been well described. We analyzed the immunophenotype of the infused T cells using multicolor flow cytometry and found it to be similar to that of activated effector T cells,[28,29] expressing CD3, CD8, and CD45RO and lacking CD62L, CCR7, and CD127 (Figure 5). As expected, patients treated with CD8$^+$ T cells derived by limiting dilution received negligible numbers of CD4-expressing cells (0.67%-4.5%), whereas patients receiving infusions of T cells grown in bulk culture received 3.4% to 38.6% CD4$^+$ cells. Infused T cells also expressed the activation marker CD95, but relatively few cells (1.3%-6.2%) expressed CD134 (OX40; Table 3). We found negligible numbers of cells expressing central memory (CD62L$^+$/CCR7$^+$/CD45RA$^-$/CD127$^+$) and effector memory (CD62L$^-$/CCR7$^-$/CD45RA$^-$/CD127$^+$) phenotypes.

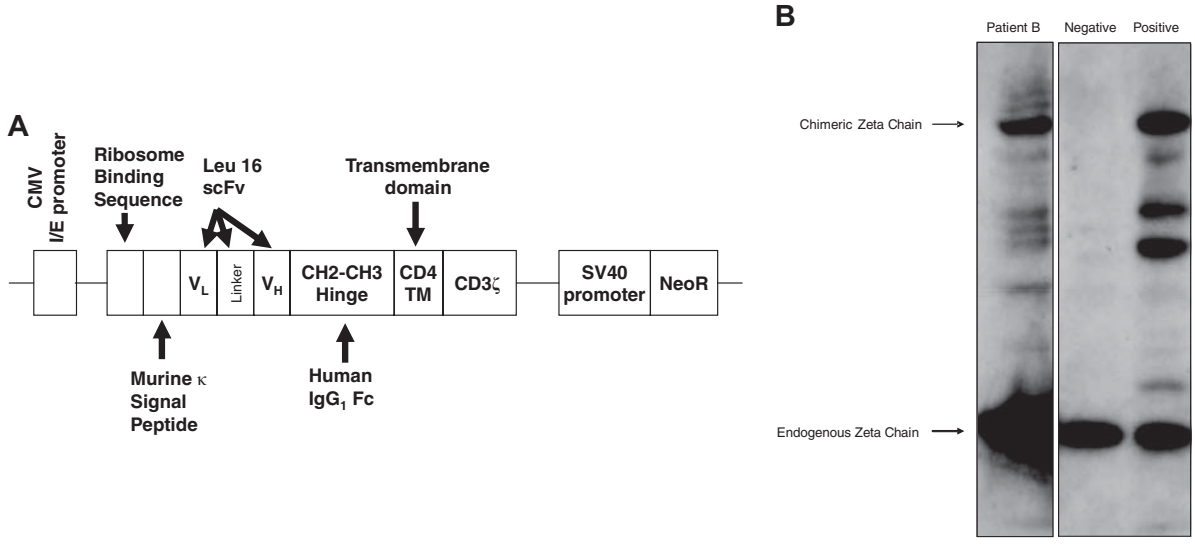

Figure 2. Expression of the CD20-specific cTCR. (A) Schematic diagram of the CD20-specific scFvFc:ζ chimeric T-cell receptor cDNA plasmid. (B) A representative Western blot analysis of cTCR expression performed using whole-cell lysates of preinfusion T cells from patient B, probed with mouse anti–human CD3ζ monoclonal Ab. Negative control was parental PBMCs, and positive control was transfected Jurkat cell line. A 21-kDa band corresponding to the endogenous CD3ζ chain and a 66-kDa band representing the expected cTCR protein were detected. The intermediate bands indicate degradation products or truncated forms of the cTCR.

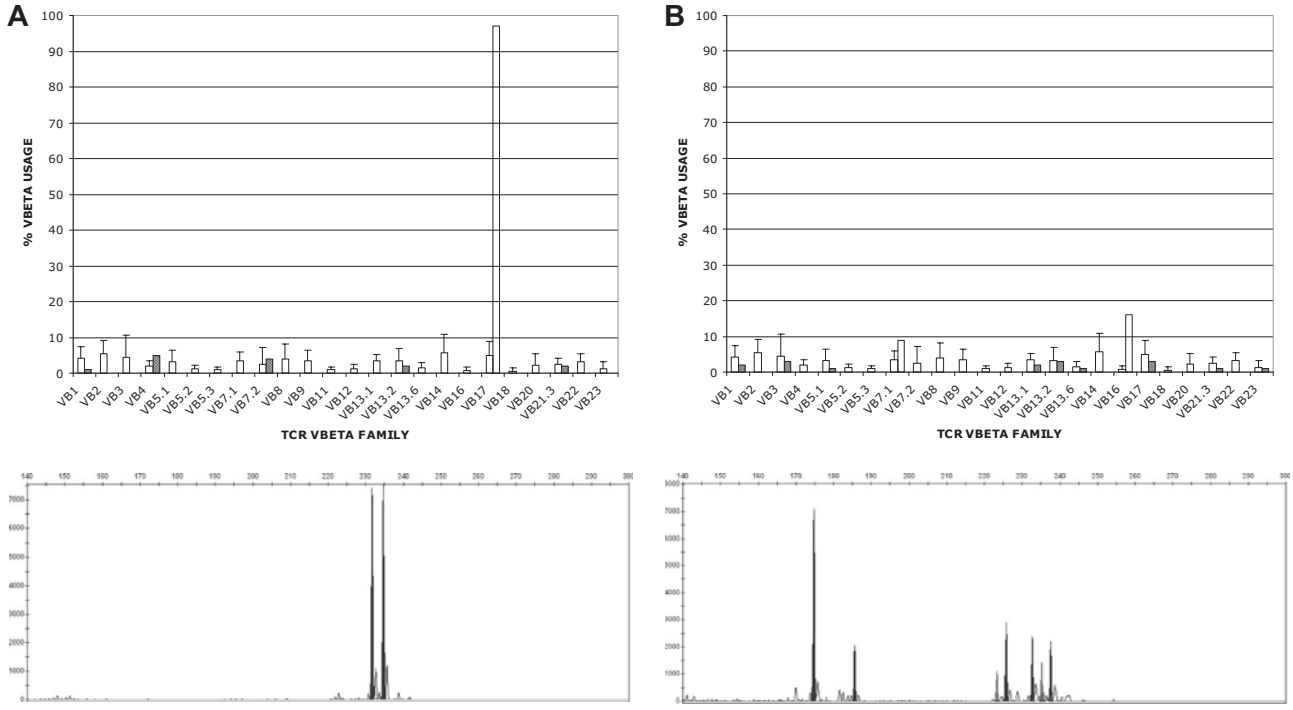

Figure 3. Clonality of T cells produced by limiting dilution and in bulk culture. T-cell clonality was determined by flow cytometric T-cell receptor (TCR) Vβ spectratyping (top) and by PCR amplification of clonal V-J rearrangements at the TCRγ locus (bottom). Representative results for T cells produced by limiting dilution (A) and in bulk culture (B) are shown. (A) T cells produced by limiting dilution (patient B), showing clonal expression of Vβ17 in 98% of $CD8^+$ T cells by Vβ spectratyping (top; ■) and showing 2 predominant TCRγ rearrangements (bottom). Because each T-cell clone can rearrange one or both of its TCRγ alleles, the 2 PCR products could represent either 1 T-cell clone with biallelic TCRγ rearrangements or 2 singly rearranged clones, although the single predominant Vβ17 clone identified by spectratyping would favor a single doubly rearranged clone. (B) T cells produced in bulk culture (patient G) showing oligoclonal Vβ expression in $CD8^+$ T cells (16% Vβ16; 9% Vβ7.1; 3% each Vβ3, Vβ13.2 and Vβ17; 2% each Vβ1 and Vβ13.1; and 1% each Vβ5.1, Vβ13.6, Vβ21.3, and Vβ23) and 7 distinct TCRγ rearrangements by PCR (bottom; ■) that could correspond to between 4 and 7 different T-cell clones, depending on the number of singly and doubly rearranged clones (see Table S1). The □ in both top panels represent the average expression levels for each Vβ chain in normal polyclonal T-cell populations.

T cells resulting from bulk culture and from limiting dilution were phenotypically similar, although the former generally contained higher proportions of $CD4^+$ cells (3.4%-38.6% compared with 0.67%-4.5%) and cells with a regulatory T (Treg)–like phenotype ($CD4^+/CD25^+/FoxP3^+$) (0.54%-1.9% compared with 0.61%-21.6%). Treg functionality studies were not performed, however. All T cells exhibited low expression of costimulatory markers CD28 (0.92%-5.4%) and CD137 (0.47%-4.4%). High proportions of cells from all patients expressed adhesion molecules such as CD11a (98.7%-100%), CD44 (99.8%-100%), and CD49d (85.8%-99.6%).

Cytotoxicity of modified T cells in vitro

Our group showed in preclinical studies that T-cell clones bearing CD20-specific cTCRs exhibit antigen-specific cytotoxicity.[20] We assessed the cytotoxicity of T cells used in this trial by coincubation with ^{51}Cr-labeled $CD20^+$ target cells (Daudi lymphoma cells and EL4 mouse lymphoma cells transfected to express human CD20), and the expanded T cells used for all 7 patients killed $CD20^+$ lymphoma cells in an antigen-specific manner (Figure 6).

Table 2. T-cell infusions

Patient	Infusion 1, cells/m^2*	Infusion 2, cells/m^2†	Infusion 3, cells/m^2‡	Fresh versus thawed cells	Time from apheresis to target cell number, d	No. of stimulation cycles§
A	10^8	10^9	3.3×10^9	Fresh	130	7
B	10^8	10^9	2×10^9	Fresh	129+	7
C					Expansion failed	5
D	10^8	4×10^8		Thawed	159+	7
E					Expansion failed	5
F	10^8	10^9	3.3×10^9	Thawed	96	6
G	10^8	10^9	3.3×10^9	Thawed	90	5
H	10^8	10^9	3.3×10^9	Thawed	81	5
I	10^8	10^9	2×10^9‖	Thawed	104	8

For patients A through E, T cells were selected and expanded by limiting dilution. For patients F through I, T cells were expanded in bulk culture. Patients C and E did not receive T-cell infusions. Patients B and D received infusions but did not reach the target cell dose.

*Target dose was 10^8 cells/m^2.

†Target dose was 10^9 cells/m^2.

‡Target dose was 3.3×10^9 cells/m^2.

§Defined as the number of times cells were stimulated with OKT3 in the presence of irradiated feeder PBMCs and LCL (repeated every 14 days, first cycle at day 14).

‖The target number of cells was generated for this patient, but nearly half of the last cell dose was lost during a quality control assay before infusion.

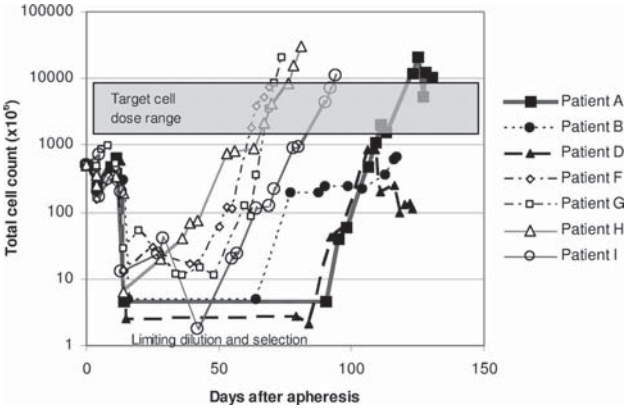

Figure 4. Growth curves of genetically modified T cells. Patient PBMCs were transfected with the scFvFc:ζ plasmid by electroporation after stimulation with OKT3. For patients A, B, and D, populations of G418-resistant T cells were generated by limiting dilution, and T-cell cultures exhibiting the most favorable cytotoxicity and growth profiles were selected for expansion to therapeutic numbers. For patients F through I, G418-resistant cells were grown as bulk cultures.

Adoptive therapy with CD20-specific T cells is safe and well tolerated

Seven patients received a total of 20 T-cell infusions. No grade 3 or 4 toxicities were seen, and no adverse events attributable to the T-cell infusions themselves were observed. Grade 2 toxicities associated with subcutaneous IL-2 injections included a flulike syndrome (1 of 4 patients), fever (1 of 4 patients), and skin reactions at injection site (2 of 4 patients) and resolved after the cessation of IL-2. Grade 1 toxicities attributable to IL-2 included chills, myalgias, dyspnea, dysgeusia, malaise or fatigue, diaphoresis, and injection site reactions (Table 4).

In vivo persistence of modified T cells

We measured the in vivo persistence of modified T cells using quantitative real-time PCR of DNA from patient PBMCs collected at serial time points after T-cell infusion. Modified T cells were detectable by PCR for 5 to 21 days in the first 3 patients receiving T cells without adjuvant IL-2. In contrast, modified T cells were detectable for 5 to 9 weeks in the last 4 patients, who received T cells produced in bulk culture and 14 days of subcutaneous IL-2 (Figure 7).

Modified T cells were also detectable by PCR in the bone marrow 24 hours after the final T-cell infusion in patients A, D, F, and G (lymphoma cells were detectable in the marrow by flow cytometry only in patients G and H at this time point). Of the 7 treated patients, only patients B and G had accessible lymph nodes for biopsy after T-cell infusions. The lymph node from patient B showed only fibroadipose tissue, and patient G's lymph node showed tumor, but no modified T cells were detectable.

Modified T cells were not immunogenic

The introduction of foreign transgenes in therapeutic vectors has resulted in immune responses against modified T cells in previous gene therapy clinical trials.[27,30] Given the significant immunosuppression present in patients with lymphoma, we hypothesized that the transgenic cells in this trial might elicit lower immune response rates, and we used 3 assays to test this hypothesis. To detect cellular immune responses, ^{51}Cr-release assays were performed with serially collected patient PBMCs that had been coincubated with irradiated T cells expressing the scFvFc:ζ and neoR gene products (Figure S1A), using transfected and nontransfected T cells as target cells. In 2 patients the antigen responsiveness of recovered T cells was confirmed using allogeneic LCL as target cells; in both cases a cytotoxic response was elicited at all time points tested (Figure

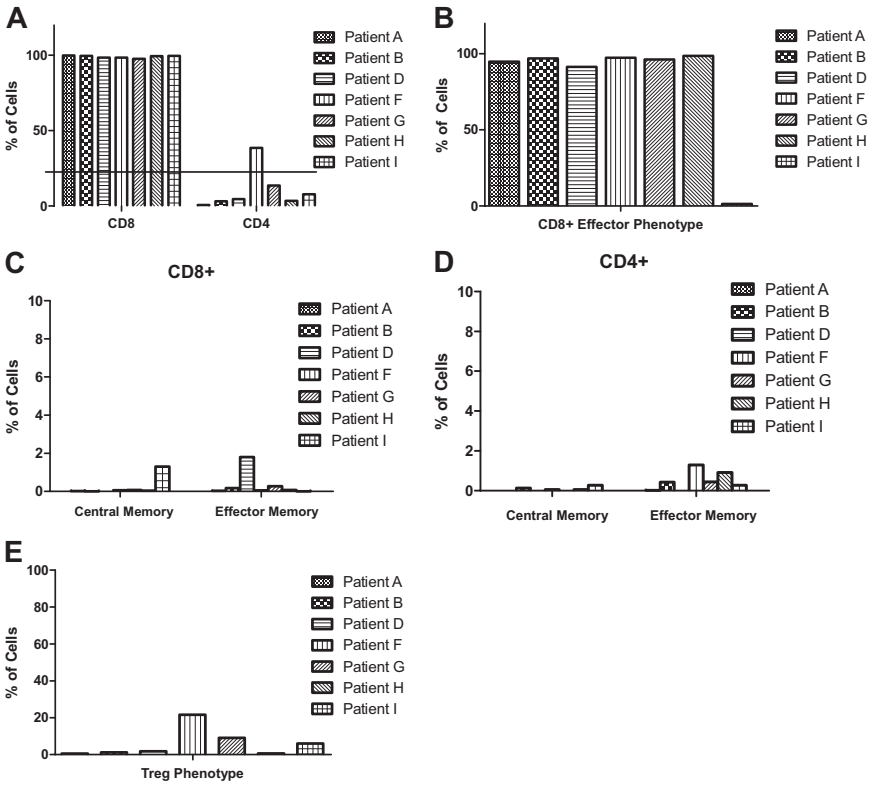

Figure 5. Immunophenotypes of infused T cells. The phenotypes shown were determined using multicolor flow cytometry and are expressed in terms of percentage of the population of infused cells. (A) $CD8^+$ versus $CD4^+$ cells, (B) cells with a $CD8^+$ effector T-cell phenotype ($CD8^+/CD62L^-/CCR7^-/CD45RA^-/CD127^-$), (C) cells with $CD8^+$ central memory ($CD62L^+/CCR7^+/CD45RA^-/CD127^+$) versus effector memory ($CD62L^-/CCR7^-/CD45RA^-/CD127^+$) T-cell phenotypes, (D) cells with $CD4^+$ central memory versus effector memory T-cell phenotypes, and (E) cells with a regulatory T-cell (Treg) phenotype ($CD4^+/CD25^+/FoxP3^+$) are shown.

Table 3. Flow cytometric immunophenotypes of infused T cells

Marker	Patient A	Patient B	Patient D	Patient F	Patient G	Patient H	Patient I
CD2	100	100	99	100	99.9	100	99.8
CD3	99	99.8	99	99.9	99.9	99.9	99.8
CD4	0.67	3.2	4.5	38.6	13.6	3.4	7.8
CD5	5.4	25.3	93	85.8	97.3	93.8	92.3
CD8	99.8	99.7	98.5	98.5	97.7	99.4	99.6
CD11a	100	100	98.7	99.9	99.8	99.9	99.8
CD25	2.4	45.9	22.1	23.2	52.5	52.7	16.6
CD28	0.92	3.2	4.4	2.6	5.4	1.9	2.7
CD44	99.8	100	99.5	99.9	100	99.9	99.9
CD45RA	61.5	14.1	2.3	3.1	9.5	2.2	23.0
CD45RO	96.8	99.3	99.1	99.4	99.4	99.2	99.8
CD49d	85.8	98.7	91.2	95.2	99.6	98.1	97.3
CD56	99.9	98.4	98.7	95.7	91.7	95.8	99.3
CD62L	1.3	6.8	4.6	2.1	6.2	2.7	2.5
CD94	98.8	67.3	38.7	32.8	7.1	17.6	33.6
CD95	99.4	99.5	99.8	98.1	98.4	99.6	98.6
CD134	1.3	2.9	6.2	4.1	5.3	3.2	4.5
CD137 (4-1BB)	0.47	1.9	4.4	1.2	3.1	0.69	3.7
CD154 (CD40L)	12.5	3.3	3.2	4.4	2.6	0.64	24.4
CD314 (NKG2D)	99.5	97.7	92.6	93.7	99.2	95.3	97.4
CCR5 (CD195)	58.7	43.1	58.1	27.9	85.9	6.0	96.8
CCR6	5.5	31.1	10.8	3.4	10.7	12.3	9.9
CCR7	0.21	2.3	4.2	2.1	4.6	0.75	5.4
CXCR3	97	89.9	91.9	95.2	97.5	93.1	98.1
CXCR4	6.5	9.6	6.1	3.9	21.1	9.7	2.6
CXCR5	1.1	3.1	3.8	2.7	6.1	2.0	3.5
TCR α/β	92.8	99.2	98.7	99	96	99.7	99.7

Values represent percentages of total cell population.

S1B). For humoral immune responses, patient serum collected at various time points after T-cell infusion was assessed in an ELISA assay in which the Leu-16 murine anti–human CD20 Ab served as an internal control (Figure S1C). Although it is theoretically possible that immune responses against other cTCR epitopes (eg, junctional regions) could be missed with this assay, the Leu-16 murine domain is expected to be the dominant immunogenic cTCR epitope because all the other domains are of human derivation. The absence of anti-cTCR Ab in patient sera at serial time points was confirmed by flow cytometry (Figure S2). No humoral or cellular immune responses were observed with these assays in any of the 7 patients who received infusions of genetically modified T cells. Two patients, however, were subsequently found to be seropositive for HAMA, 3 and 12 months after T-cell infusions, respectively. One of those patients had been previously treated with a radioiodinated murine monoclonal Ab (tositumomab).

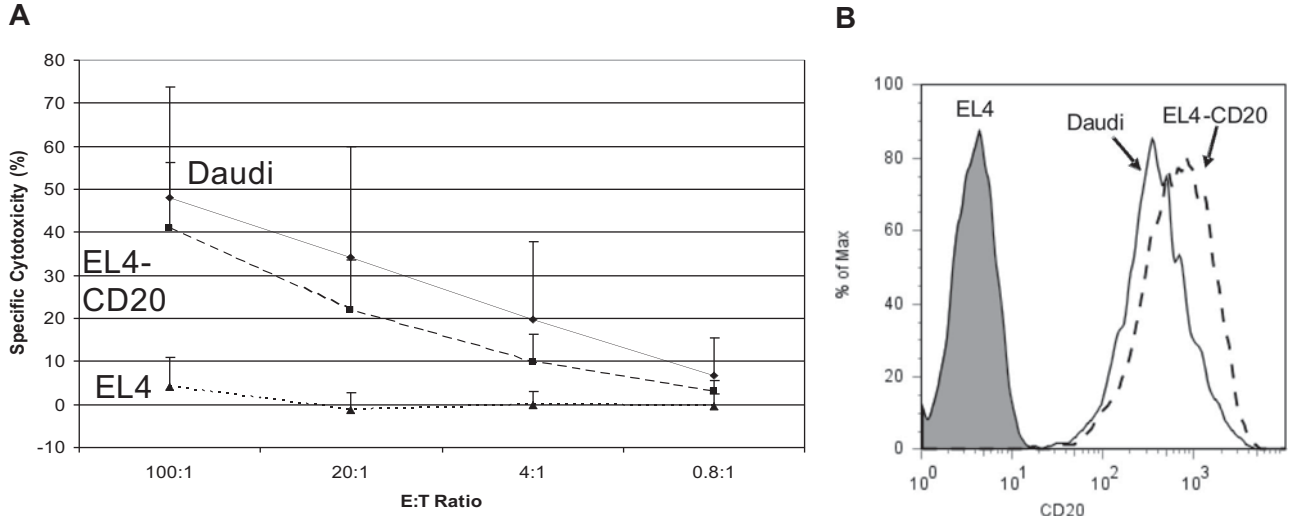

Figure 6. Cytotoxicity of modified T cells. Standard chromium release assays were performed using preinfusion-modified T cells at 8 to 12 days after restimulation, using the following MHC-mismatched target cells: EL4-CD20, a murine T-cell lymphoma line transfected to express the human CD20 molecule (····), the parental EL4 nontransfected CD20$^-$ line (···), and the Daudi CD20$^+$ Burkitt lymphoma cell line (—), at the E:T ratios shown. The calculated specific cytolysis values are displayed as percentages. (A) The CD20-specific cytotoxicity of the reinfused T cells. Data shown represent the mean combined data from all treated patients ($\pm$ 1 SD). Triplicate assays were performed for each patient. (B) CD20 expression of the target cell lines of EL4, EL4-CD20, and Daudi, as determined by flow cytometry using PE-labeled mouse anti–human CD20 Ab.

Table 4. Adverse events possibly or probably related to the treatment regimen

Patient*	Grade 1	Grade 2
A	None	None
B	None	None
D	None	None
F	Chills, myalgias, and shortness of breath (all during IL-2 treatment)	Injection site skin reaction (during IL-2 treatment)
G	None	Injection site skin reaction (during IL-2 treatment)
H	Dysgeusia, fatigue, diaphoresis, and injection site skin reactions (all during IL-2 treatment)	Flulike syndrome (during IL-2 treatment)
I	Malaise/fatigue, chills, injection site reactions, and dyspnea (all during IL-2 treatment)	Fever (during IL-2 treatment)

None of the patients experienced any grade 3 or grade 4 events.
*Patients C and E are omitted because they did not receive T-cell infusions.

Effect of adoptive T-cell therapy on native lymphocyte subsets

Anti-CD20 Ab therapy substantially depletes normal $CD20^+$ B cells,[31] and adoptive anti-CD20 T-cell therapy was therefore also expected to reduce circulating B-cell numbers. We found, however, that, although B-cell counts as measured by flow cytometry fluctuated during the period of T-cell infusions, the number of $CD20^+$ B cells remained stable or slightly increased in the months after treatment in all patients (Table S2). Other lymphocyte subsets ($CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, and $FoxP3^+$ cells) showed similar vacillations before and after T-cell infusions, but no consistent trends were noted (Table S3).

Responses to T-cell therapy

Two of the 7 patients achieved a complete response to cytoreductive chemotherapy administered before the T-cell infusions and remained disease-free 3 months and 13 months after T-cell infusions (Table 5). Another patient attained an objective partial response lasting 3 months after treatment with T-cell infusions plus IL-2. Four patients exhibited stable disease for 3, 5, 6, and 12 months. One of these patients showed decreased uptake of fluorodeoxyglucose, a marker for viable tumor metabolism, on positron emission tomographic scanning 3 months after T-cell infusions.

Discussion

MCL and indolent NHL almost invariably relapse after standard therapy despite complete clinical remissions, suggesting the existence of a minimal residual disease state in which a small number of cells resistant to chemotherapy, radiation, or Ab therapy persist and eventually lead to recurrent lymphoma. These cells appear to be susceptible to cellular immune responses, however, because patients undergoing nonmyeloablative allogeneic SCT or DLI can achieve long-term disease-free remissions, suggesting cure.[11] However, the high morbidity and mortality rates associated with these treatments,[9,32] primarily resulting from graft-versus-host disease, indicate a need for less-toxic forms of cellular therapy. One such strategy is infusion of ex vivo–expanded autologous T cells modified to recognize lymphoma-associated surface antigens.

We initiated a proof-of-concept clinical trial to test the safety and feasibility of this approach and found that autologous PBMCs from patients with lymphoma could be reliably modified to express a CD20-specific cTCR, expanded ex vivo, and reinfused with minimal toxicities. The process of generating therapeutic numbers of genetically modified anti-CD20 T cells by limiting dilution proved laborious, however, and the target cell dose was reached in only 1 of 5 patients. In contrast, expansion of transfected cells produced in bulk cultures was reliable, more efficient, and may also facilitate longer in vivo persistence in patients receiving such T cells requiring a shorter ex vivo expansion period. All patients tolerated the T-cell infusions well, with no adverse events attributable to T cells, and only grade 1 to 2 toxicities associated with IL-2 injections.

Adoptively transferred T cells presumably must survive in vivo for many weeks to effect a complete clinical response, and establishing memory cell responses may be required to produce durable remissions. We found that the administration of low-dose IL-2 after T-cell infusions prolonged in vivo persistence of modified T cells, consistent with previous observations.[33] Immunophenotypic differences between T cells produced by limiting dilution and those produced in bulk cultures were minimal, suggesting this was probably not the dominant reason for longer survival of the T cells from bulk cultures, although the shorter culture time may have contributed by decreasing replicative senescence. These questions cannot be directly assessed from our data because the change in culture methods was coincident with initiation of adjuvant IL-2 treatment. The administration of lymphodepleting chemotherapy before adoptive T-cell transfer is another factor potentially affecting in vivo persistence by enhancement of homeostatic proliferation by IL-7 and IL-15 up-regulation,[34,35] depletion of Treg cells,[36] and suppression of host immune responses to modified cells. Recent T-cell trials for melanoma incorporating aggressive lymphodepletion regimens have shown long periods of T-cell survival in some patients.[37,38] In the present trial, few compelling conclusions can be drawn as to the potential contribution of lymphodepletion to T-cell persistence because of the limited number of patients, the variability in chemotherapy regimens used, and the required 4-week interval between chemotherapy and T-cell infusions.

Recent data by Berger et al[39] indicate that $CD8^+$ T cells derived from central memory cells (T_{CM}) persist much longer in vivo than those derived from effector memory cells, raising the question of whether the longer persistence of T cells produced in bulk cultures in this study could be due to the presence of T_{CM}-derived cells among the infused T cells. Although this is theoretically possible, the low transfection efficiency by electroporation (0.002%-0.004%)[24] and the small number of clones infused make it unlikely that any infused cells were T_{CM} derived. It is much more likely that the difference in persistence in our study was due primarily to the IL-2 injections. Nevertheless, it may be important in future clinical trials to select, modify, and expand $CD62L^+$ cells to establish a population of persisting T_{CM}.

The expansion of genetically modified T cells produced by limiting dilution confers the theoretical advantage of permitting selection of cells with an in vitro cytotoxicity that exceeds the mean of a population of unselected cells in bulk culture. We observed, however, that the highest lytic activity and best proliferative capacity usually did not coincide in the same clonal population, and, consequently, expansion of the most cytolytic T cells was problematic. We believe, therefore, that the more favorable growth kinetics and apparently longer in vivo persistence of

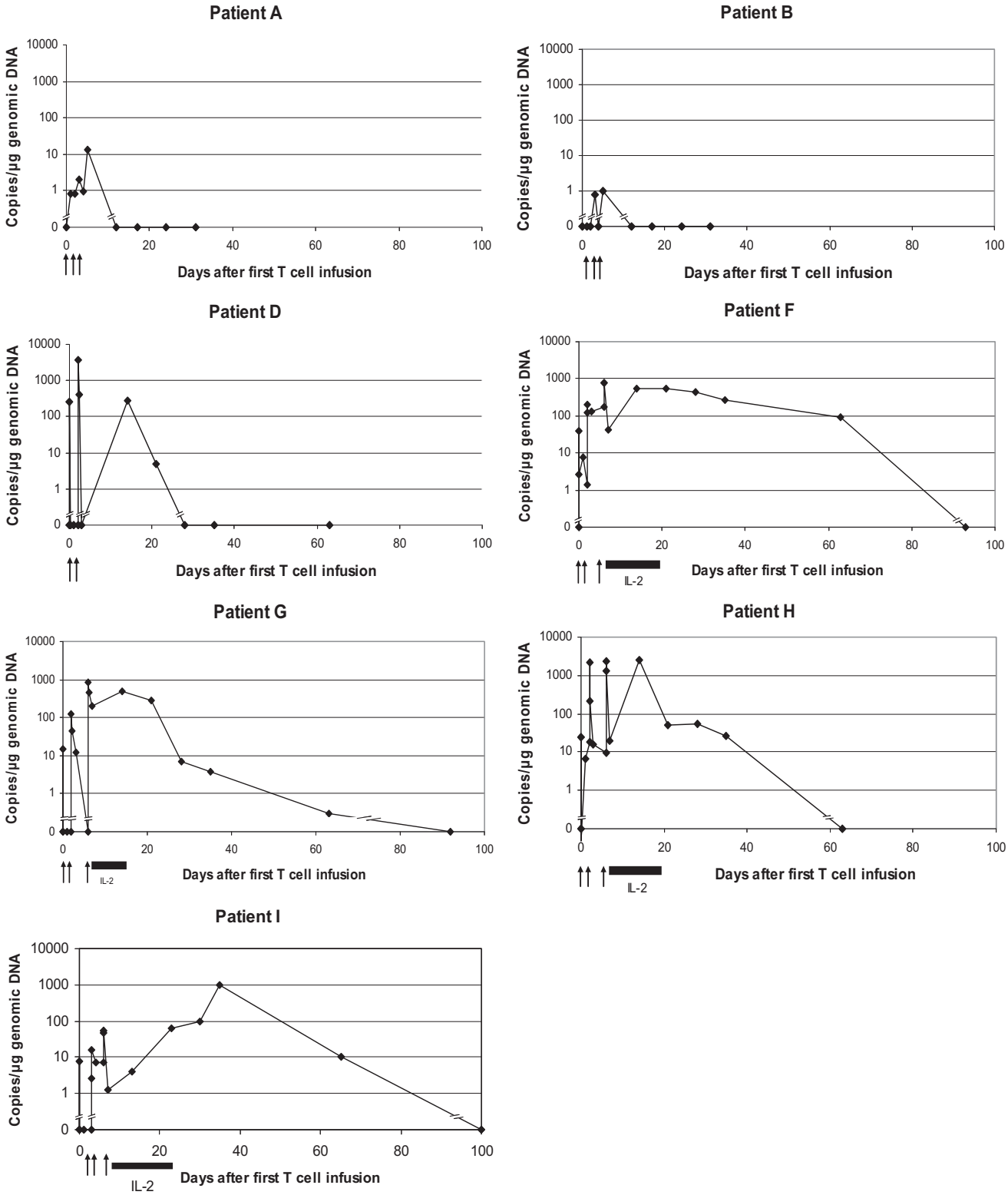

Figure 7. In vivo persistence of modified T cells. Genomic DNA was isolated from patient PBMCs collected at serial time points after T-cell infusions and used for quantitative real-time PCR using one primer within the human CD3ζ gene and the other from the adjacent CD4 transmembrane region in the scFvFc:ζ plasmid. The copy number of scFvFc:ζ-specific DNA based on quantitative reverse transcription-PCR results for all treated patients is shown. Arrows denote T-cell infusions, and horizontal black bars indicate the period of subcutaneous IL-2 injections for patients F, G, H, and I. Modified T cells were detectable for 12, 5, 21, 63, 63, 35, and 65 days, respectively, in the 7 patients.

T cells produced in bulk cultures outweighs any minor differences in cytotoxicity. T cells from all patients showed CD20-specific cytotoxicity despite the low levels of cTCR they expressed.

Efficacy was not a primary end point of this proof-of-concept study, but clearly that is the long-term goal. Objective clinical responses were modest, with one unequivocal partial remission

Table 5. Clinical responses

Patient*	Response to cytoreductive chemotherapy	Response to T-cell Infusions†	Duration of response after T-cell infusions, mo‡
A	CR	NED	13
B	PR	SD	3
D	SD	SD	12
F	CR	NED	3
G	Did not receive chemotherapy	SD (with FDG response on PET scan)	5
H	PR	PR§	3
I	PR	SD	6

CR indicates complete response; NED, no evidence of disease; PR, partial response; SD, stable disease; FDG, fluorodeoxyglucose; and PET, positron emission tomography.

*Patients C and E are omitted because they did not receive T-cell infusions.

†Response assessment was performed compared with the restaging imaging after cytoreductive chemotherapy.

‡All patients progressed after the times listed.

§Patient H achieved an additional objective PR after T-cell infusions.

among the 5 patients with evaluable disease at the time of T-cell infusions. There are many possible explanations for the limited therapeutic antitumor activity in this trial, including insufficient numbers of surviving modified T cells, CD20 antigen competition from native B cells, ineffective localization of T cells to tumor sites, inadequate cTCR surface expression leading to poor killing, and lack of costimulatory signaling from the cTCR construct. The suboptimal in vitro cytotoxicity of these cells suggests that the latter 2 factors were probably at least partially responsible. We plan to address some of these pitfalls in a subsequent trial by using T cells engineered with a "second-generation" anti-CD20 cTCR that includes CD28 and CD137 costimulatory domains and an SP163 translational enhancer. T cells transfected with this second-generation cTCR have shown superior proliferation, surface expression, and cytotoxicity in preclinical studies.[25]

Another possible explanation for the modest responses is competitive inhibition of cTCR-antigen binding by residual anti-CD20 Ab present in the serum of patients previously treated with rituximab, ibritumomab, or tositumomab. However, none of the treated patients (with the exception of patient I, who received ^{131}I-tositumomab 4 months before T-cell infusions) had been exposed to anti-CD20 Ab within 1 year of T-cell infusions. Therefore, with the exception of this patient, it is unlikely that significant circulating levels of anti-CD20 Ab were present at the time of adoptive T-cell transfer. It is somewhat surprising that significant changes in circulating B-cell levels were not seen in this study, in view of the well-documented B-cell depletion associated with rituximab treatment.[31,40] We do not know whether this reflects a resistance of normal resting B cells in vivo to the cytolytic activity of the cTCR$^+$ effector cells or a demonstration that adoptive T-cell immunotherapy with the "first-generation" plasmid as implemented in this trial has insufficient in vivo activity. This issue may be more clearly resolved after testing T cells expressing cTCR with the augmented second-generation plasmid described earlier.

In summary, adoptive immunotherapy with anti-CD20 cTCR-bearing T cells is a safe, feasible, and well-tolerated treatment for patients with relapsed or refractory indolent NHL, although clinical responses were modest. Expansion of transfected T cells produced in bulk cultures proved to be more efficient for achieving therapeutic numbers than generating T cells by limiting dilution. Furthermore, in vivo persistence of T cells was longer in patients receiving T cells produced by bulk cultures and low-dose IL-2 than in patients receiving T cells produced by limiting dilution and administered without subcutaneous IL-2. These results are encouraging but identify areas for improvement to be addressed in future adoptive T-cell therapy trials.

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Authorship

Contribution: B.G.T. enrolled patients, collected and analyzed data, and wrote the paper; M.C.J. designed the plasmid used to modify the T cells and developed the treatment protocol; J.W. performed PCR, cytotoxicity, and immune response assays; E.Y.C. enrolled patients and collected and analyzed data; B.L.W. and H.A.G. performed flow cytometry and TCR spectratyping experiments and contributed to writing the paper; X.Q. performed flow cytometry and humoral immune response assays; S.E.J. analyzed data and contributed to the design of experiments; A.R. and S.J.F. contributed to trial design; A.K.G. and J.M.P. enrolled patients and contributed to the interpretation of data; C.G.L. supervised T-cell transfection and expansion; P.D.G. and S.R.R. contributed to the design of the trial and experiments, provided the TM-LCL cell line, and analyzed data; and O.W.P. contributed to the conception, design, analysis, and interpretation of the clinical trial and experiments, wrote the protocol, and revised the manuscript.

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