From the Mouse Cage to Human Therapy: A Personal Perspective of the Emergence of T-bodies/Chimeric Antigen Receptor T Cells

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Chronicle of CAR Invention

AT THE END OF NOVEMBER 2013, *Science* magazine chose the advances made in *Cancer Immunotherapy* as the breakthrough of the year (Couzin-Frankel, 2013). The dramatic clinical outcome of patients treated with chimeric antigen receptor (CAR) T cells has been described as one of the major turning points of cancer therapy. In the framework of the annual issue of the journal of *Human Gene Therapy* that is dedicated to personal perspectives by scientists who have pioneered the field, I am honored to represent the members of my lab who have worked alongside me in establishing the CAR T cell approach (which we nicknamed “T-bodies”) and taken it from its initial proof of concept, through experimental animal studies, until its recent successful testing by our fellow clinicians in cancer patients.

When we pioneered the first CAR designs in the 1980s and early 1990s (Gross *et al.*, 1989; Gorman *et al.*, 1990), it was widely believed that T cells, while being potentially powerful effectors, have limited efficacy in the fight against cancer. Tumor infiltrating lymphocytes (TIL) are limited by virtue of their human leukocyte antigen (HLA)-restricted specificity to only a subset of patients from whom cancer-specific lymphocytes could be extracted. Similarly, tumors often silence their major histocompatibility complex (MHC) molecules as part of their escape from immune recognition (Gross and Eshhar, 1992), and many tumors do not express surface ligands to costimulatory receptors that are needed for the execution of the full antitumor potency of T cells. These factors and the immunosuppressive microenvironment induced by the tumor often render tumor-specific T cells dysfunctional in cancer patients (Eshhar *et al.*, 1993). To overcome the difficulty in activating anti-tumor T cells through the T cell receptor (TCR), we developed the CAR, which when introduced to T cells combines antibody-type specificity with effector T cell function. We demonstrated that artificially synthesized CARs effectively circumvent issues in activating the native receptor, and thereby provide an effective and potent tool for adoptive cell transfer (or therapy) of cancer. A description chronicling the milestones along the pathway of the CAR design structure and function is shown in Table 1.

Structural CAR Designs

The structure of the CAR (Fig. 1) evolved through several generations to optimize its function primarily for anti-cancer adoptive cell therapy (ACT). In fact, when we constructed the first CAR design we developed these constructs as a reagent to investigate the role of MHC recognition in the process of T cell activation. To that end, we replaced the $V\alpha$ and $V\beta$ extracellular domains of the TCR chains with their V_H and V_L immunoglobulin homologs (Gross *et al.*, 1989). The two chimeric TCR chains ($C\alpha V_H + C\beta V_L$ or $C\alpha V_L + C\beta V_H$) paired together to generate the first functional CAR and endowed the transfected T cells with antibody-type specificity. This “double-chain” configuration provided evidence that stimuli through the TCR/CD3 complex can activate T cells in an MHC unrestricted and independent manner (Gorman *et al.*, 1990; Gross and Eshhar 1992). Nevertheless, from a practical point of view, the engineering, construction, and expression of the double-chain CAR design in T cells was cumbersome. In addition, to attain full function, this construct is dependent on the immune-tyrosine activation motifs (ITAMs) that are the limiting element in the assembly of the native TCR/CD3 complex. To address these issues, we pioneered the single-chain CAR design (Eshhar *et al.*, 1993), a modular configuration that uses an extracellular single-chain Fv (scFv) (Bird *et al.*, 1988; Huston *et al.*, 1988) of a given antibody as the recognition unit, contiguous to structural and cytoplasmic elements required for T cell activation.

Our first scFv-based CAR constructs contained either the CD3 ζ or the FcR γ ITAM. For the first-generation single-chain CAR, we used scFv of the trinitrophenol (TNP)-specific Sp6 monoclonal antibody, and demonstrated its activity *in vitro* against TNP-modified murine lymphoma cells. Like the double-chain CAR, this construct recognized antigen in a non-MHC restricted manner. This first-generation CAR did not contain built-in costimulatory elements that are needed for full T cell activation. In fact, these cells could become anergized/exhausted by tumors in a manner similar to conventional T cells. We believe that such a scenario was circumvented in our first scFv CAR generation because the costimulatory signal

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TABLE 1. MILESTONES IN THE CHIMERIC ANTIGEN RECEPTOR EVOLUTION

Year(s)	Milestone	Investigator(s)
1989	First “double-chain” chimeric antibody receptor (CAR): TCRαV _H +TCRβV _L	Gross <i>et al.</i>
1993	First single-chain (scFv) CAR	Eshhar <i>et al.</i>
1993	First antitumor specific CARs (demonstrating <i>in vitro</i> cytotoxicity)	Stancovski (Her2/neu); Hwu <i>et al.</i> (a folate receptor); Gross <i>et al.</i> (B cell lymphoma idiotype)
1995	First demonstration of antitumor reactivity by redirected natural killer (NK) cells	Bach <i>et al.</i>
1996–1998	Introduction of costimulatory signaling molecules into the CAR	Hawkins <i>et al.</i> ; Finney <i>et al.</i> ; Sadelain <i>et al.</i>
2000–2006	First tumor-specific CAR in cancer patients	Kershaw <i>et al.</i> (a folate receptor, ovarian cancer); Lamers <i>et al.</i> (carboxyanhydrase-IX (CA-IX), renal cell carcinoma)
2010–2014	Pilot clinical trials showing complete remission of ALL and CLL patients	Rosenberg <i>et al.</i> ; Brenner <i>et al.</i> ; June <i>et al.</i> ; Jensen and Riddell <i>et al.</i> ; Brentjens <i>et al.</i>

ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia.

(also known as signal 2) was provided by the interaction between the endogenous CD28 receptor on the T cell surface and its CD80/CD86 ligands on the target cell surface. Subsequently, the modularly designed single-chain CAR allowed the inclusion of either one costimulatory moiety (e.g., CD28; known as second-generation CAR) (Finney *et al.*, 1998) or two costimulatory moieties (e.g., CD28+4–1BB; resulting in a third-generation CAR) (Tammana *et al.*, 2010; Hombach *et al.*, 2013). These CARs displayed superior functionality and anti-tumor responses compared to first-generation CARs (Kershaw *et al.*, 2002). In summary, the CAR is modular by design, allowing the use of any antibody as well as different combinations of signaling and cosignaling components enabling T cells to be redirected against any desired target antigen. Since antigen recognition is mediated by an antibody fragment, CAR-redirection T cells recognize their target in an MHC independent manner. Thus, a given CAR, unlike TCRs (that are MHC-restricted or dependent), can be used in any patients regardless of their MHC type. In addition, the lack of MHC restriction means CAR-redirection T cells can even recognize tumors that have lost their MHC expression.

Experimental Animal Models

Redirecting of effector T cells against tumors

The modification of T cells by CAR makes them very powerful effectors in the fight against cancer. In practice, every newly engineered CAR is tested *in vitro* following its introduction and expression in T cells, and the expected specificity is validated by a series of routine assays such as cytotoxicity and cytokine release toward specific and control target cells of human origin. Next, the *in vivo* function of human CAR T cells specific to a given tumor-associated antigen (TAA) is usually tested in immunodeficient mice, bearing a human cancer (cell line or fresh xenograft) expressing the target antigen. This setup is artificial in several respects, the major one being the allogeneic mismatch between the CAR T cells and the target tumor cells. Another difference is the xenogeneic difference between human effector CAR T cells and the murine host tissues. Thus, it is no wonder that most of these experiments resulted in tumor rejection and elimination. Close follow-up of CAR T cell recipient mice often reveals GvH-like symptoms that parallel the antitumor response. Specific antitumor responses

FIG. 1. Models of an antibody, T cell receptor, and chimeric receptors.

TABLE 2. TAKE-HOME LESSONS (FROM MURINE MODELS)

For use in adoptive cell therapy

- Use a combination of both CD4⁺ and CD8⁺ redirected naive memory T cells
- Precondition the recipient with lymphodepleting treatments
- Remove/neutralize Tregs
- Grow/infuse T-bodies in the presence of interleukin (IL)-7, IL-15, and IL-21 or a combination thereof
- Activation of naive T-bodies results in a better antitumor response
- Vaccination of recipients prior to T-body administration augments antitumor response
- Dual-specificity T cells result in longer persistence in the host
- Allogeneic T-bodies can serve as a universal source of donor cells under controlled conditions in adjuvant therapy for systemic metastases
- Cancer stem cells are optimal targets for T-body cancer therapy

To enhance safety of therapy

- Use inducible suicide genes
- Use site-specific integrating vectors

have been noted, primarily because the specific CAR modified T cells can persist longer and cause more sustained responses compared to nonrelevant CAR controls. Such behavior gave rise to the concept of “dual-recognition,” suggesting that co-expression in a cell of a tumor-specific CAR transgene together with an endogenous TCR against an alloantigen or opportunistic viral antigens (e.g. Epstein-Barr virus (EBV), cytomegalovirus (CMV) on the target cell results in an enhanced antitumor response (Kershaw *et al.*, 2002; Dutour *et al.*, 2012; Terakura *et al.*, 2012). In fact, this strategy was also applied to generate antigen-presenting cells to support the propagation of CAR T cells during their manufacture to prolong their persistence in the patient (Louis *et al.*, 2011).

Below, I describe several representative studies performed by our group investigating the antitumor activity of murine CAR T cells in immune competent murine models (Table 2). In one such experimental model relevant to systemic metastases, we systemically (i.v.) injected to syngeneic mice the Renca renal cell carcinoma, expressing the human *erbB-2* transgene. In this system, mice died of lung micrometastases with median survival time of 50 days. Mice receiving TNP-specific control CAR T cells died at the same rate. However, a single systemic administration of 10×10^6 *erbB-2*-specific CAR T cells prolonged the median life span of the treated mice to 90 days. Repeated administration of the same CAR T cells further prolonged the median lifetime to 125 days (Friedmann-Morvinski *et al.*, 2014). Using this system, we demonstrated that preimmunization of mice with heat-killed Renca cells, preconditioning the mice with lymphodepleting regimens, as well as preimmunization and preactivation of the CAR T cells by Con-A, significantly prolonged the survival of the treated mice to more than 200 days. Using this experimental system, Dr. Assaf Marcus (Marcus *et al.*, 2011) treated the tumor-bearing host that created a time window that allowed allogeneic CAR T cells to reject the otherwise lethal systemic cancer metastases. This was achieved by infusion of allogeneic *erbB-2*-specific

CAR T cells that combine both allograft rejection with the antitumor effector reactivity of the CAR T cells. To overcome the potential host-versus-graft (HvG) response and transiently suppress and delay the recipient's HvG, the mice were sublethally irradiated and treated with the sphingosine monophosphate antagonist, FTY720, which sequesters the host T cells in the lymphoid organs (Matloubian *et al.*, 2004).

Until recently, most of our experimental murine models were based on transplanted tumors. A more suitable model that faithfully mimics cancer patients is that of spontaneously developing cancers in immune-competent mice. In a most recent study performed by Dr. Anat Globerson-Levin (Globerson-Levin *et al.*, 2014) in my group, we compared the antitumor response of *erbB-2*-specific CAR T-cells in the Her2NG transgenic mouse, which overexpresses the human *erbB-2* transgene under the mouse mammary tumor virus (MMTV) promoter (Finkle *et al.*, 2004). Female Her2NG mice progressively develop mammary tumors in almost all their mammary glands. Comparing the antitumor reactivity of *erbB-2*-specific CAR modified T cells under various therapeutic settings, either prophylactically, prior to tumor development, or therapeutically, after tumor(s) became apparent in one or more mammary glands, we found that repeated administration of CAR modified T cells is required to eliminate and control spontaneously developing mammary cancer. Systemic, as well as intratumorally administered, CAR modified T cells accumulated at tumor sites and eventually eliminated the malignant cells.

Interestingly, within a few weeks after a single administration of CAR modified T cells and rejection of the primary lesion, tumors usually relapsed both in the treated mammary gland and at remote sites; however, repeated injections of CAR modified T cells were able to control the secondary tumors. In fact, one mouse remained cancer-free for more than 500 days following treatment. Analyses of T cells isolated from this mouse revealed that about 40% of the T cells in the blood, 50% in the spleen, and 4% in the bone marrow expressed the *erbB-2*-specific CAR T cells. Out of the blood-borne CAR T cells, 47% had a naive phenotype, 21% were effector memory cells, and 4% had central memory phenotype. Systemically injected luciferase-labeled CAR T cells specifically accumulated within 2 days after systemic injection at the mammary glands where tumors developed; however, the in-vivo imaging system (IVIS) signal disappeared about 2 weeks after CAR T cell administration. Notably, anti-idiotypic antibodies could be detected in the sera of the treated mice, and we believe that this is associated with the disappearance of the IVIS signal. Interestingly, expression of low levels of *erbB-2* were reported in healthy organs such as lungs, pancreas, and liver of the Her2NG mice (Finkle *et al.*, 2004); however, no histological damage has been noted in these organs. Intratumoral injection of the *erbB-2*-specific CAR modified T cells had a systemic effect: the engineered T cells rejected not only the mammary tumor at the injection site, but also at remote glands, and retarded the appearance of tumors in others. Finally, this unique model allowed us to study the prophylactic potential of CAR T cells. Indeed, a single systemic injection of 10×10^6 CAR T cells before the appearance of any mammary tumor became apparent could significantly delay the appearance of mammary tumors for several months.

In an as yet unpublished study, we used the CEABAC-10 strain of transgenic mice expressing the human carcinoembryonic antigen (CEA) gene under its native promoter (Chan and Stanners, 2004). In these mice, we could induce colitis-associated colorectal cancer, and succeeded to alleviate tumor load and severity through two approaches, suppressing the intensity of experimental colitis using CEA-specific CAR-modified regulatory T cells (see below) and by colonoscopy-guided intrarectal tumor injection of CAR-modified T effector cells.

Redirection of regulatory T cells

Regulatory T cells (Treg) can potentially be applied for the suppression of inflammatory immune conditions such as autoimmunity, graft rejection, and graft versus host responses. The main problem in employing Treg for the treatment of these indications is the scarcity of naive Treg cells. A few years ago, we embarked on a series of studies aimed at redirecting naive Treg (nTreg) to sites of immune inflammation. In the first set of studies, we isolated nTregs from a transgenic strain of mice that we generated expressing TNP-specific CAR (Friedmann-Morvinski *et al.*, 2005; Elinav *et al.*, 2008), and tested their ability to alleviate acute colitis induced by dinitrobenzene sulfonic acid (DNBS) in a syngeneic wild-type strain. The data obtained were clear-cut: adoptive transfer of the TNP-specific CAR Treg cells rescued a large proportion of the colitic mice from death. The effect was specific; it did not affect disease caused by a nonspecific sensitizing agent (e.g., oxazolone), and CAR Treg of irrelevant specificity had no effect. The adoptively transferred TNP-CAR-Tregs accumulated at the inflamed colon, and upon their interaction with the colitogen, propagated and secreted TGF β and interleukin-10 (IL-10) (Elinav *et al.*, 2008). The fact that a significant component of the Treg mediated suppressive effect was mediated through suppressive cytokines explains the “bystander” effect we observed at the inflamed site. We found that the Treg need not necessarily be directed at the pathogen or inducer of the immune-inflammation. Rather, one can redirect the Treg with CAR recognizing tissue-specific markers in the inflamed site, which, upon interaction with the CAR, will induce the release of suppressive cytokines and, in turn, will also alleviate inflammatory responses initiated by different targets.

To test the effect of CAR-redirected Treg on a chronic autoimmune disorder, we chose the chronic colitis model in the CEA transgenic mice (Chan and Stanners, 2004). We used two models of colitis, first the classical dextran sodium sulfate (DSS) model, in which the level of CEA expressed in the colonic epithelia was elevated similarly to that in human patients. The second model was that of cell-induced colitis, in which the CD4⁺, CEA-specific CAR-Teff inducer cells were adoptively transferred to the CEA transgenic mice. In these two models, the CEA-specific CAR Treg significantly alleviated the colitis, further supporting the therapeutic potential of the CAR-Treg strategy (Blat *et al.*, 2014).

CAR Made It to the Patients' Bed

Early steps

About a decade ago, a few CARs specific to tumor-associated antigens had been introduced into cancer patients.

First one was by Lamers *et al.* (2005) who started a phase I/II CAR T cell therapy study of metastatic renal cell carcinoma (RCC) using autologous T lymphocytes transduced with a CAR specific to carbonic anhydrase IX (CAIX), which is overexpressed by RCC cells. The kinetics of CAR T cell counts suggested loss of surface CAR expression on adoptive transfer. At the first trial, no effect had been noticed. At a later point in time, the CAIX specific CAR T cells were administered to 12 RCC. Patients were treated in three cohorts with a maximum of 10 infusions of a total of 0.2 to 2.1×10^9 CAR T cells. Toxicity grade 2–4 liver enzyme disturbances occurred, necessitating cessation of treatment in four out of eight patients. Liver biopsies revealed CAIX expression on bile duct epithelium with infiltration of T cells, including CAR T cells. In a subsequent trial (Lamers *et al.*, 2013) patients' pretreatment with CAIX monoclonal antibody prevented the CAR-specific liver toxicities; nevertheless, no clinical responses were recorded. This report provided inpatient proof that the observed “on-target off-tumor” toxicity is antigen-directed and can be prevented by blocking antigenic sites in off-tumor organs and allowing higher T cell doses.

In parallel, another phase I study (Kershaw *et al.*, 2006) was conducted to assess the safety of adoptive immunotherapy using gene-modified autologous T cells for the treatment of metastatic ovarian cancer. Autologous T cells expressing CAR against the ovarian cancer-associated antigen alpha-folate receptor (FR) were administered to two cohorts. One received a dose escalation of T cells in combination with high-dose IL-2, the second received dual-specific T cells (reactive with both FR and allogeneic cells) followed by immunization with allogeneic peripheral blood mononuclear cells. Patients in the first cohort experienced some grade 3 to 4 treatment-related toxicity that was probably due to IL-2 administration. Patients in cohort two experienced relatively mild side effects with grade 1 to 2 symptoms. No reduction in tumor burden was seen in any patient. Tracking labeled CAR T cells in the first cohort revealed a lack of specific localization of T cells to tumor except in one patient. Kinetic analysis showed that gene-modified T cells quickly declined to be barely detectable 1 month later in most patients. An inhibitory factor developed in the serum of half of the tested patients. This pioneering study taught us that CAR T cells can be safely given to patients, but these cells do not persist in large numbers long term.

CD19-specific CAR T cells cure B cell malignancies

The breakthrough of the CAR strategy emerged in the beginning of second decade of this century when several groups have embarked on treating B-cell lymphomas and leukemias, as well as neuroblastoma. It is detailed and summarized in an excellent review by the University of Philadelphia group (Maus *et al.*, 2014). The first group to report on significant responses was led by Dr. Rosenberg at the National Cancer Institute's (NCI's) surgery branch (Kochenderfer *et al.*, 2010, 2012, 2014). The most recent publication that summarizes 4-year's treatment of end-stage patients with chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies has been recently reported (Kochenderfer *et al.*, 2014). The outcome of

the phase I/II study treating patients with advanced B cell malignancies using CD19-specific, autologous CAR T cells resulted in 12 of 13 treated patients that came with objective response rate (92%), 8 patients came with complete remission (67%), and 4 of 7 patients with diffuse large b-cell lymphoma (DLBCL) are having ongoing complete remissions, one of whom is disease free for more than 22 months.

The second group to report on substantial outcomes in the treatment of B-CD19 leukemias and lymphomas is led by Dr. Carl June from Abramson Cancer Center at the University of Pennsylvania (Kalos *et al.*, 2011; Porter *et al.*, 2011; Grupp *et al.*, 2013; Maus *et al.*, 2014). In another contribution to the pioneers in gene therapy of this *Human Gene Therapy* series, Dr. Carl June presents achievements in chronic lymphocytic leukemia (CLL) and acute lymphocytic leukemia (ALL). As of November 2013, this group has been employing third-generation CD19-specific CAR T cells. Within the last 4 years they have treated 33 CLL patients, 23 of whom (70%) with objective response and 17 (53%) with complete response. Twenty-two ALL patients have been similarly treated; 18 (82%) came with objective responses, out of whom 13 (59%) had complete remission.

About seven clinical research and clinical institutions in the United States and at least two abroad are currently involved in the treatment of patients with CAR T cells; almost all of them are active in the search for new CARs for new indications. To date, there are more than 60 clinical studies listed at the NIH registry, a large proportion of which are open and recruiting patients. Big biopharma as well as start-ups are joining the scene. The space of this review is too short to list all innovations and contributions to the field. For broader, updated, and detailed reviews of the field, the article of Maus *et al.* (2014) is highly recommended.

CAR in Prospective

Starting with a basic scientific question through research in experimental animal models, the clinical data achieved using the CD19 CAR T cell supersedes any other treatment available today for patients with end-stage hematopoietic cancers. By combining the two major arms of adaptive immunity, the T-body strategy has already proved efficient in the treatment and therapy of terminal lymphoma and leukemia. A major future challenge in cancer immunotherapy is to translate this success to solid tumors. Initial attempts, however, have fallen short of this goal.

Hematological malignancies and solid tumors lack genuine cancer cell specific antigens. Both healthy and cancer cells coexpress a similar set of target antigens and are thereby susceptible to toxicity mediated by CAR redirected T cells. Hematologic cells, however, will eventually regenerate from the hematopoietic stem cell reservoirs. Hands-on experience gained from recent clinical studies has accumulated during the last 5 years, enabling practitioners to overcome and reverse the adverse effects resulting from cataclysmic damage to cancer cells and toxicity to healthy tissues, including cytokine release storm, tumor-lysis syndrome, as well as B cell aplasia. In parallel, efficient procedures have been developed to manufacture the CAR modified T cells (e.g., addition of antigen-presenting cells, use of EBV-specific T cells, as well as by supplementing the growth media with interleukins and growth factors that in-

crease the persistence of the CAR T cells in the recipients). In practice, these manipulations increase the specific anti-tumor activity of the CAR T cells, enabling their administration to patients in smaller numbers. In fact, the curative dose of CAR T cells is currently around 5 million per kg body weight. To date, there are more than 60 clinical studies listed in the National Institutes of Health (NIH) registry, a large proportion of which are open and recruiting patients; big biopharma as well as start-ups are joining the scene.

Lessons learned from the lymphoma/leukemia trials are being applied in animal models to the more difficult problem of solid tumors. The major challenge is to avoid or overcome toxicity to essential nonregenerating healthy tissues. Some promising approaches have been discussed above and include: a combination of CAR T cell treatments with immunomodulatory protocols to overcome the immunosuppressive microenvironment induced by solid tumors, the development of new CAR designs to achieve more selective killing of the malignant tissue, and aiming at cancer target antigens to avoid tumor escape and relapse.

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