Second-Generation Anti--Carcinoembryonic Antigen Designer T Cells Resist Activation-Induced Cell Death, Proliferate on Tumor Contact, Secrete Cytokines, and Exhibit Superior Antitumor Activity In vivo: A Preclinical Evaluation

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Abstract

Purpose: This report describes the development and preclinical qualification tests of second-generation anti-carcinoembryonic (CEA) designer T cells for use in human trials.

Experimental Design: The progenitor first-generation immunoglobulin-T-cell receptor (IgTCR) that transmits Signal 1 only effectively mediated chimeric immune receptor (CIR) directed cytotoxicity, but expressor T cells succumbed to activation-induced cell death (AICD). The second-generation CIR (termed “Tandem” for two signals) was designed to transmit TCR Signal 1 and CD28 Signal 2 to render T cells resistant to AICD and provide prolonged antitumor effect in vivo.

Results: A CIR was created that combines portions of CD28, TCR, and a single chain antibody domain (sFv) specific for CEA into a single molecule (IgCD28TCR). As designed, the gene-modified Tandem T cells exhibit the new property of being resistant to AICD, showing instead an accelerated proliferation on tumor contact. Tandem T cells are more potent than first generation in targeting and lysing CEA+ tumor. Tandem T cells secrete high levels of interleukin-2 and IFNγ on tumor contact that first-generation T cells lacked, but secretion was exhaustible, suggesting a need for interleukin-2 supplementation in therapy even for these second-generation agents. Finally, second-generation T cells were more effective in suppressing tumor in animal models.

Conclusion: An advanced generation of anti-CEA designer T cells is described with features that promise a more potent and enduring antitumor immune response in vivo. These preclinical data qualify the human use of this agent that is currently undergoing trial in patients with CEA+ cancers.

The use of tumor-infiltrating lymphocytes (TIL) in patients with melanoma or renal cell carcinoma possessed a rationale based on a native but weak response of the host to the tumor before therapeutic intervention that was augmented by ex vivo manipulation and re-administration with interleukin-2 (IL-2) supplementation (1). However, this recognition was imperfect, or the tumor would have regressed on its own, and the degree and durability of the responses were infrequently satisfactory, whether due to induced tolerance or antigen modulation.

A rational approach to build on the encouraging results in these two tumor systems (melanoma and renal cell carcinoma) would be to provide patient T cells the power to recognize tumor-associated antigens by design. Such therapies applying chimeric immune receptors (CIR) have been variously termed T-bodies, universal receptors, or designer T cells (reviewed in ref. 2). In one configuration, these molecules are fusion products of an antibody binding domain (immunoglobulin, Ig) with the ζ signaling chain of the TCR, to form IgTCR. When expressed by gene therapy techniques in recipient T cells, the resulting designer T cells are redirected by the neo-specificity of the CIR to attack tumors expressing the surface antigen recognized by the immunoglobulin. This strategy is designed to bypass a major drawback of cancer immunotherapies, which have been hampered by the fact that most “tumor antigens” are normal self-proteins to which the patient is already tolerized.

These protocols have the advantage that one may design therapies to attack other types of tumors than the limited set susceptible to TIL therapies. Any cancer can be targeted that has a known cell surface marker that is expressed in a tumor-
restricted fashion. This strategy has the advantage that peripheral blood cells may be used without resort to tumor sampling and the associated challenges of tumor-induced propagation of T cells ex vivo. In chimeric receptor approaches, the receptors are MHC independent and the vector to modify autologous T cells is therefore an off-the-shelf reagent available to all patients irrespective of HLA-type. Moreover, being non–MHC dependent means that the T cells are not thwarted by mutations affecting antigen processing (TAP proteins) or down-regulation of MHC that can lead to tumor evasion (3, 4). Additionally, transgene modification of CD4+ T cells in bulk peripheral blood mononuclear cells provides a mechanism for generating CD4 T helper cell activity that is typically missing from classic TIL protocols. Finally, the weak antitumor response among TIL in patients that is sampled for later TIL therapy may be improved on by the stronger, engineered interactions that foster ‘more effective’ T-cell responses (e.g., ref. 5). In principle, the antitumor T cells so designed may respond to the tumor as in an organ rejection, with cytokine secretion, proliferation, and cytotoxicity against antigen-expressing targets. These features constitute the rationale for the designer T-cell approach for cancer therapy.

In parallel with these developments, an evolution in our understanding of T-cell biology was taking place. It had been noted that T cells would gradually die after engagement of the TCR (Signal 1), although killing many target cells in the interim. This death process in T cells was termed antigen-induced cell death (AICD). Investigations showed early on that AICD was a form of apoptosis, mediated through the so-called death receptor pathway (6). AICD was associated with DNA fragmentation, phosphotidylserine inversion (measured by Annexin V binding), and an abbreviated survival that was not rescued by any cytokine supplementation. In contrast, when TCR Signal 1 was supplemented with costimulatory Signal 2 by CD28 engagement on the T cell, there was a suppression of AICD and its associated correlate measures with an accompanying proliferation and improved survival (7, 8).

We previously created the so-called first-generation Signal 1–only anti–carcinoembryonic antigen (CEA) designer T cells (9) and instituted a clinical trial in colorectal and breast cancers. Biological responses were observed, providing proof-of-principle of antitumor immune activity, but the responses were transient in nature (10). Thereupon, we embarked on laboratory studies that confirmed loss of modified T cells in an antigen-dependent manner over a period of 1 week in vitro (11), corresponding to the time of expiration of immune activity in vivo. Like normal T cells, first-generation designer T cells received only Signal 1 on antigen contact, supplied by the grafted TCR, which would be predicted to render the designer T cells susceptible to AICD. In a test of the potential of CD28 Signal 2 to rescue cells from loss after Signal 1, designer T cells were stimulated by tumor cells with B7 artificially coexpressed to engage T-cell CD28 coreceptor; as a result, T-cell death was blocked and an accelerated T-cell proliferation ensued with prolonged tumor cell killing.

These data (11) provided the impetus and justification for a further modification to the original design to incorporate CD28 signaling into the chimeric receptor. In comparison with the first-generation IgTCR, the second generation exhibits induced proliferation on contact with tumor and resistance to AICD, superior cytotoxicity and cytokine production, and improved activity in animal tumor models.

Our goal is to create an effective, self-sustaining immune attack on recurrent or refractory CEA-expressing cancers, including colorectal, breast, lung, and others, in a therapy that combines the advantages of antibody specificity with the homing, tissue penetration and target cell destruction capabilities of T cells. Between 100,000 and 150,000 people die of CEA+ tumors per year in the United States alone who could be helped by this new therapy. The product of this study is the next iteration in the cycle of bench-to-bedside-to-bench-to-bedside toward achieving this goal. This second-generation designer T-cell product is presently in human clinical trials under Food and Drug Administration BB-IND 10791. The following describe the supporting preclinical data for this application.

Materials and Methods

Mice. Six- to 8-week-old female BALB/c nude mice were purchased from Harlan Sprague-Dawley and maintained under a protocol approved by the Institutional Animal Care and Use Committee.

Cell lines. The human colorectal cell line MIP101 was derived from a CEA-negative, poorly differentiated tumor, and the MIP CEA line created by introducing the full-length human CEA gene (12). The B7.1-positive derivatives of MIP101 (MIP101 B7.1) and MIPCEA (MIPCEA B7.1) were generated by introducing the full-length human B7.1 gene (11). Cell lines were maintained in DMEM and 10% FCS (Invitrogen) supplemented with 100 units/mL penicillin/streptomycin (Cellgro).

Antibodies and flow cytometric analysis. The humanized anti-CEA antibody hMN14 (13) and its anti-idiotypic antibody WI2 were obtained from Immunomedics. The WI2 antibody was prepared as a phycoerythrin conjugate (WI2-PE; Molecular Probes) used to detect CIR expression on transduced T cells. Negative control antibody UPI-10 (Sigma Chemical) is an IgG2a mouse antibody against ρ-2-6-linked fructosan (Sigma). Expression of the CEA and B7.1 transgenes in the MIP101 and MIPCEA cell lines was monitored by flow cytometric analysis using hMN14 or anti-B7.1 (Caltag) as previously described (11).

Generation of chimeric receptors. The first-generation anti-CEA IgTCR fusion receptor that was cloned into the MFG retroviral backbone was previously described (9). The Tandem molecule (pTandem) was generated by molecularly fusing the hMN14 sFv-CD8 hinge segment of the IgTCR (IgCEA) in the MFG retroviral backbone with a hybrid CD28/CD3ε molecule. CD3ε and CD28 fragments were obtained from human peripheral blood mononuclear cells by reverse transcription-PCR with suitably designed primers for direct cloning into assembled intermediate constructs. The pTandem construct was verified by restriction digestion and sequencing. Supplementary Fig. S1 indicates the boundaries of the included parent proteins in the final product.

Retroviral supernatant was produced using the PG13 vector producer cell line to generate gibbon ape leukemia virus pseudotyped viral particles as described (14).

Lymphocyte culture and transduction. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of whole blood or leukocyte filter washes onto a Histopaque 1077 (Sigma) cushion. The PBMC T-cell fraction was activated by treatment with the anti-CD3
monoclonal antibody OKT3 (Ortho Biotech) at 20 ng/ml in the presence of 300 units/ml of human IL-2 (Chiron). After 3 to 5 days of culture, the majority of cells are T lymphocytes with few natural killer cells, typically 0% to 2%. At 2 to 3 d postactivation, T cells were transduced two to five times over 1 to 2 d using retroviral supernatant or control medium and then maintained in 100 IU/ml IL-2 as described (14). After 48 h, cells were analyzed for CIR expression by flow cytometry (above).

Western blot. Transduced T cells (1 × 10^7) were processed to membrane fractions of protein as described (9). Samples with the equivalent of 10^6 cells were prepared in nonreducing or reducing buffer and run on polyacrylamide gradient gels, transferred, and analyzed using antihuman ζ-chain monoclonal antibody 8D3 (Pharmingen) followed by horseradish peroxidase-conjugated second antibody (Caltag). Immunodetection was done using enhanced chemiluminescence (Amersham) and X-ray film exposure.

Proliferation and expansion assays. Untransduced, IgTCR, and tandem bulk T-cell populations were counted and aliquoted at 7.5 × 10^7 per well of a 24-well plate in GM plus 100 IU/ml IL-2. To each well, 1 × 10^6 irradiated (10,000 rad; Nordion gammacell 40 Exactor Irradiator) target cells from MIP101, MIP101 B7-1, MIPCEA, or MIPCEA B7-1 cell lines were added to assess the ability of the untransduced, IgTCR, and Tandem transduced T cells to proliferate in response to antigen-specific stimulation. Viable T cells were counted on days 3 and 7 for the shorter-term “expansion” cultures and on days 3, 6, 9, 12, and 15 for the longer-term “proliferation” cultures.

To further characterize the proliferation of the transduced T cells, 5 × 10^6 bulk T cells from the IgTCR and Tandem groups were seeded in small flasks. The T cells were cocultured with irradiated MIPCEA or MIPCEA B7-1 target cells. The T-cell cultures were restimulated every 3 d with fresh irradiated target cells. The selective effect of antigen on proliferation was assessed by the changing CIR+ fraction of T cells by flow cytometry. The pre-experimental histogram for IgTCR+ cells in Fig. 2C was 5% (as shown), but was further diluted with untransduced activated T cells to make a final percentage of 2% for the proliferation experiment. The final adjusted T-cell mix was not reanalyzed before initiating the coculture. The IgCD28TCR+ cells were 2% on their preanalysis and were used directly without further adjustment.

Estimation of growth rate constant change. The basal growth rate constant for the activated but non-reconstituted untransduced T cells is specified as k1, in which the doubling time is ln2/k1. The growth rate constant for the restimulated Tandem T cells is specified as k2. The following equations apply, in which C1 and C2 are the number of untransduced and Tandem T cells, respectively, and C10 and C20 are their starting numbers:

\[
C_1 = C_10 \times \exp(k_1 t)
\]

\[
C_2 = C_{20} \times \exp(k_2 t)
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Three conditions in Fig. 2C allow unique solution of these simultaneous equations: the initial condition of C20/C10 = 0.33; and specification of simultaneous equations: the initial condition of C20/C10 = 0.02; the condition at 14 d of C2/(C1 + C2) = 0.33; and specification of the basal expansion k1 rate = 0.23 day⁻¹ for a nominal 3-d doubling time. When solved with these values, k2 ≈ 2 × k1, or 200% of the basal rate (k1).

Chromium-51 release assays (CTL). PMBCs were activated and transduced with IgTCR or Tandem retroviruses and expanded in IL-2 for 3 d. The effector cells were harvested and then rested overnight without IL-2 to decrease nonspecific lysis in the assay. Serial dilutions of the rested effector cells were incubated in a V-bottomed 96-well plate with 5 × 10^3 labeled MIP101 or MIPCEA target cells in a 4-h assay. The percent lysis was calculated as (experimental cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm) × 100.

Cytokine secretion by ELISA. MIP101, MIP101 B7-1, MIPCEA, and MIPCEA B7-1 target cells were seeded at 5 × 10^5 per well and allowed to adhere for 6 h. Effector cells (1 × 10^5) were added and cocultured for 16 to 20 h in the presence of 20 units/ml IL-2 (1.1 ng/ml). Supernatants were analyzed for IL-2 and IFNγ by ELISA (eBioscience).

In vivo tumor model. To generate tumors, 2.5 × 10^5 MIP101 and 5 × 10^6 MIPCEA cells were injected s.c. on opposing flanks into BALB/c nude mice as described (15) to yield comparable tumor growth rates. Small (1-d) tumor assays: After 24 h, a small nodule was palpated at each tumor injection site and the T cells (5 × 10^6), unmodified, IgTCR, or Tandem, were administered via tail vein. Sizes are in square millimeter as the product of the largest and smallest transverse dimensions.

Established (6-d) tumor assays: Tumors were allowed to grow for 6 d until ~7 mm in diameter. Some animals had only MIP-CEA injected to allow long-term monitoring of responding tumors. T cells were injected as above, and IL-2 treatment was initiated (2.5 × 10^5 IU s.c. every 12 h for 14 d) because of the anticipated longer T-cell survival needed to eliminate the larger tumors.

Results

Vector construction

Model studies previously showed that costimulation could be engaged in a similar antigen-dependent fashion as the IgTCR, either as IgCD28 or in a colinear structure with TCR signaling molecules (16–18). We examined CIR constructs with IgTCR + IgCD28 in the same retroviral vector, with an intervening internal ribosome entry site. However, these proved unstable on infecting the vector producer cells, undergoing recombination deletion between the immunoglobulin domains to generate an IgCD28 without IgTCR. To maintain the goal of a single viral vector for gene expression, we turned our focus to adopting a colinear design. We reengineered our first-generation IgTCR (9) by inserting the CD28 transmembrane domain plus a portion of the CD28 extra-cellular domain (Fig. 1B) that maintains CD28 dimerization (ref. 18; the molecular junctions of the chimeric molecule are represented in Supplementary Fig. S1). This molecule was termed “Tandem” to signify the two signaling regions (Signals 1 + 2) in the same molecule. Whereas the IgTCR has a TCRβ transmembrane domain that directs it to assemble into the native TCR, the CD28 transmembrane domain allows Tandem to express on the T cell independently of the TCR.

Expression

Supernatants from gibbon ape leukemia virus– pseudotyped vector producer cells were used to infect activated normal human T cells that were modified to 20% to 50% after two to five rounds of infection (Fig. 1C). Surface expression of the IgCD28TCR transgene per cell was at a level comparable to or higher than the corresponding first-generation IgTCR (data not shown). Western blot confirmed a disulfide-linked dimer under native conditions of apparent MW of 120 kDa (Fig. 1D). Under reduced conditions, a monomer molecular weight of 64 kDa was observed versus a calculated 56 kDa for the mature protein, with glycosylation accounting for the difference. An expected size increment of 10 kDa is seen in relation to the prior IgTCR version that is 46 kDa by calculation.
Insertion of the chimeric TCR into T cells genetically instructs these cells to respond in an MHC-unrestricted manner to immobilized (but not soluble) CEA with activation of T-cell effector functions (9, 19). Three mechanisms are assayed relevant to antitumor activity of the second-generation versus the first-generation product.

(a) Proliferation/survival of T cells. To examine the role of costimulation in T-cell proliferation and death, we created a panel of tumor lines that could provide to the T cells either Signal 1, by engaging the TCR, or Signal 2, by engaging the CD28 costimulatory molecule. Because epithelial tissues and cancers do not express B7, the natural ligand for CD28, there was no opportunity for the modified T cells to receive Signal 2 on tumor contact. To examine the dependency of modified T cells on costimulation, we created tumor targets that includes the costimulatory portion of CD28. This latter form does not show dimers in the NR lane, apparently cleaved proximal to the dimerizing cysteine in the CD28 extracellular domain. These data suggest that the transmembrane Cys in CD28 does not participate in disulfide bonding as does its counterpart in TCRζ.

IgTNo signal (MIP101), Signal 1 (MIPCEA), Signal 2 (MIP101-B7), or Signal 1 + 2 (MIPCEA-B7). Our earlier studies showed that Signal 1 alone led to death in first-generation designer T cells in a process compatible with AICD, but that the addition of Signal 2 via MIPCEA-B7 was sufficient to rescue the modified T cells from cell death and induce their superproliferation (11). In the following, we apply the term AICD to mean T-cell death that is selectively mediated via antigen stimulation. Prior studies have repeatedly correlated this means of inducing T-cell death with diverse molecular markers of apoptosis (6–8), including Annexin V staining, which we reproduce below.
IgCD28TCR+ cells over a 2-week period (2% to 31%). Like the MIPCEA or MIPCEA-B7 with a 15-fold fraction enrichment of On the other hand, Tandem T cells show equal enrichment on MIPCEA-B7, with a level below that with Tandem T cells. This confirms our prior results with AICD in first-generation IgTcr T cells (11) and establishes the activity of second-generation Tandem T cells to obtain Signal 1 + 2 and resist AICD from binding CEA on tumor, but without need for B7 coexpression. A more prolonged assay (Fig. 2B) with a second restimulation at day 7 confirmed the pattern of selective expansion of Tandem T cells and selective depletion of IgTcr-modified T cells on the same CEA+B7− tumor cells over a 2-week interval. Interestingly, the close juxtaposition of Signal 1 and Signal 2 in the same molecule (IgCD28TCR) is more efficient than their same molecule (IgTCR + B7-CD28). This effect in the colinear molecule is apparently also at saturation for costimulation inasmuch as the addition of B7-CD28 to IgCD28TCR across several experiments produced no reproducible increment in the proliferative response. With a mixed population of unmodified and IgCD28TCR+ T cells, tumor antigen–induced AICD or proliferation predicts depletion or enrichment of the IgCD28TCR+ fraction. An experiment was designed analogous to Fig. 2B, but using a very small starting proportion of designer T cells and monitoring by fluorescence-activated cell sorting for modified cells (Fig. 2C). There was a selective loss of first-generation designer T cells on MIPCEA, with Signal 1 alone, but enrichment/expansion on MIPCEA-B7, with Signal 1 + 2, confirming our prior results (11). On the other hand, Tandem T cells show equal enrichment on MIPCEA or MIPCEA-B7 with a 15-fold fractional enrichment of IgCD28TCR+ cells over a 2-week period (2% to 31%). Like the proliferation results (Fig. 2A and B), second generation alone regularly exceeded that which was attainable with first generation plus B7 costimulation (2% to 22%). To account for this degree of enrichment, calculations (Materials and Methods) indicate that the growth rate constant for the Tandem T cells after antigen stimulation was increased to >200% of the basal expansion rate of the unmodified cells. In two similar experiments with different donors beginning with 5% Tandem fractions, the final fractions after 2 weeks were 39% and 41%.

Finally, the presumed correlation of cell loss with AICD in the first-generation IgTcr construct was tested by Annexin V binding, a procedure that marks the externalization and exposure of phosphatidyl serine on the cell surface, an early event in apoptotic cell death. Flow analysis of Annexin V staining after 72 hours on contact with CEA+ targets showed 30% apoptosis of IgTcr+ cells by this assay, but without any effect on IgCD28TCR+ cells in the same test (see Supplementary Fig. S2). This confirms the susceptibility of Signal 1-only designer T cells to AICD and the resistance to the same of Signal 1 + 2 designer T cells, in accord with expectation (6–8). These selective death and survival effects were reflected in decreasing or increasing fractions of CIR-modified T cells in this 3-day assay (Supplementary Fig. S2C), as seen more prominently in longer-term assays in Fig. 2C.

(b) Cytotoxicity. The single most critical feature of designer T cells in antitumor therapies is their antigen-directed killing capacity. Binding of antigen on tumor cells by first-generation CD8+ designer T cells resulted in cytotoxicity against CEA+ tumor cell targets. This feature was preserved in the second generation version. In multiple tests, second-generation (Tandem) designer T cells were actually more potent than first generation (Fig. 3), although the basis for this superiority is presently uncertain. For lower effector-to-target (E/T) ratios, the second-generation T cells killed approximately twice as many targets. When expressed in terms of lytic units per cell, the Tandem T cells were ~8-fold more potent on a per cell basis (compare activity with E/T = 3 for IgTcr to E/T = 0.38 for Tandem). Thus, the second goal of preserved cytotoxicity was achieved and surpassed.

(c) Cytokine secretion. Cytokine secretion by T cells in support of their own growth and activity is a characteristic of T lymphocyte biology. IL-2 is an essential growth factor for T-cell survival, and secretion of IFNγ has been correlated with tumor rejection. Other studies showed that IL-2 is secreted principally by CD4 helper T cells and that IFNγ may be secreted by both CD4 and CD8 cells on antigen stimulation (e.g., ref. 20). We assayed for IL-2 and IFNγ in our bulk unfractonated modified T cells to assess whether these cytokines could be detected after antigen stimulation. The IgTcr first-generation designer T cells were inert on MIPCEA for cytokine secretion, producing IL-2 only if tumor artificially also provided B7 antigen for stimulation of native CD28 on the T cells. In contrast, insertion of the IgCD28TCR gene into CD4+ T cells leads to cellular activation and IL-2 secretion on MIPCEA (Fig. 4A). The second-generation designer T cells also secreted high levels of IFNγ (Fig. 4B), marking these T cells as potent Th1/Tc1 effectors (21).

We then examined the effect of IL-2 secretion on T-cell survival. Activated before transduction and maintained in IL-2, all T cells expanded rapidly (e.g., Fig. 2A and B). When subjected to IL-2 withdrawal in the absence of antigen stimulation, however, all cells died within 7 days (Fig. 4C). These data confirm that designer T cells, first and second generations alike, remain absolutely dependent on IL-2 for their survival.

For Tandem T cells trafficking to tumor with activation on contact with tumorous CEA, there may be local secretion of IL-2, possibly at levels sufficient for an

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4 T cells must be activated and induced to proliferate before infecting them with retrovirus. The basal expansion is that of the T-cell population after this initial stimulus and may continue for 2 to 4 weeks, after which T-cell numbers decline, even with IL-2 present at saturating levels (data not shown).

5 Proliferation tests longer than 2 weeks were not done with Tandem, but the principle of an advantage of two-signals for proliferation was established for a minimum of 30 days in our earlier tests (11).

6 Prior first-generation IgTcr assays of IL-2 secretion [9] included phorbol ester (phorbol 12-myristate 13-acetate) as a Signal 2-like agent, affording evidence for Signal 1, but not producing IL-2 in a manner relevant to in vivo needs.
antitumor immune response. We sought to derive a functional in vitro test of this hypothesis.

In vivo, the IL-2 that is secreted diffuses out of the tissue and into the blood where it is cleared by renal filtration with an abbreviated half-life of minutes to hours (22). In vitro, the culture flask artificially retains the secreted IL-2 and sustains activity that would otherwise dissipate in vivo. To model better the in vivo setting, therefore, we periodically exchanged the flask with fresh medium (−IL-2) twice per week, each time also feeding with fresh CEA+ tumor cells. This forces the T cells to depend for their survival on IL-2 that was resecreted with each stimulation.

Under −IL-2 conditions (Fig. 4D), normal untransduced and first-generation T-cell cultures maintained viability and basal expansion and Tandem T cells exhibited accelerated proliferation, as previously shown. Under the −IL-2 condition (Fig. 4E), control unmodified and first-generation T cells died quickly, as in Fig. 4C. In contrast, Tandem T cells on CEA+ tumor exhibited an initial period of expansion, indicating that the IL-2 secreted by the Tandem T cells was sufficient for growth in the first stimulations (Fig. 4E). This expansion is in contrast to death of Tandem T cells over the same period when placed in −IL-2 medium without stimulation (Fig. 4C). However, with subsequent restimulations, this proliferation began to lag behind that observed in the presence of added IL-2: by day 9, there was only one third as many T cells in the −IL-2 culture (Fig. 4E) as in the control Tandem culture +IL-2 (Fig. 4D; note scale difference), after which there was a progressive T-cell decline.

When testing IL-2 production, we confirmed an exhaustion of autogenous IL-2 secretion, even with the advantage of the costimulation (Fig. 4F). Because CD28 costimulation is stated to up-regulate Bcl family proteins (23) that oppose apoptosis on cytokine withdrawal (24), it was conceivable that the Tandem T cells would be IL-2 independent and not require either autologous or supplemented IL-2 for their in vivo survival. However, our results of T-cell death indicates that any Bcl up-regulation after CD28 coactivation (23, 24) was insufficient to render the T cells immune to IL-2 deprivation. This scenario presents us with the likelihood of a value for IL-2
supplementation in human therapies for extended in vivo efficacy of second-generation designer T cells.

Animal Model

Finally, the effect of the second-generation versus first-generation designer T cells was examined in a mouse model with human tumor xenografts (Fig. 5). First-generation designer T cells delayed CEA+ tumors relative to CEA– control or untransduced T cells, but all tumors grew. In contrast, second-generation T cells suppressed tumors in all animals (P < 0.02).7 By 7 days, masses that were initially palpable at 2 to 3 days were no longer detectable and no tumors were evident at 2 weeks when animals were sacrificed due to large CEA– control tumor size. No toxicity was evident in any animal.

In pilot tests with established tumors (Fig. 6A), there was also an immunotherapeutic benefit. Before designer T-cell injection, these established tumors were allowed to grow 6 days instead of 1 day as in Fig. 5. Because of an expected longer duration of activity required of the infused T cells for cure in the established tumor setting, we added IL-2 to in vivo to support the T cells beyond the 3 to 6 days of their anticipated survival in the absence of IL-2 as seen in Fig. 4C. Animals treated with activated but unmodified T cells had equivalent growth of CEA– and CEA+ tumors at 10 days (Fig. 6B). With designer T cells, CEA+ tumor was reduced and CEA– tumor unaffected (Fig. 6C). In a comparison among animals carrying just CEA+ tumors (Fig. 6), there was an initial delay of tumor growth by activated normal T cells supplemented with IL-2, whereas actual tumor suppressions required the anti-CEA specificity of the modified cells, seen with both IgTCR and Tandem, as also seen in Fig. 5. However, the response duration was longer for Tandem than for IgTCR to result in smaller mean tumor size at 12 days (50 versus 210 mm2; P < 0.05) despite a lower fraction of modified T cells in the Tandem treatment group (25% versus 40% for IgTCR). (At the same 12-day time point, the difference was nonsignificant between IgTCR and control unmodified T cells.) Among the Tandem-treated group with established tumors, one of four animals was without tumor at 12 days (in contrast to four of four without tumor with small tumors), which was still tumor-free at 70 days, potentially representing cure (Fig. 6D).

Discussion

To date, CIRs have been generated to a variety of tumor-associated antigens (2). The target to which this agent is directed, CEA, is expressed at high levels on tumor cells of many colorectal, breast, and lung carcinomas and other cancers (25). CEA is expressed on normal cells of gastric fovea, colonic epithelium, and elsewhere in the gastrointestinal tract, but mainly on microvilli on luminal surfaces that should be topologically sequestered from attack. Furthermore, tumor cells typically express quantitatively much higher levels of CEA (estimated at 35-fold), which enhances discrimination between normal and tumorous expression of the protein (26). MN14 (27) is a Primus class III antibody, defined as reacting only with CEA in the family of CEA-related proteins. We acquired a humanized version of MN14 for engineering human T cells (13), with the advantage of reducing or eliminating host responses that only rarely target the small retained murine segments in the antibody binding domain (28).

Designer T cells have had a number of clinical tests, all first generation (10, 29–32). One agent induced hepatotoxicity by action against normal expression of antigen on bile duct (32), but others have been well tolerated, to date also without major responses. Our own first-generation anti-CEA clinical trial (10) showed adequate patient tolerance and proof-of-principle antitumor immune activity, but that was limited by lack of sustained effect. Evolving understandings of the normal role of costimulation in T-cell biology led to a belief that AICD was at least contributory to the inadequacy of these early products (11, 33, 34). Signal 1 through the T-cell receptor (TCR) or the chimeric receptor (IgTCR) was adequate for T-cell cytotoxicity but leads to little or no IL-2 production and promotes anergy or apoptosis without effect cell expansion. In contrast, Signal 1 in conjunction with CD28 (Signal 2) induces robust IL-2 production and up-regulation of antiapoptosis genes such as Bcl-2, Bcl-xL, and cFLIP (23, 24, 35–37). Accordingly, researchers undertook to create fusion molecules that conferred on the engrafted T cells a functional CD28 signal after encountering specific antigen (16–18).

The data in this report validate core premises of our motivation for creating second-generation anti-CEA designer T cells. This modification results in improved features of blocked AICD and tumor-induced T-cell proliferation that seemed to be key in the face of time-limited efficacies in clinical testing of our first-generation construct. We previously showed that IgTCR Signal 1-only designer T cells die over a period of days while nonetheless killing CEA+ targets. In the present report, we confirm that this death is apoptotic as supposed for AICD and that Tandem T cells were resistant to such death. Further, the incorporation of CD28 in the CIR was confirmed to lead to augmented proliferation on antigen contact, with

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7 P = 0.014 by Fisher exact test (0/4 with tumor at 15 days for Tandem, versus 4/4 with tumor for IgTCR). When tumor sizes are examined by two-tailed t test, Tandem has better response at P < 0.002.
a doubling of growth rates above the basal expansion of the previously activated unmodified T cells. In correlative reconstruction studies, the effect of CD28 recruitment in our Tandem constructs was to induce resistance to both AICD and fas apoptosis, which are linked via FLIP (38), but not to render T cells IL-2 independent, a function ascribed to Bcl family members (24). Tandem T cells were also more potent in lytic units per cell than the first-generation construct. Additionally, Tandem T cells exhibited heightened secretion of cytokines IL-2 and IFNγ on tumor contact. Finally, in an animal model, Tandem T cells with their improved survival, greater cytolytic potency, and heightened cytokine secretion were superior in in vivo tumor therapy models with cures of small tumors and some established tumors.

Although improved cytokine production is widely reported for Tandem-type designer T cells, including an earlier anti-CEA construct (39), our functional data are the first to examine whether effective cytokine production by T cells is sustainable, from which there seems to be a limited capacity on restimulation. This raises significant question of the ability of modified T cells in this format to support their own growth with autogenous IL-2 that may require yet other signals for persistent secretion (e.g., lymphocyte function–associated antigen 1 (LFA-1); refs. 33, 34). The benefit of this improved, albeit limited, feature of increased cytokine production is less critical because the designer T cells may be supported by infusional IL-2 in patients. By contrast, beyond being a marker for Th1/Tc1 cells (21), the therapeutic role of IFNγ is less clear. IFNγ has been touted as stimulating up-regulation of MHC to increase efficiency of TCR target recognition (40), but this would not assist designer T cells, which are MHC independent. Even in situations where T cells are dependent on MHC for cytotoxic targeting, clinical data have shown no correlation of T-cell IFNγ production with clinical benefit (41). IFNγ has been cited to oppose the action of transforming growth factor β in tumor microenvironment (42, 43). In mouse models, IFNγ

Fig. 4. Cytokine secretion. A and B, high cytokine production with IgCD28TCR. Designer T cells (20% modified) were cultured with tumor cells overnight, and supernatants harvested and assayed for IL-2 (A) and IFNγ (B) by ELISA. All cultures were negative for cytokine production on MIP101 and MIP101-B7 (data not shown). By the international unit definition (WHO standard), 30 ng/mL of IL-2 corresponds to 450 IU/mL. C, Tandem T cells die without IL-2. T cells from A during log-phase expansion in IL-2 were washed and placed in −IL-2 medium without tumor cells. T cells were replaced with fresh medium −IL-2 on day 3. D–E limited survival of Tandem T cells when depending solely on their own stimulated IL-2 secretion. At time zero and each time point, T cells, as in C, were fed irradiated MIPCEA tumor cells and placed in fresh medium +IL-2 (100 IU/mL; D) or −IL-2 (E). Note 5 scale difference, with lower cell numbers for −IL-2 (data in D reproduced in part from Fig. 2B). F, exhaustion of IL-2 secretion with restimulation. Tandem T cells were incubated with MIPCEA cells as in E, with medium change and replating of viable T cells with fresh irradiated MIPCEA tumor every 48 h. IL-2 measured at each 48-h time point by ELISA. IL-2 was unmeasurable after the third stimulation (days 4–6).

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8 Gomes et al., in preparation.

9 E. Gomes and R. Junghans, unpublished results.
was critical to the therapeutic elimination of tumor by second-generation designer T cells (20, 44). Incorporation of Signal 2 was separately shown to render designer T cells resistant to regulatory T-cell and transforming growth factor-β suppression (45, 46), and the expression of IFNγ that is secreted may plausibly mediate this resistance that correlates with improved antitumor activity in model systems. This has not as yet been directly addressed.

Cytotoxicity was improved in our second-generation versus first-generation designer T cells. Calculated on a basis of lytic units per cell, it translated into a 4- to 8-fold improvement. In some prior reports, CD28 inclusion improved killing over first generation (47, 48), but most comparisons showed no difference in killing potential (39, 44, 49, 50). We have also seen this advantage of second generation in our GD3 constructs (51). The basis for an improvement is not presently understood and was not explored further.

Finally, animal tests corroborated an improvement with Tandem T cells in vivo that was seen in vitro. Animal models have, in general, shown benefit to adding costimulation when compared with first-generation T cells (44, 50), paralleling still earlier studies of bispecific antibodies with T cells that recruited CD28 costimulation (52).

For small tumors, treatment with first-generation T cells showed specificity and growth delay in all CEA+ tumors, but no cures, whereas second-generation T cells cured all animals. The tumor ablation was not immediate: tumor grew for 2 days after Tandem infusion before regressing. This shows that the relatively few designer T cells that will traffic to tumor can suppress a much larger number of malignant cells. For larger (~7 mm) tumors, second-generation T cells exhibited the same relative benefit over first generation, but with a lower rate of cure than in small tumors.

Given that our therapeutic goal with Tandem in suppressing AICD is precisely to secure prolonged T-cell persistence in tumors, the differences in immune environments (complement, ligands, adhesion molecules, etc.) that limit human T-cell persistence in mice will be detrimental to this same goal in mice. If the T cells cannot survive long periods in vivo, the full benefit of these modifications to suppress large tumors will not be appreciated. Further, it has been shown that IFNγ is critical to the cure of established tumors in mice, whereas the human cytokine released by the designer T cells does not interact with the murine IFN receptors to recruit cooperating host cellular activities for tumor elimination (20, 44). As suggested by Moeller et al. (20), there are "several problems" with testing the efficacy of human T cells in mice. This likely dictates that further product advances for treating solid tumors in humans that depend on human T-cell persistence and expansion will not be shown in this mixed-species in vivo model using human T cells in mice. This likely dictates that further product advances for treating solid tumors in humans that depend on human T-cell persistence and expansion will not be shown in this mixed-species in vivo model using human T cells, but that purely murine systems will continue to be applied for proof-of-principle demonstrations. It is our hypothesis, therefore, that responses with this agent will be much better in the autologous setting in humans than was observable with human T cells in our mice, where we already proved that systemically administered second-generation designer T cells can cure established tumors. The cure of established (e.g., stage IV) tumor in any patient with CEA-positive colon, breast, or lung cancer will be a revolutionary outcome. Hence, for this reason especially, clinical trials of these advanced agents are awaited with anticipation for the display of their full potential.

With documentation of tumor-induced T-cell expansion, the second-generation designer T cells offer the perspective of a self-sustained antitumor immune response in human anti-CEA cancer therapies. Because the Tandem CIR activates T cells through the normal TCR and costimulation pathways, this same feature is also self-limiting for T-cell survival in the absence of antigen, a key safety feature for human use: When tumor is gone, the proliferative impulse is absent, after which the T cells gradually plateau and then decline in number over a period of a couple of weeks. Further, the designer T cells are

Fig. 5. Treatment of in vivo tumor model. Nude mice (four per group) were injected s.c. with 5 × 10⁶ MIP101 (A) and MIPCEA (B) tumor cells on opposing flanks. After 1 d, a small nodule was palpable at each site, and 5 × 10⁷ T cells were injected via tail vein (arrow). The modified T-cell fraction was 20% to 22% of total cells for both IgTCR and Tandem. Tumor size was tracked over 2 wk. (average ± SE), when animals were sacrificed due to large size of control tumors.

10 A. Lo and R.P. Junghans, submitted for publication.
entirely dependent on IL-2 for their survival. In vivo, the elimination of tumor will withdraw any stimulus for autogenous IL-2 production, and termination of IL-2 infusion at that time should therefore lead to decline of the surviving second-generation designer T cells in human therapies, providing a further safety assurance. With elimination of antigen and IL-2, the CEA-specific T cells will die off, with a small fraction returning to a resting memory state, potentially maintaining an immune surveillance against recurrent tumor (20).

The first generation version of the anti-CEA designer T cells was applied in a phase I clinical trial without and with IL-2 coadministration that showed adequate safety and proof-of-principle biological responses, but which were time limited in their activity (10). By our laboratory correlate studies, it was inferred that AICD and a deficiency of costimulation contributed to the decline of T-cell activity in vivo in our trial (11). These studies provided the impetus to engineer a second-generation (Tandem) version of this clinical product that could resist AICD and therefore persist longer in vivo to induce a meaningful response in human therapies.

These preclinical tests establish essential data concordant with the intended features of a second-generation reagent. Clinical trials are under way in which the hypotheses of extended antitumor function of second-generation designer T cells will be assessed for actual human clinical utility. Future directions include the possibility of coexpressing additional molecules, supplementing Tandem with FLIP and/or Bcl-xL for the potential to augment the resistance of designer T cells to AICD and/or to render them immune to cytokine deprivation, obviating the need for systemic IL-2 supplementation (24, 35, 36). Other maneuvers to include Signal 3 molecules (e.g., LFA-1) for more robust T-cell activation and sustained IL-2 secretion may be additional options (33, 34). Such third-generation configurations would require additional tests of safety and efficacy before human translational studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Second-Generation Anti–Carcinoembryonic Antigen Designer T Cells Resist Activation-Induced Cell Death, Proliferate on Tumor Contact, Secrete Cytokines, and Exhibit Superior Antitumor Activity In vivo: A Preclinical Evaluation

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