SII-1

Bypassing Immunization: Optimized Design of Designer T Cells against CEA expressing Tumors

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INTRODUCTION

A major focus of modern cancer research has been to explore the potential of immune system components to combat malignant disease. This strategy has exploited, on the one hand, the humoral immune system as represented by antibodies, and, on the other, the cellular immune system as represented by lymphokine-activated killer cells and tumor-infiltrating lymphocytes. In general, antibodies are able to supply specificity but suffer from lack of potency. In contrast, cellular therapies have the requisite potencies, sufficient to induce lethal consequences if not closely regulated, but typically lack specificity for the self-proteins that predominate among tumor-associated antigens. The IgTCR approach, demonstrated first by Kuwana et. al. in 1987, attempts to combine the specificity of antibodies with the efficient cytotoxic effector properties of T cells, essentially bypassing immunization. Model systems subsequently showed that IgTCR can direct T cells to respond to antigen targets in a specific, MHC-unrestricted manner, generating IL-2 secretion, cellular proliferation, and cytotoxicity, the hallmarks of an effective, self-sustaining immune response. It came to be an appropriate juncture at which to extend these studies to a human system of wide clinical relevance, to explore the clinical potential of this technology for cancer therapy.

In preparation for direct human application, several features of the design of chimeric IgTCR were identified as subjects for optimization. The concept of IgTCR was established using antibody V genes fused to TCR α and β constant domains. Subsequently, other IgTCR joined antibody V regions directly to signaling chains, TCR ζ or the homologous FcεR-I γ chains. Two basic studies showed that ζ was 300% more potent than γ, both by IL-2 secretion and cytotoxicity, for which reason we considered only native TCR chains for our chimeric receptors. Several studies created ζ chimeras with modified or irrelevant TM domains that prevent assembly into the complete TCR. We wished instead to exploit the available multichain TCR apparatus in our constructs and therefore preserved the native TM domains. Additionally, no direct functional comparisons between TCR-ε and -ζ chains have been reported in the context of an intact TCR complex. This is of particular interest because TCR-ε has the longest history as the principal target for antibody-mediated T cell activation (e.g., via OKT3) and for redirected killing via bifunctional antibodies, to which the IgTCR is structurally and functionally analogous.

Further, all recent IgTCR used sFv antibody V gene constructs. The preparation of

sFv requires its own engineering, expression in soluble form, and Scatchard analysis to ascertain affinity preservation, and then molecular fusion to the TCR chain. Many times this effort fails, and appropriately stable sFvs may not be generated. It was therefore of interest to examine alternatives for integrating antibody binding domains into the IgTCR:Fab constructs, by contrast, maintain the monovalent binding affinity of the original antibody because the native juxtaposition of VH and VL is undisturbed, stabilized through CH1:CL interactions.

A potential concern for all antibody and IgTCR therapies, regardless of structure, is the presence of high levels of soluble antigen that frequently accompany expression of cancers that may inhibit specific interactions with target cells. It was therefore pertinent to examine whether the multivalent binding between designer T cells and tumor targets would overcome this potential source of interference.

For the clinical application of this technology, we selected CEA as the target antigen. One of the best characterized of human tumor-associated antigens, CEA is expressed on tumors in 60-94% of patients with metastatic and recurrent colorectal cancer and in 30-60% of metastatic carcinomas of the breast, pancreas, and other organs. In the United States alone, ~150,000 patients die per year with CEA-expressing malignancies. Therefore, a therapy effective against such tumors could have a major impact on the clinical and financial consequences of cancer.

To address these several hypotheses, we used a humanized antibody as sFv or Fab joined via IgG $\gamma 1$ hinge or CD8α hinge domains to CD3 ε and ζ chains to derive an optimal IgTCR structure for future anticancer designer T cell therapies. Specific gene modifications were implemented to enhance IgTCR stability and surface expression. We show that all combinations of anti-CEA IgTCRs are well expressed on the surface of T cells, they recognize CEA, they bind tumor cell targets, and they efficiently transmit TM signals for T cell activation in an antigen-specific a manner. Potent cytotoxicity was demonstrated by chimeric IgTCR when expressed in normal human T cells, with persistent T cell killing and recycling over a period of at least several days. The T cell responses were independent of soluble CEA at concentrations that far exceed *in vivo* levels in patients. This report represents the completed preclinical optimizations for a new therapy directed against CEA-expressing tumors in humans. Phase I clinical trials are currently under way.

RESULTS & DISCUSSION

Design of Anti-CEA IgTCR.

As part of the optimization plan, different IgTCRs were designed to test hypotheses related to their potential for activity in *in vivo* therapies. Chimeric receptors were prepared using sFv and Fab antigen-binding domains of humanized anti-CEA antibody hMN-14 joined with both ζ and ε chains of the TCR (Fig. 1).

To create our sFv molecule, we modified the canonical $(GGGGS)_3$ linker by introducing a second serine in the repeat, $(GGSGS)_3$, to increase hydration. Several prior sFvs have failed to preserve affinity. We supposed that this more "hydrated" linker would be less

likely to invade the hydrophobic domains between the VH and VL that could otherwise disrupt the appropriate VH:VL apposition for antigen binding.

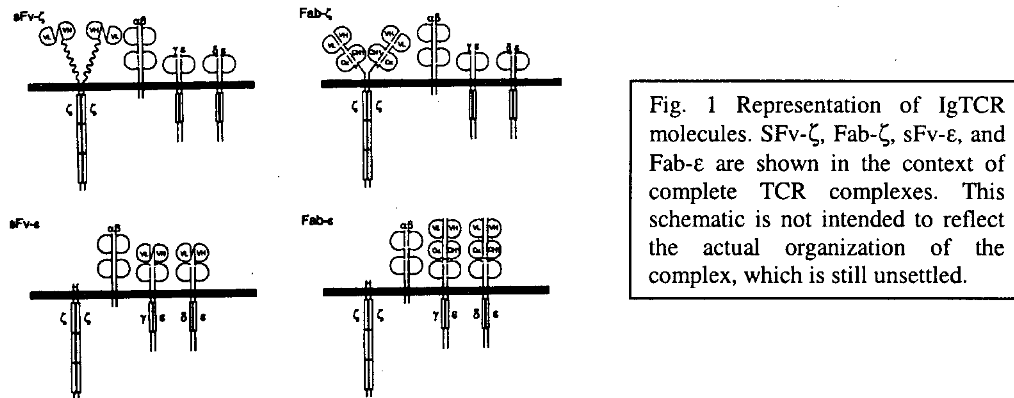

Fig. 1 Representation of IgTCR molecules. SFv-ζ, Fab-ζ, sFv-ε, and Fab-ε are shown in the context of complete TCR complexes. This schematic is not intended to reflect the actual organization of the complex, which is still unsettled.

For the sFv-ζ IgTCR construct, we introduced 46amino acids of the CD8α hinge between the sFv and the TCRζ chain to add an extra spacer between the antigen-binding moiety and the membrane surface (sFv-CD8α hinge-ζ). With the aim to increase surface expression of CD8α hinge-containing molecules, we engineered the hinge to remove the two cysteines that are normally involved in CD8αα and CD8αβ dimerization. For the Fab-ζ construct, the CD8α hinge was not inserted because the Fab constant region (CH1 and CL) was thought to serve as a natural spacer, analogous to the Cα and Cβ of the TCR (Fig. 1). A natural human IgG H chain γ1 hinge region was inserted to link the CH1 domain of the Fab H chain to the ζ chain (H-γ1 hinge-ζ). The γ1 hinge provides sufficient spacing between the terminal β-sheet hydrophobic domain of the CH1 and the membrane attachment to allow suitable immunoglobulin-domain protein folding.

For the ε constructs, it was supposed that no extra spacer would be required because the immunoglobulin-like extracellular domain of ε could serve as a natural spacer (Fig. 1). Hence, the sFv was directly linked to the ε chain without the CD8α hinge. Fab-ε used a γ1 hinge linker as for Fab-ζ joining, but it now contained two membrane spacer domains: ε extracellular domain and the Fab constant domain.

To express Fab-ζ and Fab-ε chimeric proteins, genes for H-TCR and L chain were coexpressed from different vectors for the purpose of these experiments. There is an ER retention sequence in the CH1 domain of the Fab that prevents surface transport of the H-ε/ζ constructs unless associated with L chain that masks this sequence. Hence, no H-TCR forms could reach the surface as incomplete Fab lacking L chain.

Expression of Chimeric Receptors.

The human T helper cell line Jurkat was used as a model system in which to express anti-CEA receptors and test their ability to initiate T cell activation in response to specific antigen. To confirm the expression of each molecule, membrane fractions were analyzed by Western blotting using either anti-ζ or anti-ε antibody (Fig. 2). In the reduced condition using anti-ζ antibody, monomers of sFv-ζ chain, and the H-ζ molecule from the transgene were detected (Lanes 2 and 3) as well as the 16-kDa endogenous ζ chain. On the other hand, only

the endogenous ζ chain was detected for sFv/Fab-ε transformants (Lanes 4 and 5) and for Jurkat cells transfected with empty vector (V, Lane 1). When the same reduced samples were probed with anti-ε antibody, the sFv-ε chain, and the H-ε molecule (of similar mass when L chain is stripped from Fab-ε) as well as endogenous ε chain of 23 kDa were detected (Lanes 9 and 10). For sFv/Fab-ζ transformants (Lanes 7 and 8), and the negative control (Lane 6), only the signals corresponding to the endogenous ε chain were observed.

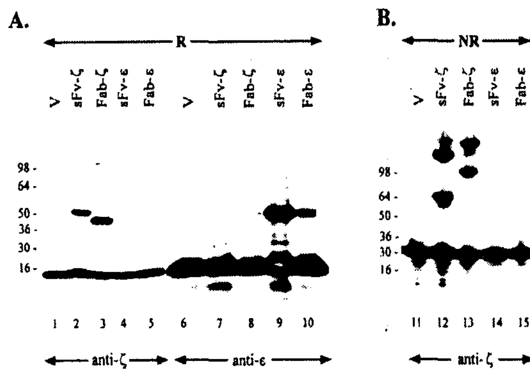

Fig.2 Membrane-associated IgTCR show expected structures. Proteins were detected with anti-ζ (Lanes 1-5 and 11-15) or anti-ε (Lanes 6-10) antibody after blotting. A, apparent molecular masses of sFv-ζ, H-ζ, sFv-ε, and H-ε molecules were 53, 47, 51, and 51 kDa. B, homodimer and heterodimer of sFv-ζ were detected as 100- and 65-kDa bands, respectively. Fab-ζ homodimer and heterodimer were observed with an apparent molecular mass of 145 and 89 kDa in Lane 13.

To confirm expression of IgTCR on the surface of the modified T cells, Jurkat transductants were also examined by flow cytometry. In Fig. 3, cells were stained with anti-idiotypic antibody to the hMN14 anti-CEA antibody, termed WI_2. All IgTCR-modified Jurkat clones showed comparably high expression, although this feature was variable in different transductions. Cells expressing Fab-TCR were stained similarly by both anti-idiotype and anti-κ L chain antibodies, indicating that the chimeric molecules retain L chain and form appropriate antigen-binding sites, corroborating the results of Western blotting (Fig. 2, nonreduced).

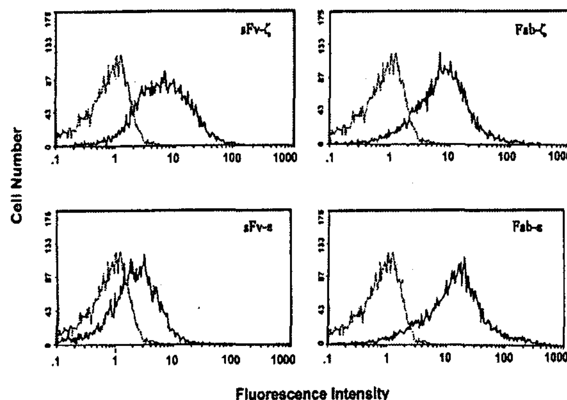

Fig. 3 Chimeric IgTCR molecules are expressed at high levels on cell surfaces. Transduced (solid line) and control nontransduced (dotted line) Jurkat cells were analyzed by FACS staining with anti-hMN14 idiotypic antibody WI_2.

In T cells, the coexpression of endogenous TCR chains normally masks the ER retention signal present in the cytoplasmic domain of ε chain, permitting ε transport out of the ER and onto the cell surface (Fig. 3). Correspondingly, initial DNA transfection studies with the human embryonic kidney cell line 293, which lacks any TCR chains, showed no surface expression of sFv-ε or Fab-ε, despite clear detection by Western blot of whole cell lysates. In contrast, the sFv-ζ and Fab-ζ constructs were well expressed even on the surface of 293 cells, confirming that surface transport of ζ chains is less dependent on associating with the complete TCR complex.

IgTCRs Direct Antigen-specific Binding by Modified T Cells.

To trigger signaling, IgTCR-modified T cells must contact antigen in such a way that the receptors are crosslinked or aggregated. Specific binding of IgTCR-modified T cells was confirmed by microscopy with immobilized target antigens (Fig. 4). Unmodified Jurkat T cells did not adhere to plastic surfaces coated with BSA or CEA (Fig. 4, A and B), showing only rounded cells resting on the plate bottom. IgTCR-modified cells did not adhere to BSA-coated plastic (not shown, but appearance like A and B) but did bind to CEA, with firm adhesion that led to progressive cellular flattening on the plate surface over time (Fig. 4C). Identical effects were observed with all four IgTCR Jurkat transformants.

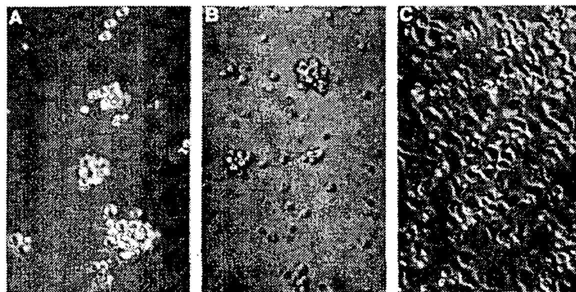

Fig. 4 Anti-CEA IgTCR-modified cells bind to immobilized CEA. A, Jurkat V (transduced with empty vector) with BSA; B, Jurkat V with CEA; C. Jurkat Fab-ζ with CEA. IgTCR-modified T cells did not bind to BSA-coated plates (not shown).

We then examined the ability of IgTCR to induce association of effector cells with antigen-expressing cellular targets. Cells were differentially stained and examined by fluorescence microscopy (Fig. 5). Target cells (orange) were either CEA-negative (MIP-101) or CEA-positive (MIP-CEA), and were previously seeded on plates to create adherent colonies. Effector T cells (green) were added, which are nonadherent to plastic. After incubation, the nonadherent T cells were washed away and complexes were visualized. Conjugates with MIP-CEA cells were not observed in the absence of IgTCR (Fig. 5A, unmodified Jurkat), and conjugates did not form between MIP-101 cells and any of the IgTCR+ Jurkat (Fig. 5B). However, conjugate formation was observed when MIP-CEA cells were coincubated with all IgTCR-modified T cells (Fig. 5C). Virtually all CEA+ tumor cells could bound by CEA-specific T cells.

Serum CEA to 1000 ng/ml or more may occasionally be observed in patients versus the normal level of <5 ng/ml. Incubation in the presence of soluble CEA at 10,000 ng/ml did not interfere with conjugate formation (Fig. 5D), or with binding to immobilized CEA (not

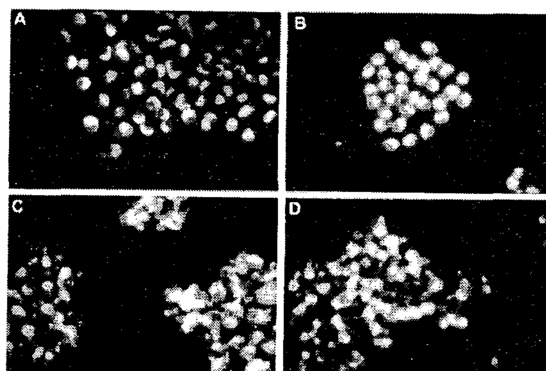

Fig. 5 Anti-CEA IgTCR-modified cells bind to CEA-expressing tumor cells. Fluorescence microscopy was used to distinguish targets (orange) from Jurkat effectors (green). A, Jurkat V with MIP-CEA; B, Jurkat sFv-ζ with MIP-101; C, Jurkat sFv-ζ with MIP-CEA; D, Jurkat sFv-ζ with MIP-CEA in the presence of 10,000 ng/ml soluble CEA.

ng/ml CEA (not shown). This resistance to soluble CEA is attributed to the avidity enhancement of multivalent cell-cell binding versus that of monovalent ligand.

IgTCR Directs T Cell Activation with IL-2 Secretion by Helper T Cells.

Once it was shown that anti-CEA IgTCR-modified T cells can bind specifically to CEA antigen, the activity of chimeric IgTCRs as functional receptor molecules was examined next (Fig. 6). As the most used model for T cell activation, human Jurkat CD4+ helper T cells are typically monitored by IL-2 secretion in response to stimuli. As positive controls, cells were treated with ionomycin as a nonspecific activator, and with immobilized OKT3, an anti-CD3 antibody, to cross-link TCR complexes: each provides the maximum known stimulus to activate all T cells, with equal signals for unmodified and IgTCR-modified Jurkat alike. Negative controls were nonspecific antibody (UPC) or BSA (not shown), which show no stimulation on any of the T cells. IL-2 was detected after incubation with immobilized anti-idiotypic antibody (WI_2) and immobilized CEA for all four of the IgTCR+ constructs but not for unmodified Jurkat. With non-CEA expressing MIP-101 cells, no T cells produced any detectable IL-2, as expected. These data confirm that all constructs assemble and are able to recognize and signal T cells on contact with antigen.

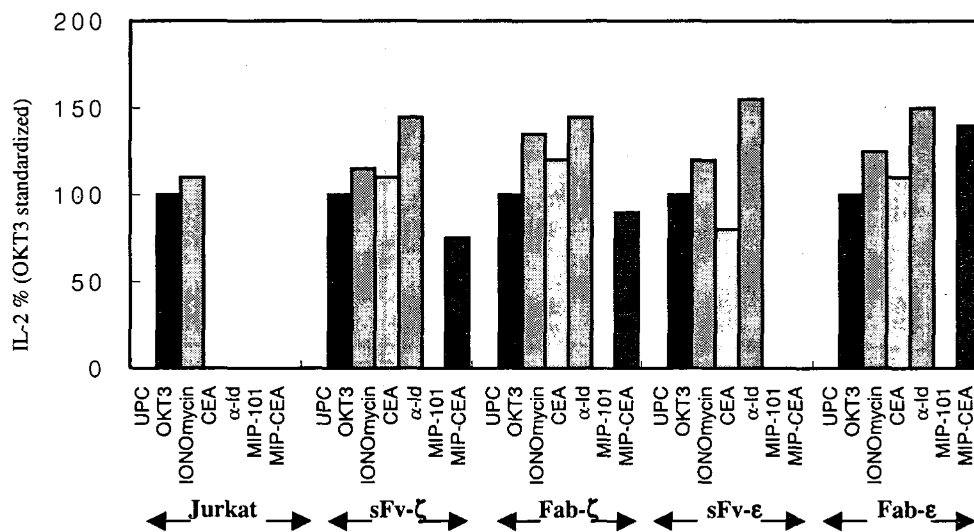

Fig. 6 IgTCR transduce CEA-specific activation of T cells. Jurkat CD4+ T cells with different IgTCR modifications were incubated for 24 h with various stimuli, and the supernatants tested for IL-2. IL-2 levels were standardized to OKT3-stimulated secretion for each group, and ranged from 200 to 350 IU/ml. Stimulators were: UPC, nonspecific antibody; OKT3, anti-TCR antibody; ionomycin; CEA; α -Id, WI_2 anti-idiotypic antibody for hMN14; MIP-101, CEA-negative colon cancer cell line; MIP-CEA, CEA-positive colon cancer cell line. The use of no stimulator or BSA gave absent signals that were equivalent to UPC. SE±20%.

Free CEA by itself binds to all IgTCR but does not activate gene-modified T cells (not shown), presumably because there is no opportunity for TCR cross-linking via the monomeric protein. To examine the possibility that soluble CEA could instead block CEA-specific T cells activation, free CEA was added in various concentrations to the assay. Little or no inhibition of IL-2 production was observed even on addition of 10,000 ng/ml soluble

CEA, whether responding to immobilized CEA (Fig. 7A) or to CEA-expressing target cells (Fig. 7B). The resistance of activation to high levels of soluble antigen parallels the lack of impact on the binding assays above (Fig. 5D).

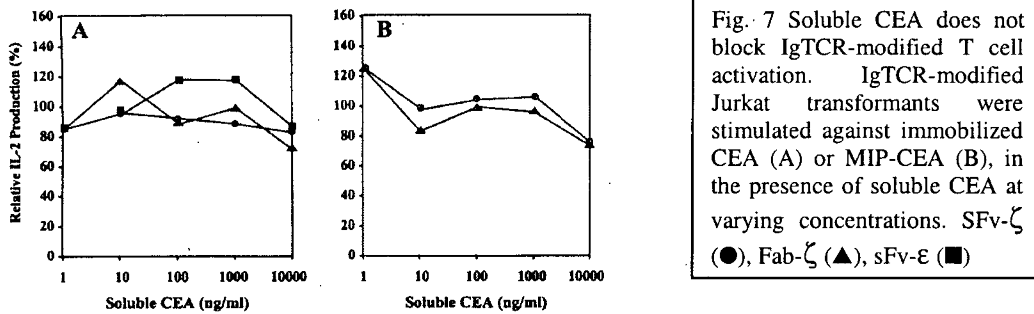

Fig. 7 Soluble CEA does not block IgTCR-modified T cell activation. IgTCR-modified Jurkat transformants were stimulated against immobilized CEA (A) or MIP-CEA (B), in the presence of soluble CEA at varying concentrations. SFv-ζ (●), Fab-ζ (▲), sFv-ε (■)

IgTCR Directs Cytotoxic T Cells in Efficient and Specific Antitumor Cytotoxicity in Vitro.

All of the four IgTCR configurations appeared equivalent by the prior criteria of T cell signaling on CEA+ tumor cells. Of these, the sFv-ζ construct was selected for clinical application based on its simplicity as a single-chain construct and was applied in the final assays for cytotoxicity. Because convenient, immortal human CD8+ CTL cell lines were not available to perform cytotoxicity studies, normal human T cells were instead used as a source of cytolytically active cells. The sFv-ζ was transferred into recently available clinical retroviral vectors and used under high efficiency conditions to modify normal human T cells without drug selection. After two rounds of infection, 30-60% of normal human T cells were transduced with IgTCR and were enriched to 100% transduced cells by panning on immobilized CEA or WI_2 anti-idiotype antibody.

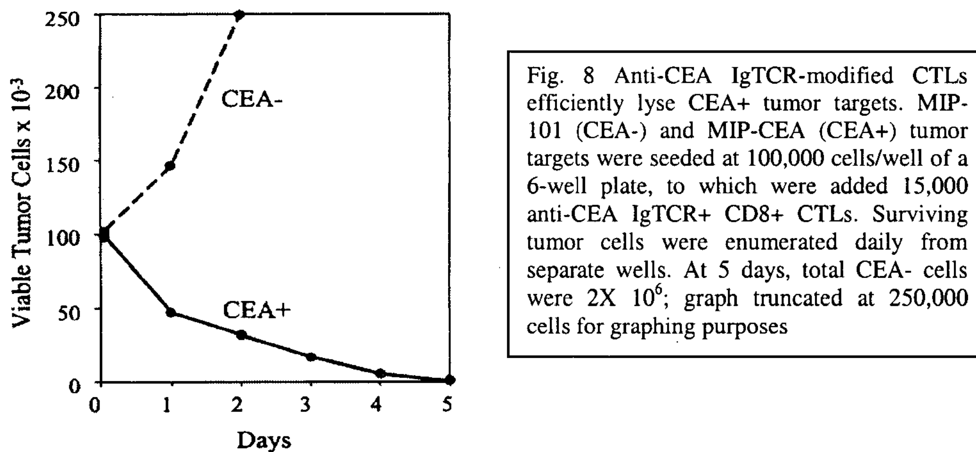

Fig. 8 Anti-CEA IgTCR-modified CTLs efficiently lyse CEA+ tumor targets. MIP-101 (CEA-) and MIP-CEA (CEA+) tumor targets were seeded at 100,000 cells/well of a 6-well plate, to which were added 15,000 anti-CEA IgTCR+ CD8+ CTLs. Surviving tumor cells were enumerated daily from separate wells. At 5 days, total CEA- cells were 2X 10^6; graph truncated at 250,000 cells for graphing purposes

For an *in vitro* model of human tumor therapies, IgTCR-modified normal human T cells were incubated with CEA+ tumor cell targets to generate a CD8+ CTL E:T ratio of 0.15:1, under conditions of sparse seeding [15,000 cytotoxic T cells and 100,000 tumor cells/well (≈960 mm^2) of a 6-well plate], and surviving tumor cells were visually counted at daily intervals. Although these T cells had been in continuous *ex vivo* culture for >3 months without TCR restimulation, they still displayed potent CEA-specific antitumor cytotoxicity. The tumor cells in the coculture were progressively and completely eliminated over a period of 5 days; no

single tumor cells survived at the end of the assay period, indicating a reversion rate to CEA negative of <1:10^5 tumor cells (Fig. 8). CEA-negative cells incubated in parallel with the same effectors expanded 20-fold, yielding a net 6-log difference in surviving cells at the end of the 5-day assay period. Identical expansion of CEA+ tumor cells was seen in the presence of an equal ratio of unmodified, mock-transduced effector T cells (not shown), indicating that the cytotoxicity is specific.

We have pursued a strategy to optimize the design of chimeric IgTCR for use in treatment of a wide range of CEA-expressing adenocarcinomas. We have shown that the sFv and Fab antibody forms are equally effective in engaging target antigen on tumor cells and that the ε and ζ TCR signaling chains are equivalent when activated in the context of the TCR under the functional tests of this study. With all four constructs in hand, the hMN14 sFv-CD8α hinge-TCRζ was selected based on its single-chain structure as the construct for CEA-expressing cancer therapy. However, the effort to create and test the sFv and modified CD8α hinge made this the most laborious of all to engineer, and Fab-ζ or Fab-ε might have been preferred for *de novo* preparations. Other studies not shown indicate that CD4+ helper and CD8+ cytotoxic normal human T cells are gene modified by retroviral transduction with equal efficiency and equal levels of IgTCR expression. With the optimization of IgTCR structure for expression and function, demonstrating IL-2 secretion and cytotoxicity, this open the potential for a self-sustaining autogenic response against CEA-expressing tumors as a new therapeutic modality against this major class of cancer.