Cancer Therapy: Preclinical

Clinical Cancer Research

Anti-GD3 Chimeric sFv-CD28/T-Cell Receptor ζ Designer T Cells for Treatment of Metastatic Melanoma and Other Neuroectodermal Tumors

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Abstract

Purpose: The aims of this study are to compare antitumor activities of two generations of GD3-specific chimeric antigen receptors (CAR) in human primary T lymphocytes *in vitro* and to evaluate the antitumor efficacy of using a combination of systemic infusion of interleukin-2 (IL2) and designer T cells to eradicate subcutaneous established $GD3^+$ melanoma in nude mice.

Experimental Design: Antitumor activities were compared for two generations of designer T cells, the progenitor first-generation with immunoglobulin T-cell receptor (TCR) with Signal 1 and the second-generation designer T cells with Signal 1+2. Osmotic IL2 pumps were used to deliver the maximum tolerated dose of IL2 to enhance the antitumor effects of designer T cells on subcutaneous established melanoma in nude mice.

Results: Melanoma is associated with high expression of ganglioside GD3, which has been targeted with modest effect in antibody therapies. We previously showed that an anti-GD3 CAR (sFv-TCRζ) will recruit T cells to target this non–T-dependent antigen, with potent killing of melanoma cells. Here, we report the addition of a CD28 costimulation domain to create a second-generation CAR, called Tandem for two signals. We show that this Tandem sFv-CD28/TCRζ receptor on T cells confers advantages of improved cytokine secretion, cytotoxicity, proliferation, and clonal expansion on tumor contact versus the same CAR without costimulation. In an adoptive transfer model using established melanoma tumors, designer T cells with CD28 showed a 50% rate of complete remissions but only where IL2 was supplemented.

Conclusions: As a reagent for clinical development, the second-generation product is shown to have superior properties to warrant its preference for clinical designer T-cell immunotherapy for melanoma and other tumors. Systemic IL2 was required for optimal activity in an established tumor model. *Clin Cancer Res; 16(10); 2769–80. ©2010 AACR.*

Tumors have several potential mechanisms for immune evasion, such as downregulation of MHC, lack of costimulatory signal, deficiency of antigen processing or expression, and creating an immunosuppressive microenvironment by production of tumor cytokines or by induction of regulatory T cells (1). Adoptive T-cell therapies using lymphokine-activated killer (LAK) cells and tumor-infiltrating lymphocytes (TILs) have had limited success (2). Such therapies can enhance the immune system by secretion of type I cytokines, but they fail as a general solution to the problems of the immune evasion by tumors. Efforts are under way to improve the effectiveness of adoptive T-cell therapy by the identification and amplification of antigen-specific T cells possessing *in vivo* cytotoxic activity, by overcoming challenges of trafficking of the transferred T cells to the tumor bed, by overcoming activation-induced cell death (AICD) resulting from Signal 1–only stimulation, and by overcoming apoptosis resulting from growth factor deprivation.

Gangliosides are a group of monosialic acid–, disialic acid–, and trisialic acid–containing glycosphingolipids on cell membranes and are expressed in high density in embryonic tissues and adult neural crest-derived tissues (3). Glycoantigens have been implicated in cell growth, differentiation, malignant transformation, invasion, and immunosuppression (3). Monosialo GM3 is the major ganglioside expressed in epithelial cells of both normal and neoplastic prostate, bladder, and melanocytes. Another (disialo)ganglioside, GD3, has low expression on retina

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Translational Relevance

The human ganglioside antigen GD3 is overexpressed on melanoma and other tumors of neuroectodermal origin. We previously published first-generation anti-GD3 designer T cell with T-cell receptor Signal 1 that showed specific targeting of $GD3^+$ tumor cells. We have now prepared a second-generation version adding Signal 2 costimulation that is superior with accelerated T-cell expansion and increased potency *in vitro* and *in vivo*. The second generation has the *in vitro* feature of high interleukin-2 (IL2) production on tumor contact, an essential growth factor for T-cell survival. However, the benefits of the second-generation designer T cells against established tumors *in vivo* were revealed only with coadministered systemic IL2, indicating that two-signal production of IL2 by the T cells is insufficient. This validates these second-generation anti-GD3 designer T cells as the preferred format for human clinical trials and provides a rationale for IL2 supplementation in designer T-cell therapies.

pigment cells, central nervous system, and normal melanocytes (4). On the other hand, GD3 has high expression on tumor cells of >80% of melanomas and also has increased expression on neuroectodermal tumors (neuroblastoma and glioma) and on epithelial origin tumors, including cervix, lung, prostate, breast, head and neck, colon, and ovary. The growth rate and metastatic status of malignant melanomas, renal cell carcinoma, and head and neck tumors are correlated with excessive synthesis of GD3 (5, 6). The highly restricted expression of GD3 on select tumor types makes it an attractive antigen for immunotherapeutic targeting. As such, it has been used for passive (7, 8) and active immunotherapy of melanomas and small cell lung cancers (9, 10).

Chimeric antigen receptors (CAR) have the capacity to bypass native tolerance to redirect T cells ("designer T cells") against preselected tumor antigens by an MHC-independent, antibody-type recognition, with broad off-the-shelf applicability for all patients. CARs have been designed to incorporate sFv antibody recognition domains targeting a wide range of tumor antigens (11), including non–T-type ganglioside antigens (12–15). T cells with these modified CARs activate on contact with specific tumor antigen, leading to secretion of Th1 cytokines, including IFNγ, interleukin-2 (IL2), granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-α, which had been shown to contribute to retard tumor growth in mouse models *in vivo* (11).

More recently, the focus of many investigations has been shifted to incorporating the endodomains from T-cell costimulatory molecules such as CD28, OX40, and 4-1BB to overcome problems of inefficient effector function and anergic status of intratumor T cells, including resistance to AICD that results from isolated T-cell receptor (TCR) stimulation ("Signal 1 only"; ref. 16). In a number of studies, expression of recombinant costimulatory immunoreceptors in T cells has shown superior antitumor responses with advantages of T-cell proliferation and clonal expansion *in vitro* and *in situ* (17–19).

In this report, we compare the functional activities of two generations of a GD3-specific chimeric TCR construct to select one version for clinical development. In this, we confirm the advantages of costimulation for cytokine secretion, cytotoxicity, proliferation, and clonal expansion. Finally, we show that the systemic delivery of IL2 was essential to realization of benefits of antitumor activity of second-generation designer T cells *in vivo*. This work represents the preclinical characterization of this agent that is in the process of development for human therapies under Food and Drug Administration Investigational New Drug status.

Materials and Methods

Cell lines. MB3.6 is an anti-GD3 monoclonal antibody (mAb) developed by Dr. R.A. Reisfeld (Scripps Research Institute, La Jolla, CA) that was obtained in human-mouse chimeric form from Repligen. Human melanoma cell lines M21 ($GD3^+$) and M24 ($GD3^-$) were obtained from Dr. G. Gammon (John Wayne Cancer Institute, Santa Monica, CA).

Vector and packaging cell line construction and retroviral transduction of human lymphocytes. The first-generation GD3 immunoglobulin TCR (IgTCR; sFv-TCRζ) retroviral DNA construct sFv (243 amino acids)–CD8α hinge (46 amino acids)–ζ chain (transmembrane domain, 24 amino acids; intracellular domain, 112 amino acids) was previously made and characterized (15). The second-generation (Tandem) chimeric receptor (sFv-CD28/TCRζ) was created as sFv (243 amino acids)–CD8α hinge (46 amino acids)–nucleotides 334 to 660 (109 amino acids) of CD28 [a portion of extracellular domain (44 amino acids), transmembrane (24 amino acids), and intracellular domain (41 amino acids)]–ζ chain (intracellular domain, 112 amino acids) by replacing the anti–carcinoembryonic antigen(CEA) sFv in the second-generation anti-CEA CAR with MB3.6 sFv via *Not*I and *Hin*dIII sites. The vector backbone was the MFG retroviral vector (gift of Dr. R. Mulligan, Children's Hospital, Boston, MA). Vector producer cells were generated, and high-titer vector was harvested and applied to OKT3-activated human peripheral blood mononuclear cells as described to create designer T cells. The transduced T cells were expanded in 10% fetal bovine serum in AIM-V medium (Invitrogen) containing 100 IU/mL IL2 with changes every 3 or 4 days.

Flow cytometric analysis. Expression of GD3-specific CAR on T cells was detected by staining with anti-idiotype antibodies against MB3.6 rabbit polyclonal antibody or mouse mAb 4B10 as described (15).

Western blot analysis of CAR expression. Equivalent of 1 million transduced and nontransduced T cells (1×10^6)

were prepared and analyzed by Western as described (20). The membrane was incubated with 1:2,000 primary antibody, anti-human ζ-chain mAb 8D3 (BD Pharmingen), and then with 1:3,000 secondary antibody horseradish peroxidase (Caltag). Immunodetection was done using the ECL Plus Western blotting detection system (GE Healthcare).

Proliferation and clonal expansion. For antigen-stimulated proliferation and clonal enrichment experiments, irradiated tumor cells were seeded 5×10^5, 2.5×10^5, and 1.25×10^5 per well, and T cells were added at 1×10^6 for effector-to-target (E:T) ratios of 1:0.5, 1:0.25, and 1:0.125. T cells were split to maintain suitable density and restimulated with tumor cells weekly. The number of T cells was counted every 3 or 4 days for 2 weeks. The percentage expression of CAR^+ T cells was determined weekly by fluorescence-activated cell sorting (FACS). The different E:T experiments showed indistinguishable enrichments and were pooled for analysis in Fig. 2Bii.

Cytokine secretion. Melanoma cell lines M21 ($GD3^+$) or M24 ($GD3^-$) and also B7.1 expressed in M21 or M24 were used as stimulators. Irradiated (3,000 rads) tumor cells were seeded overnight at 1×10^6 per well in 24-well plate, and 1×10^6 untransduced or transduced T cells were added per well. After overnight incubation, the supernatants were harvested and assayed for IL2 and IFNγ by ELISA (eBioscience).

Chromium release assay. ^{51}Cr-labeled melanoma $GD3^+$ M21 target cells (1×10^4) were mixed with varying ratios of effectors in wells of a 96-well round-bottom plate in a 4-hour chromium release assay per standard (20).

Treatment of melanoma xenografts in nude mice. Animal experiments were done in compliance with guidelines of the Beth Israel Deaconess Animal Care Committee. Six- to 8-week-old female BALB/c nude mice (Harlan Sprague Dawley) were inoculated s.c. with 5×10^6 $GD3^+$ M21 melanoma on the flank as indicated. For therapeutic experiments, on day 7 after tumor inoculation, the mice were injected i.v. by tail vein with 5×10^7 nontransduced or IgTCR- or Tandem-transduced T cells. Half of the mice were implanted s.c. with Alzet 7-day microosmotic pumps (Durcet Corp.) filled with IL2 on the day of T-cell injection. The pump was set to deliver at a rate of 10,000 IU/h (550 pg/h), near the maximum tolerated dose (MTD) for continuous infusion IL2 in mice (see Supplementary Data). Tumor size was measured by caliper in two dimensions, and the tumor volume was calculated as (width × $(\text{length})^2$) × $\pi/6$. Mice in remission were followed for recurrence of disease for 90 days. Other mice were sacrificed on reaching tumor size allowances: tumor reaching 15-mm diameter or 2,000 mm^3 volume. For H&E staining, the tumors or tissues were harvested, fixed in 10% formalin solution, and

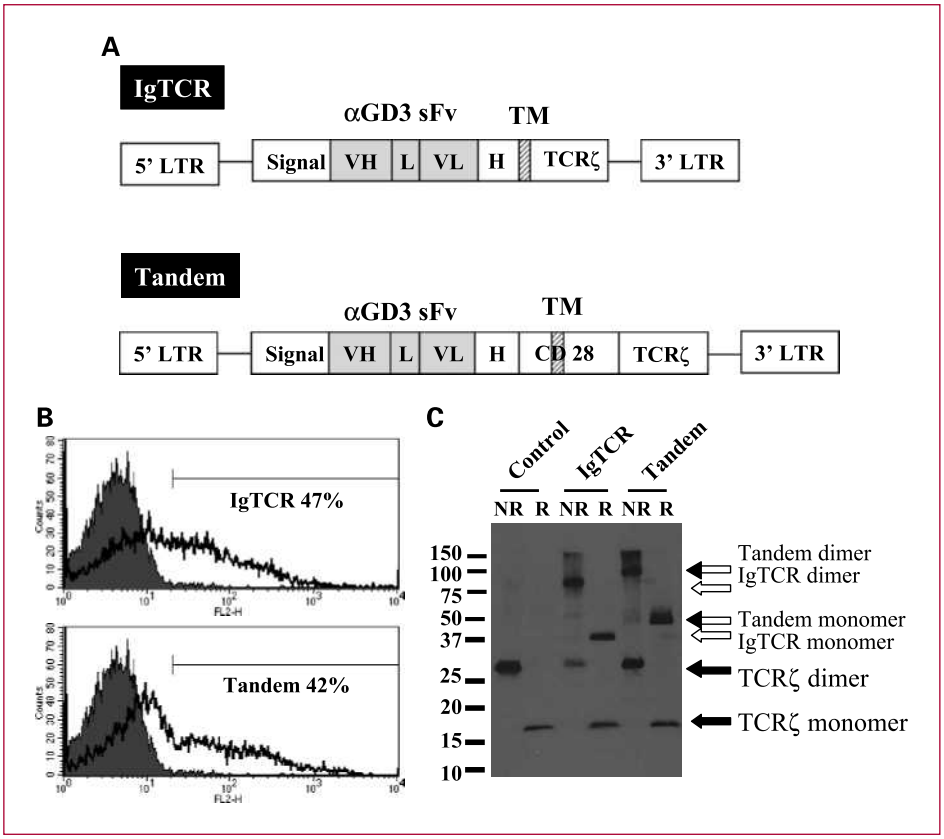


Fig. 1. Construction and expression of GD3-specific CARs. A, schematic diagram of two generations of GD3-specific CAR. B, expression of CARs by FACS. Activated primary human T cells were transduced with retrovirus to express GD3-specific CARs and assessed by flow cytometry. Numbers in panels indicate percentage of cells positive for CAR versus a negative control of untransduced T cells. C, sizes of CARs by Western blot. Lysates of untransduced and transduced T cells (~40%) were prepared under nonreducing (NR) and reducing (R) conditions and evaluated by immunodetection with anti-TCRζ antibody. The locations of monomer and dimer of Tandem, IgTCR, and endogenous TCRζ are indicated.

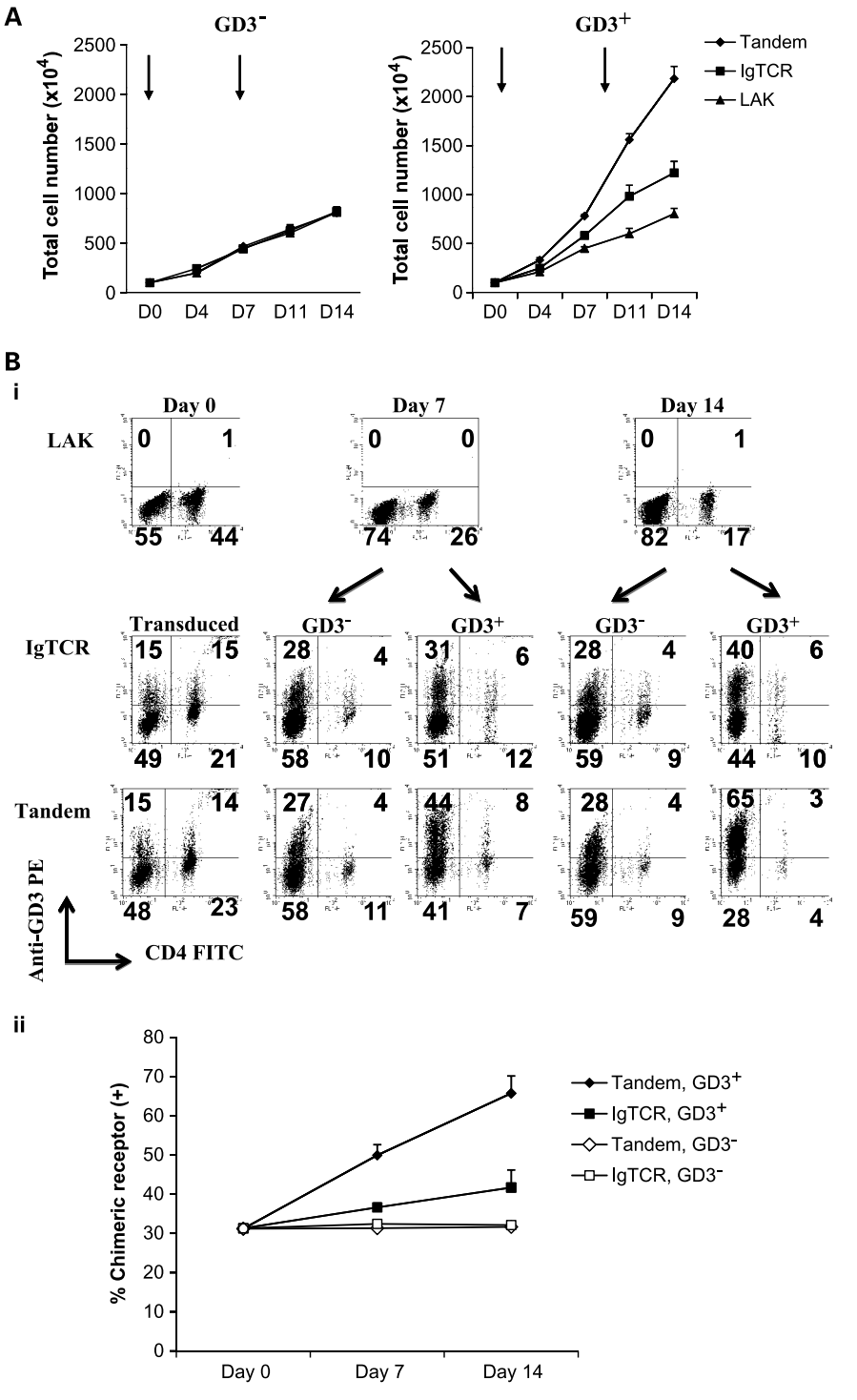


Fig. 2. Clonal expansion of CAR$^+$ T cells after tumor contact. A, proliferation. CAR-modified and untransduced T cells were plated at 10^6/mL at ratios of 1:0.125, 1:0.25, and 1:0.5 with irradiated GD3$^+$ tumor M21 and GD3$^-$ tumor M24, with weekly tumor stimulation. T-cell number was counted every 3 or 4 d in triplicate from two separate wells. Similar results were obtained in the three experiments and pooled for analysis. B, clonal enrichment. i, flow cytometry. Cultures from CAR-modified and untransduced T cells as in A, shown here with the E:T ratio of 1:0.5, were assayed on days 0, 7, and 14 by flow cytometry for expression of CAR for CD4$^+$ T-cell subset. The CD4$^-$ population corresponds to the CD8 subset in these graphs in parallel tests (data not shown). More than 98% of cells are CD3$^+$ T cells (data not shown). Similar results were obtained in all three E:T experiments. ii, summary of enrichment data. Profiles of percentage for CAR on days 7 and 14 as in Bi are averaged from three different experiments versus CAR$^+$ T cells without antigen stimulation as control.

submitted to the Beth Israel Deaconess Medical Center Pathology Core Facility.

Statistical analysis. Statistical significance was determined using the two-tailed Student *t* test, the χ^2 test, and the Mann-Whitney rank-sum test.

Results

Construction and expression of GD3-specific chimeric receptors. The aim of this study was to compare antitumor activities of two generations of GD3-specific CARs in

human primary T lymphocytes to select a preferred agent for clinical development. MB3.6 mAb was chosen for sFv design and for specific antigen recognition because of its reported high affinity and cytotoxic activity (21). The first-generation of GD3-specific CAR, also known as IgTCR, is composed of a GD3-specific sFv fragment and CD8α hinge linker fused with transmembrane and intracellular signaling domains derived from TCRζ. To deliver costimulatory signals, the second generation of GD3-specific CAR ("Tandem" for two signals) was generated to comprise a GD3-specific sFv fragment, CD8α hinge linker, and partial extracellular-transmembrane-intracellular domains of CD28 coupled to the signaling domains of TCRζ (Fig. 1A).

Anti-CD3 (OKT3) antibody was used to stimulate peripheral blood lymphocytes for 3 days, and the cells were subjected to retroviral transduction. Three days after transduction, expression of CAR in human T lymphocytes ranged from 35% to 60% by FACS analysis with comparable levels of surface expression (Fig. 1B). On Western blot, the sizes were 46 kDa for IgTCR and 56 kDa for Tandem under reducing condition versus 16 kDa for endogenous TCRζ. Under nonreducing conditions, these two fusion receptors both form homodimers (Fig. 1C).

In the following, designer T cells were not purified or fractionated into CD4 or CD8 subsets but used as a mixed population for all assays, reflecting current clinical practice to use bulk modified cells in patient infusions (11).

Improved* in vitro *proliferation in designer T cells with GD3$^+$ tumor. The first-generation Signal 1–only CARs were previously shown by us (22, 23) and others (18) to render designer T cells susceptible to clonal elimination on tumor contact via a process of AICD. The key new feature hoped for with the second-generation modification to incorporate CD28 costimulation was to resist AICD with an active proliferation on tumor contact until tumor elimination.

For proliferation and clonal expansion experiments, nontransduced and transduced (20-30% modified) T cells were admixed weekly with GD3$^+$ or GD3$^-$ melanoma in the presence of 100 units/mL human IL2. To provide putatively different levels of antigen stimulation to fixed numbers of T cells, three E:T ratios were used: 1:0.125, 1:0.25, and 1:0.5. From each culture, T-cell numbers were counted by trypan exclusion and designer T-cell fractions were assessed by flow cytometry. Without antigen stimulation, the transduced T cells incubated with GD3$^-$ melanoma grew at the same baseline rate as the untransduced T cells, increasing 8-fold from start. When placed on GD3$^+$ melanoma, however, the transduced cell populations proliferated at an accelerated rate, with IgTCR increased up to 12-fold and Tandem up to 22-fold at 2 weeks. All E:T ratios produced the same proliferative outcome, and results from the three E:T arms were pooled for this analysis (Fig. 2A).

To confirm that the increased growth was due to the enhanced expansion of the modified T-cell fractions,

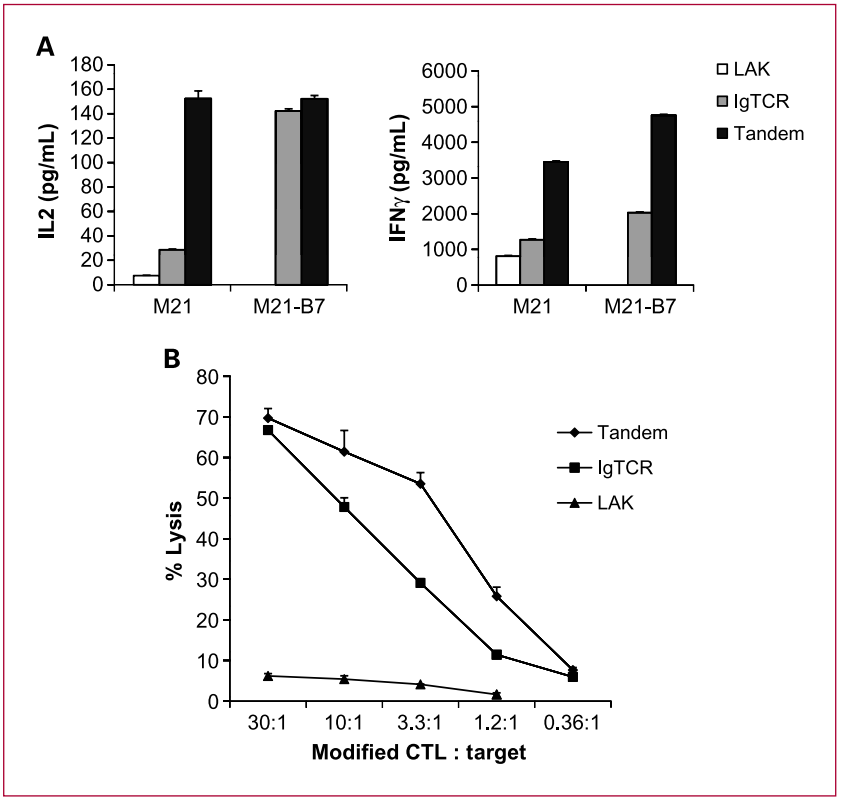
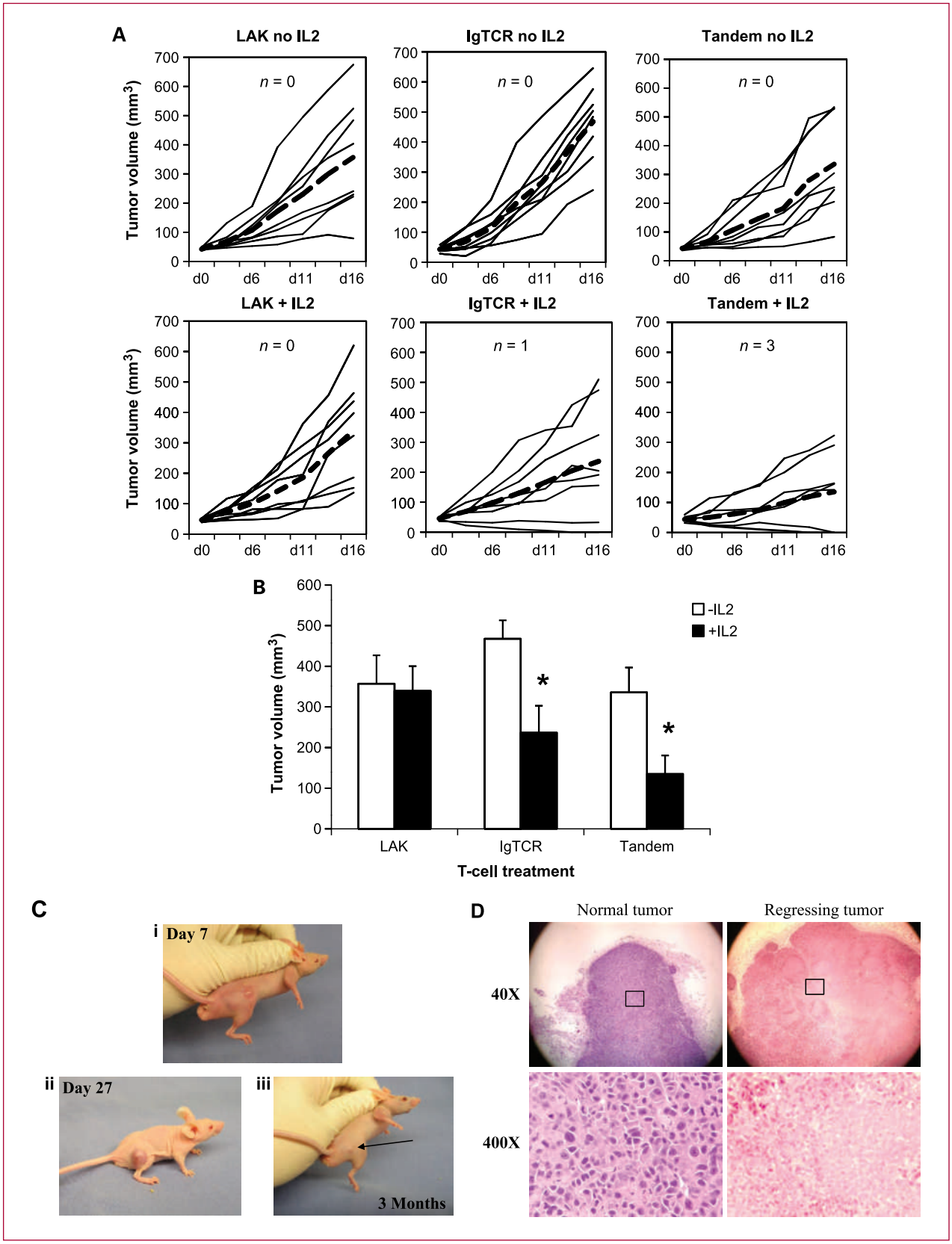


Fig. 3. T-cell effector functions. A, cytokine production. Transduced T cells were stimulated overnight with GD3$^+$ M21 tumor cells or with B7.1$^+$ M21 tumor cells at E:T ratio of 1:1. IL2 and IFNγ in medium are presented as the average of two wells (pg/mL). Coefficient of variation between duplicated wells was <10%. Similar results were obtained in three experiments. B, specific antitumor cytolysis. Transduced T cells were assayed in a 4-h killing assay at different E:T ratios with ^{51}Cr-labeled GD3$^+$ M21 target cells. The transduced fractions for IgTCR and Tandem were 42% and 37%, respectively. Control cells were untransduced activated T cells (LAK). Similar results were obtained in four experiments.



expression of CAR on the cells in Fig. 2A was measured on days 7 and 14 by flow cytometry as in Fig. 2Bi. On $GD3^-$ tumor, there was no change in the percentage of modified cells among the expanding cell populations. On $GD3^+$ tumor, however, the same T cells showed an enrichment of CAR^+ cells. Expression of IgTCR CAR was enriched from 30% on day 0 to 37% on day 7 to 46% on day 14. For Tandem-modified T cells, CAR was enriched from 29% on day 0 to 52% on day 7 to 68% on day 14 (Fig. 2Bi). Again, comparable CAR staining results were obtained under the three levels of antigen stimulation with variable E:T ratios, and these data were averaged and shown in Fig. 2Bii. A second independent experiment using the same three E:T ratios and another donor's T cells showed a similar pattern of enrichment on $GD3^+$ cells for IgTCR (from 26% on day 0 to 40% on day 7 to 48% on day 14) and for Tandem (from 22% on day 0 to 52% on day 7 to 74% on day 14), again with equivalent results for different ratios.

The equivalence of activation across a 4-fold range of targets by proliferation and clonal enrichment in two independent sets of experiments suggests that stimulation was maximal even at the lowest ratio, estimated at 1 target per 2.5 T cells (adjusted for 30% modification at the first stimulation) versus 1.3 targets per T cell at the highest, for IgTCR and for Tandem, respectively.

Calculations showed an increase of 22-fold for IgTCR and 74-fold for Tandem T cells over the period versus 8-fold increase for nonspecific T cells in the same test or for designer T cells placed on antigen-negative tumor. Expressed in expansion rate constants (i.e., cell divisions per day; ref. 23), that for Tandem on tumor was 200% of control-activated cells without antigen stimulation and IgTCR was 150% of control, or 100% and 50% above control, respectively, indicating a net 2-fold benefit of Tandem versus IgTCR.

Activation is known to promote a relative enrichment of CD8 cells in culture because activated CD8 cells proliferate faster than activated CD4 cells. The same effect was observed in restimulations across these two sets of experiments. By the first day of restimulation (day 0), 6 days after initial activation, the CD8/CD4 ratios were typically increased from the blood ratio of 0.5:1 to a starting level of 1.5:1 to 2:1. By day 14, this could increase to 2:1 to 6:1 in the absence of antigen restimulation. With antigen restimulation, the CD8 advantage translated into heightened CD8/CD4 ratios of 12:1 to 20:1 for Tandem fractions at 14 days, with lesser increments for IgTCR.

Similarly, the CAR^+ fractions were altered in this period according to CD4 and CD8 status. CD4 and CD8 cells were equivalently modified by transduction and stable throughout the expansion without stimulation, each remaining at 32% modified at day 14. With GD3 restimulation, both CD4 and CD8 fractions were enriched but the more rapid acceleration of the CD8 cells resulted in a higher overall CAR-modified fraction in the Tandem CD8 (68%) than in the CD4 cells (48%) by the end of the experiment. Similar trends were apparent in a second set of experiments (data not shown). Therefore, most of the enrichment in CAR^+ T cells from GD3 restimulation was contributed by the CD8 fraction, with lesser enrichment from stimulation in CD4 fraction that also lagged in total cell numbers. In the end, the designer T cells that started 50% or more as CD4 could represent <5% of the designer cells after 2 weeks with repeated stimulation.

Enhanced cytokine secretion by transduced T cells on contact with $GD3^+$ melanoma. A second potential benefit of incorporating costimulation into second-generation constructs is improved cytokine secretion. *In vitro* cytokine assays were done to measure the activation status of transduced T cells after CAR-directed signaling on contact with tumor. Nontransduced and transduced T cells were incubated with tumor overnight and the supernatants were harvested. With E:T ratio of 1:1, Tandem-transduced T cells secreted 5 times more IL2 and 2.3 times more IFNγ than IgTCR-tansduced T cells (Fig. 3A).

Because Tandem T cells were designed to generate Signal 2 on antigen contact, it was of interest to determine whether the CAR could fully substitute for the native CD28 interactions. For this test, target cells were modified to coexpress B7.1. IgTCR T cells plated on $B7^+$ $GD3^+$ tumor cells now produced as much IL2 as Tandem, showing equivalent benefits of costimulation by CAR or native ligands. Tandem on $B7^+$ $GD3^+$ was unchanged, indicating that IL2 signaling in the Signal 1+2 CAR was already maximal. The effect of B7 coexpression on IFNγ secretion was less dramatic for IgTCR, increasing IFNγ 1.6-fold, but still 40% below that of Tandem on targets without B7, showing superiority of the second-generation CAR over native CD28 stimulation plus Signal 1. In contrast to IL2, IFNγ increased 1.4-fold after culture of Tandem on $B7.1^+$

Fig. 4. Inhibition of established human tumor xenografts by designer T cells. A, treatment. Nude mice were s.c. implanted with 5×10^6 $GD3^+$ M21 melanoma on the right flank. On day 7, with tumors of ~50 mm^3, the mice received i.v. injection of 5×10^7 nontransduced (LAK), IgTCR-transduced, or Tandem-transduced T cells. A parallel set of mice also received continuous IL2 via micro-osmotic pump placed s.c. Tumor was measured every 3 or 4 d by caliper, and volume was estimated as described in Materials and Methods. Results are pooled from two experiments of four mice per group, and a total of six groups was used. Thin lines, individual mice; heavy dashed lines, average for group. In the panels, "*n*" is number of animals (of eight) remaining tumor-free for duration of experiment (3 mo). B, tumor response. Tumor size for each group of mice in A was compared on day 16 to assess the effect of IL2 and T-cell modification on tumor suppression. *, $P < 0.02$, between groups. C, tumor response. i, established tumor. Tumor with volume ~50 mm^3 on day 7 is shown when adoptive therapy was initiated. ii, nonresponding tumor. On day 27, mouse injected with LAK –IL2 showing no retardation of tumor was sacrificed. iii, regressed tumor. Mouse treated with Tandem +IL2 showing tumor regression with only tiny nodule remaining at site. No tumor regrowth was detected during the 3-mo observation period, with only scar tissue detected microscopically at time of sacrifice. D, tumor necrosis. Histology of cells in progressing and regressing tumors by H&E staining.

targets, indicating a continuum of costimulation without an obvious saturation effect for this cytokine (Fig. 3A). Culture on M24 GD3$^-$ tumor, with or without B7, yielded no cytokine with any T cells (data not shown).

Specific cytolysis of tumor cells via CAR signaling in transduced T cells. Cytolysis, the third characteristic of designer T cells, is well expressed in first-generation IgTCR T cells and was not necessarily expected to be improved by costimulation. Both GD3-specific CAR-expressing T cells specifically lysed GD3$^+$ M21 melanoma (Fig. 3B) with only low background lysis by untransduced T cells. Using different E:T ratios, Tandem-transduced T cells (37% CAR$^+$) gave a higher killing of GD3$^+$ tumor cells than IgTCR-transduced T cells (42% CAR$^+$; Fig. 3B). Expressed in lytic units per cell, the Tandem T cells were 4-fold more potent than the IgTCR T cells. IgTCR- and Tandem-transduced T cells showed only low-level cytolysis on GD3$^-$ M24 melanoma comparable with untransduced T cells (data not shown).

Suppression of established tumor by designer T cells. Antitumor effects were addressed with an established subcutaneous tumor model. On day 7 after implantation, with typical tumor size of ~5 × 5 mm, T cells (nontransduced and transduced) were injected i.v. A dose of 5×10^7 T cells was selected, corresponding on a per kilogram basis to twice the maximum dose used in our human T-cell trials (10^{11} cells). To follow prolonged tumor growth, only GD3$^+$ melanoma cells were implanted without coimplantation of the comparable GD3$^-$ melanoma cells that could overgrow before study end.

To assess the value of IL2 supplementation, parallel groups of mice were implanted with 7-day micro-osmotic pumps to deliver IL2 by continuous infusion near the MTD (see Supplementary Data). For the purpose of evaluating IL2-supported immune activity, we selected a day (day 16), ~1 week to 10 days after the nominal expiration time of the IL2 pumps (7 d), to allow for pump discharge of residual IL2, any local GD3-dependent secretion of IL2 by designer T cells, and T-cell survival after IL2 depletion. Preliminary experiments showed that GD3$^+$ melanoma in the absence of treatment grew equally with or without IL2 on day 16 (473 ± 133 mm^3 versus 406 ± 143 mm^3; n = 3 per group; P = nonsignificant, by two-tailed t test; mean ± SE) or on day 27 when the observation period ended (1,110 ± 170 mm^3 versus 1176 ± 62 mm^3; P = nonsignificant). Pilot tests with small numbers of animals (n = 2-5 per group) suggested a benefit of +IL2 and of using an advanced CAR (data not shown). A larger study was designed to obtain adequate statistics.

Adoptive T-cell therapy was done in mice (n = 8 per group), treated with LAK, IgTCR, or Tandem designer T cells, with or without IL2 coadministration. Figure 4A shows individual tumor growth curves. In the absence of IL2, transduced T cells did not show suppression of tumor growth versus nontransduced T cells at the 16-day evaluation point (all comparisons nonsignificant; Fig. 4B). When added to T-cell therapy, systemic IL2 provided no benefit in this experiment where T cells were not specific (LAK +IL2: 339 ± 61 mm^3, −5% tumor volume versus LAK no IL2; P = nonsignificant). In contrast, IL2 addition conferred a substantial benefit when the T cells carried antitumor specificity, with a reduced mean tumor size for IgTCR +IL2 (237 ± 66 mm^3, −51% tumor volume versus IgTCR no IL2; $P < 0.02$) and Tandem +IL2 (135 ± 46, −60% tumor volume versus Tandem no IL2; $P < 0.02$; Fig. 4B). At the $P < 0.02$ level, this establishes the importance of IL2 for obtaining clinical responses with designer T cells.

Within the +IL2 groups, the Tandem-treated tumors were 60% smaller than LAK-treated tumors at 16 days ($P < 0.02$). IgTCR induced reduction also (~30%) that was nonsignificant by the same comparison ($P > 0.2$), although the tumor growth curves (data not shown) were significantly delayed by the IgTCR treatment ($P < 0.02$, Mann-Whitney). Comparing the chimeric receptors, tumors were 43% smaller treated with Tandem than with IgTCR at 16 days (135 ± 46 mm^3 versus 237 ± 66 mm^3). The difference at this single time point did not achieve statistical significance ($P = 0.2$, two-tailed t test), but the tumor growth curve with Tandem (data not shown) was significantly more delayed ($P < 0.02$, Mann-Whitney).

Similarly, complete regressions (CR) were not obtained by any test without IL2. With IL2, however, some tumor CRs were induced but only by modified T cells: zero of eight (0%) for LAK, one of eight (13%) for IgTCR, and three of eight (38%) for Tandem (Fig. 4A), significant for a trend of more CRs with more signals (zero, one, or two signals; $P = 0.044$, χ^2 test for trend). The early tumor regressions that were complete were always evident by 7 days after T-cell therapy and remained in remission for the full 3 months of observation (four of four; Fig. 4C). In our shorter term pilot tests (above), CRs were also observed, again solely in the +IL2 +CAR groups, with second generation > first generation. When these data are pooled for analysis with those of Fig. 4, the higher numbers result in increased confidence of the relationship of more CRs with more signals at the $P < 0.001$ level, with 0 of 15 (0%) for LAK, 2 of 16 (13%) for IgTCR, and 8 of 16 (50%) for Tandem (Table 1). Comparing CR rates for Tandem from this pool, +IL2 (8 of 16) versus −IL2 (0 of 16), the importance of IL2 for Tandem-induced responses was again significant at the $P = 0.001$ level.

Table 1. Frequency of complete responses increases with more signals

	LAK 0 signal	IgTCR 1 signal	Tandem 2 signals
Complete response	0	2	8
Not complete response	15	14	8

NOTE: Mice from +IL2 experiments of Fig. 4 plus animals from two further pilot tests were scored for response at 15 d, with regressed tumors termed as complete responses. "χ^2 for trend" (more complete responses with more signals) significant at $P = 0.0006$.

In one experiment, regressing and progressing tumors (three of each) were excised 1 week after T-cell injection and processed for H&E staining. Viable melanoma cells were present in growing tumor, whereas major regression was accompanied by predominantly necrotic tissue within the residual mass (Fig. 4D).

Discussion

The population-based mortality of melanoma has increased dramatically, ~4% per year in the United States and >20-fold since 1930, with >60,000 new cases and >8,000 deaths by 2009. In terms of numbers, another neuroectodermal tumor, small cell lung cancer, far exceeds melanoma as cause of death (25,000 per year) and is also $GD3^+$ and a suitable target of therapy and for which no effective treatment currently exists (24). Further $GD3^+$ malignancies include glioma, neuroblastoma, and others that could similarly be attacked by this strategy (3).

There is no standard curative treatment for advanced melanoma, which remains poorly responsive to chemotherapy, biotherapy, and radiotherapy (25). However, melanoma is one of the most immunoresponsive of human cancers and has served as prototype for the development of a number of different immunotherapies (26). Immunotherapy of melanoma typically uses one or several strategies: antitumor antibody, TILs, cytokines, vaccines (peptide and dendritic cell), and their combinations.

The antimelanoma (anti-GD3) antibodies MB3.6 and R24 fix complement and induce antibody-dependent cell-mediated cytotoxicity (21, 27, 28), and anti-GD3 antibody has shown therapeutic benefit in a fraction of patients with advanced disease (three major and two mixed responses in 12 patients), with modest overall side effects (27). This experience was a primary motivation for our selection of GD3 for designer T-cell targeting. Melanoma therapies have also been developed based on recruitment of the cellular arm of the immune system with IL2 supplementation with modest benefit [e.g., lymphokine activated killer (LAK) cells: 6 complete responses and 25 partial responses in 198 patients (29) and TILs: 1 complete response and 14 partial responses in 39 patients (30)]. IL2 without cells, with or without IFN, with or without chemotherapy, may itself induce long-term clinical remissions among a small number of melanoma patients (29). More recently, TIL therapies have been coupled with chemotherapy conditioning to foster TIL engraftments with much improved results (31). Although a high fraction of the patients experienced major responses, most were still destined to die of their diseases. Finally, a recent report summarizing cancer vaccine trials indicated a low overall objective response rate among 1,306 treated patients (3.3%; ref. 32), which were not improved by engraftment protocols (33), implying that current vaccines are not efficient to elicit effective antitumor immunity in the therapeutic setting.

To this array of melanoma strategies, we propose to add the new option of anti-GD3 designer T cells. Our strategy bypasses the challenges of immunization by directly engineering the T-cell responses. Because it is an MHC-independent mechanism, it also avoids the HLA restriction of recent TIL TCR-based gene therapy approaches (34, 35) that treat only restricted subsets of patients and that can be thwarted by mechanisms of lost antigen presentation.

We previously reported creation of first-generation designer T cells that recognize GD3 antigen via a non–MHC-restricted pathway and provide signal (Signal 1) for T-cell activation and cytotoxicity (15). To improve on these results and increase our chances for an important effect in human applications, we created our second-generation version. Alvarez-Vallina and Hawkins (36) were the first to show that a costimulatory signal (Signal 2) could be added to a single chimeric TCRζ/CD28 fusion immunoreceptor, endowing the modified T cells with enhanced IL2 secretion and proliferation. This was confirmed in later studies along with clonal expansion, suppressed AICD, and tumor localization and regression (12, 18, 19, 23, 37, 38).

With provision of costimulation Signal 2, Tandem designer T cells were enhanced in all functions: clonal expansion/proliferation, cytokine secretion, and cytotoxicity. On a lytic units per cell basis, Tandem T cells were 4-fold more potent than IgTCR T cells. Prior studies have been split on this observation: some showing unaltered levels of cell killing and others showing improvement with the addition of costimulation (cf. ref. 23 for discussion). To date, there is no explanation for these different results. Finally, the Tandem T cells were more potent in suppressing tumor in an *in vivo* model, with delayed tumor progression versus IgTCR ($P < 0.02$) and a much improved CR rate (50% versus 13% for IgTCR; $P = 0.027$).

For the proliferation assay, Tandem T cells were expected to display clonal expansion, but that IgTCR did also was unanticipated. Observations with our anti-CEA IgTCR designer T cells (22, 23) and other examples (18) showed that first-generation Signal 1–only T cells underwent AICD with reduced cell counts, and the second-generation Signal 1+2 T cells resisted AICD and expanded. A plausible explanation for expansion of our first-generation anti-GD3 designer T cells lies in the high antigen density on the melanoma targets, wherein GD3 is at least 10-fold greater by FACS than CEA on the CEA^+ tumors (data not shown). We previously showed that very high antigen expression (high Signal 1) can bypass the need for costimulation (Signal 2) to activate designer T cells for proliferation (22). Still, Tandem was the superior agent for proliferation on tumor contact, with double the incremental clonal expansion rate as IgTCR.

Interestingly, even with a shortage of targets (i.e., with 1 target per 2.5 T cells; adjusted for percent modification), stimulation appeared maximal and equivalent to a setting with excess targets (1.3 targets per T cell). This could reflect the well-documented mobility of T cells to confront multiple targets and to share targets as well (39). No further attempt was made to titrate to lower target ratios to define a target minimum for reactivation of the population. The saturation of stimulation with different "doses" of targets

Table 2. Comparison of activity of first- and second-generation designer T cells

Function	First generation	Second generation	Comparison
Proliferation, tumor induced	+	++	Improved
Cytotoxicity	+	++	Improved
Cytokine secretion	+/–	++	Improved
Animal tumor model	+	++	Improved

for IgTCR and Tandem alike implies that the residual differences in proliferative potential between the constructs is not a simple matter of quantitative signals but qualitative differences conferred by the addition of the CD28 signaling moiety.

Beyond preventing AICD, incorporation of CD28 signaling in designer T cells confers the advantage of protection from suppression by regulatory T cells and their inhibitory cytokine, transforming growth factor-β, due to the enhanced NF-κB activation through the phosphoinositide 3-kinase–Akt pathway (40, 41). This resistance to suppressive mechanisms could be an important added benefit of this modification for cancer immunotherapy.

The CD4 cytokine IL2 is important in immunotherapy for survival, proliferation, Th1-based cellular immunity, and cellular effector functions of T and natural killer cells. IL2 combined with CD28 costimulation is required for complete T-cell activation, synergistically enhancing T-cell proliferation, clonal expansion, and upregulation of the antiapoptotic molecules cFLIP, bcl-xL, and bcl-2 that suppress activation-induced T-cell death (42, 43). The addition of CD28 costimulation markedly enhanced IL2 secretion as shown in many studies and also increased IFNγ, which has been correlated with tumor response (44, 45). However, we have shown elsewhere that such autogenous IL2 production is transient and thus quickly depleted even in the two-signal designer T-cell format (23).

A number of studies have shown that designer T cells mediate suppression of tumor in minimal disease settings without IL2, wherein an abbreviated period of T-cell activity may be sufficient for tumor elimination. To suppress larger tumors, however, longer periods of T-cell action may require IL2 growth factor for sustained viability; activated T cells and first- or second-generation designer T cells all perish by 6 days without IL2 (23). With first-generation designer T cells against lymphoma (46) and established solid tumor (19), systemic IL2 was shown to be necessary for optimal response in direct comparisons. However, all tests of second-generation designer T cells with established solid tumors were either –IL2 (47) or +IL2 (23, 48), with no direct comparisons. In a murine 5-day lung metastasis model, Moeller et al. (45, 49) showed that a high ratio of second-generation CAR-modified CD4 cells was necessary for optimal tumor responses, that only the CD4 cells secreted IL2, and that anti–IL2 antibody reversed the CD4 benefit, suggesting the importance of IL2 in this model. Typical bulk expanded cultures used for therapy are dominated by CD8 with few CD4 designer T cells, as in our tests. To our knowledge, the present report is the first to test directly the hypotheses (*a*) of the sufficiency of autogenous IL2 from typical bulk preparations of second-generation designer T cells for supporting responses against established tumors and (*b*) of the ability of exogenous IL2 to replace the CD4 contribution.

For IL2 delivery, we used micro-osmotic pumps to sustain steady growth factor levels over a prolonged period of time. These pumps are easily loaded and then placed s.c., minimizing discomfort and handling of the mice as needed for repeated IL2 administration by injection. IL2-induced production of IL5 and GM-CSF leads to increased eosinophils and neutrophils, respectively, in the circulation of treated patients (50), confirmed in our own observations in the mice (Supplementary Data) and establishing that biologically relevant IL2 levels were achieved by this delivery method. Continuous i.v. IL2 infusion was also shown to recruit maximal numbers of effector lymphocytes with LAK and natural killer activity (51). In our ongoing clinical trial in prostate cancer with anti–prostate-specific membrane antigen designer T cells plus continuous infusion IL2, 75,000 IU/kg/d, compatible with an outpatient MTD, yielded serum levels in a range of 500 to 2,000 pg/mL.[3] Here, we show that 7-day IL2 delivery by osmotic pump (10,000 IU/h) near the murine MTD generated comparably high levels of IL2 in plasma ($n = 4$, 671 ± 109 pg/mL; Supplementary Data). Finally, to show the maximum T-cell therapy effect, we chose a high T-cell dose, 5×10^7 per mouse, corresponding by weight to $\sim 2 \times 10^{11}$ T cells in a 70-kg human, roughly twice the top T-cell dose in our clinical trial and comparable with TIL and LAK doses used in melanoma patients in the Surgery Branch (30).

Continuous delivery of IL2 in our therapeutic model showed enhanced antitumor effect, with tumor size reduced by 60% and significant growth delays ($P < 0.02$ versus no IL2). CR of established melanoma tumors was obtained in 50% of mice (8 of 16) treated with Tandem T cells, but none (0 of 16) using Tandem without IL2 ($P = 0.001$, χ^2 test). Yet, IL2 with this tolerated regimen had no effect by itself unless used in conjunction with T cells, and then only with those that were engineered with antitumor specificity (Fig. 4B). We conclude therefore that IL2 administration continues to have benefit even in the Tandem format that includes costimulation, thus falsifying

[3] H. Koon, E. Gomes, and R. Junghans, unpublished data.

the hypothesis of the sufficiency of autogenous IL2 with a second-generation designer T cell in the therapeutic setting[4] and supporting the hypothesis of the ability of exogenous IL2 to replace this normal CD4 contribution.

Although our results in mice are encouraging, it was previously noted that the murine environment is nonoptimal for human T cells (45), limiting tumor responses in this model versus what may be possible in humans. T-cell survivals may be restricted by interspecies differences in ligands and cytokines in the host. Nevertheless, the short duration of our assay was sufficient to show *in vivo* antitumor activity of the designer T-cell product and the value of IL2 coadministration. We could foresee still more potent responses in human therapies wherein the continued contact with antigen in the tumor bed in a native human host environment would sustain clonal expansion of the Tandem T cells *in situ*.

In summary, we have shown efficacy of second-generation designer T cells that correlated with the cumulative effect of superior cytotoxic potency per cell, improved tumor–induced proliferation *in vitro*, and improved suppression of tumors *in vivo*, making this new designer T cell the preferred agent for anti-GD3 therapy in melanoma and other tumors (Table 2). Local cytokine secretion was enhanced but still required IL2 supplementation for benefit in an *in vivo* model. In humans, if tumor-induced T-cell clonal expansion under IL2 support increases the pool of these effectors *in situ*, a sustained tumor attack and remission may be envisioned following T cell infusion. After tumor elimination, this same response should dissipate, with residual designer T cells then passing to resting memory state in peripheral sites (49, 52, 53), from where they may potentially maintain antitumor surveillance in the posttherapy period.

[4] Whereas "humanized" models have recently been developed (54) that showed costimulation to yield complete regressions of large established tumors without IL2, this model is complicated for interpretation: these animals are subjected to a concurrent smoldering graft-versus-host disease (55). This may bring additional stimulatory features to the immune response that are unrepresentative of the human host, including the possibility of systemic cytokines including IL2 that arise from the graft-versus-host disease. There is as yet no humanized mouse model that equals the human environment for therapy with human T cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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