Adoptive T cell therapy as treatment for Epstein Barr Virus-associated malignancies : strategies to enhance potential and broaden application

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Chapter 6

A Chimeric T-cell Antigen Receptor that Augments Cytokine Release and Supports Clonal Expansion of Primary Human T-cells

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Abstract

The transduction of primary T-cells to express chimeric T–cell receptors (cTCR) for redirected targeting of tumor cells is an attractive strategy for generating tumor-specific T-cells for adoptive therapy. However, tumor cells rarely provide co-stimulatory signals and hence cTCRs that transmit just a CD3ζ signal can only initiate target cell killing and Interferon-γ release, and fail to induce full activation. Although incorporation of a CD28 component results in IL-2 release and limited proliferation, T-cell activation remains incomplete. OX40 transmits a potent and prolonged T-cell activation signal and is crucial for maintaining an immunological response. We hypothesize that the CD28-OX40-CD3ζ tripartite cytoplasmic domain will provide a full complement of activation, proliferation and survival signals for enhanced anti-tumor activity.

Introduction

Adoptive immunotherapy with T-cells appears effective for treatment of certain malignant and infectious disorders.[1-4] There are however, considerable barriers to expanding the scope of this approach, not least in generating high affinity T-cells with appropriate anti-tumor effector function. This problem may be overcome by the use of chimeric T-cell receptors (cTCRs). cTCRs are fusions between an antigen-recognizing ectodomain and a signaling endodomain (Figure 1a).[5] In their simplest form, these chimeric molecules connect the antigen-recognition properties of antibodies with the signaling endodomain of CD3ζ. T-cells transduced with cTCR are redirected to almost any target, providing an attractive strategy to generate large numbers of tumor-specific T-cells. CD3ζ-based cTCRs have been tested in clinical studies to target HIV infected cells[6-8]. Although findings from these studies may not be directly applicable to cancer immunotherapy strategies, it is notable that cTCR-transduced T-cells did not appear to proliferate significantly , and that they only had a marginal therapeutic effect.

Tumor cells rarely express co-stimulatory molecules. Moreover, the lack of an innate inflammatory response to most tumors means that chimeric T-cells do not receive co-stimulatory cues from accessory immune cells. Instead cTCR-expressing T-cells targeting a tumor must function and survive solely on signals received through the chimeric receptor. Although signals generated by CD3ζ cTCRs can recruit the cellular killing machinery[9], these constructs failed to fully activate resting T-cells.[10,11] As a consequence T-cells fail to divide, lose activity and perform poorly. Improved function requires signaling through the antigen receptor to be accompanied by co-stimulatory stimuli.[12] CD28 signaling is a critical initial co-stimulatory signal, and incorporation of the endodomain of CD28 leads to proliferation and IL-2 release.[13,14] Nonetheless, activation remains suboptimal, so that T-cells fail to maintain their expansion and function following engagement of their chimeric receptor.

Physiologically, optimal activation requires CD28 engagement to be followed by co-stimulation through other T-cell signaling molecules. Amongst the most important of these is X40 (CD134)[15], which is expressed on T-cells 24 hours after antigen and CD28 stimulation.[15-17] The ligand for OX40 (OX40L) is expressed on antigen-presenting cells several hours to days after their own activation.[18-20] *In vitro* and studies have detailed how OX40 signaling can further augment CD28-activated T-cell responses, enhancing proliferation, cytokine secretion and survival[21-23]. Hence, CD28 signaling provides the initial co-stimulus for proliferation, while subsequent OX40 signaling allows effector T-cells to survive and continue proliferating.

We hypothesized that antigen engagement of a chimeric antigen receptor linked to an endomain supplying CD3ζ, CD28 and OX40 signals *in cis* would produce sustained activation, proliferation and effector function in resting T-cells. This would allow chimeric receptor mediated recognition of a tumor cell lacking costimulatory molecules, but expressing the target antigen, to result in transmission of a full proliferative signal. We used a model of human T-cells transduced with a GD-2 targeting ectodomain. When coupled to a conventional CD3ζ endodomain, these T-cells kill GD2+ neuroblastoma lines but fail to proliferate or sustain their function. We now show that inclusion of endodomains capable of acting sequentially in the T-cell activation cascade lead to greatly increased proliferation, cytokine release and effector function.

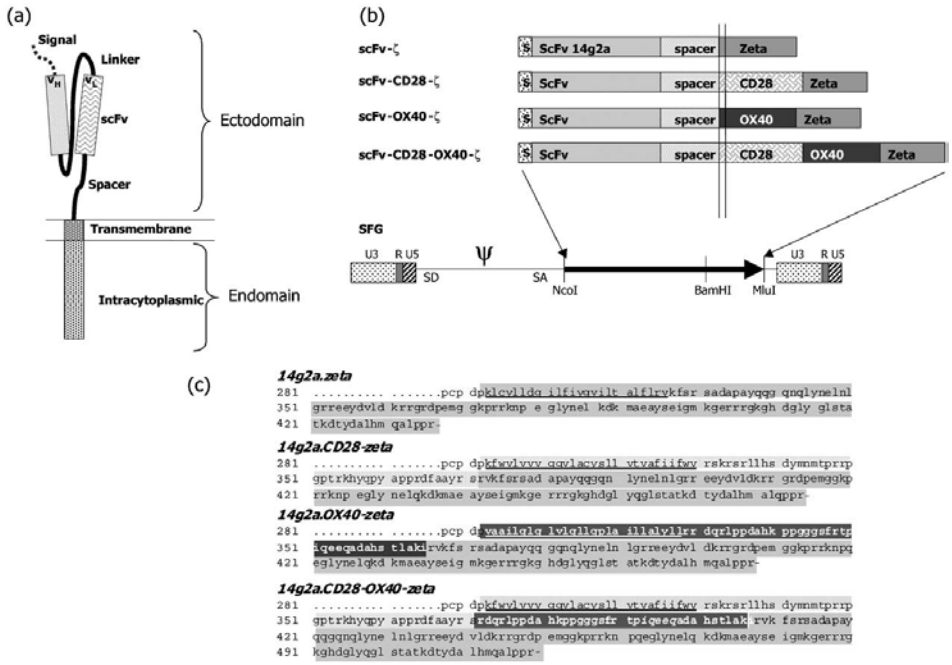

Figure 1. Receptor and vector design.

(a) *Schematic diagram of cTCR. The ectodomain is a single-chain variable fragment (ScFv) derived from a monoclonal antibody 14g2a linked to a flexible spacer derived from IgG1 hinge. The ectodomain recognizes and binds target antigen diasialoganglioside (GD2). The endodomain transmits intracellular signals and is connected to the ectodomain via a hydrophobic transmembrane region. The lipid bilayer isolates the two domains.* **(b)** *Maps of cTCRs and retroviral vector. SFG is a splicing MoMLV based retroviral vector. Gag/pol start codon has been mutated out.* **(c)** *amino-acid sequence of transmembrane and endodomains of different receptors. Underlined residues form the transmembrane domain. Residues piqee form the TRAF-2 consensus binding domain.*

Methods

Constructs

cTCR were generated using splicing by overlap PCR. The scFv derived from 14g2a has been described previously.[24] The coding sequence for CD28 was derived from Jurkat cDNA, while the coding sequence for the OX40 endodomain was derived from IMAGE clone 4334567 (Invitrogen, CA). Annotated amino-acid sequences for all constructs are provided in Figure 1c. All constructs were cloned into SFG NcoI site[25] and were all identical down to the BamHI site, which marks the junction between the ectodomains and the transmembrane domain (Figure 1b). The NFκB-luciferase reporter retrovirus was constructed by PCR cloning NFκB-RE-luciferase (Clontech, CA) as a BamHI/HindIII fragment into the SIN retroviral vector pSuper.retro (Oligo-engine, WA). FLAG-tagged TRAF2 was generated by cloning a TRAF2 coding sequence PCR product as HindIII/BamHI fragment into pFLAG3xCMV (Sigma, MO). cTCR V5-tagged constructs were generated by TOPO cloning PCR fragments of the entire receptor except the stop-codon into pCDNA3.1-V5-His expression plasmid (Invitrogen, CA). All PCR products were generated using Phusion polymerase (MJ Research, MA). All PCR generated cloned fragments were sequenced by primer extension sequencing (ABI Prism 3300). RDF – an expression plasmid for RD114 retrovirus envelope was a generous gift of Mary Collins. PeqPam3(-env) – an expression plasmid for Moloney Leukemia virus gagpol was a generous gift of Elio Vanin.

Cell lines

NIH 293T, LAN-1 and LAN-5 cells were obtained from ATCC and cultivated in IMDM (BioWhittaker) supplemented with 10% heat-inactivated FCS (Hyclone, UT) and 5 mM L-glutamine. Peripheral blood (PB), obtained from normal donors with informed consent was processed by centrifugation over Ficoll gradients. PB mononuclear cells (MC) and T-cells were cultured in 45% RPMI (Hyclone, CA), 45% Click's(Irvine, CA) medium and 10% heat-inactivated FCS and 5 mM L-glutamine.

Immunoprecipitation

$1.5x10^6$ NIH-293T-cells were transfected with 1 µg of pFLAG3xCMV-Traf2 and 8 µg of V5-tagged receptors. Transfection was facilitated by GeneJuice transfection reagent (Calbiochem, CA). 24 hours after transfection, cells were co-cultured 1:1 with GD-2 positive LAN-1 cells to cross-link the receptor. Twelve hours later, cells were lysed and proteins precipitated with anti-V5 antibody (Invitrogen, CA) using a co-immunoprecipation kit (Sigma, MO). The immunoprecipitate was run on a SDS-PAGE gel and blotted with anti-FLAG peroxidase conjugated antibody and M5 antibody. Input lysate was also analysed on the same gel and blotted for V5 and FLAG.

Retroviral supernatant

Transient retroviral supernatant was produced by co-transfection of NIH 293T-cells with the MoMLV gagpol expression plasmid PeqPam3(-env), the RD114 env expression plasmid RDF and SFG vectors at a ratio of 2:3:3 respectively, with a total of 10 µg DNA. The transfection was facilitated with gene-juice reagent (Calbiochem, CA). The supernatant was harvested 2 and 3 days after transfection, snap-frozen and stored at –80°C in 5 ml aliquots.

Transduction
Non-tissue coated 24-well plates were coated with 1 mg/ml retronectin (Takara Biochemicals, Shiga, Japan) overnight. The wells were coated with retroviral supernatant. Subsequently peripheral blood T-cells stimulated with Phytohemagglutinin (1 µg/ml) for 3 days and IL-2 (Proleukin, Chiron Corporation, Emeryville, CA, 100 U/ml) for 1 day were added. Cells were incubated for 2 days on the virus-coated plate in the presence of 100 U/ml IL-2. Then T-cells were transferred to a tissue-culture treated plate, facilitated by cell-dissociation medium (Sigma, MO).

Luciferase assay
PHA and IL-2 stimulated T-cells were first transduced with transient supernatants containing vectors coding for pNFKB->Luc. Twenty-four hours after transduction cells were divided and transduced again with supernatant containing vector coding for 14g2a-ζ, 14g2a-CD28-ζ, 14g2a-OX40-ζ or 14g2a-CD28-OX40-ζ. T-cells were rested for 5-7 days post-transduction without IL-2. Next, T-cells were stimulated at a 1:1 ratio with LAN-1 cells. Forty-eight hours later, the cells were harvested and a protein lysate from an equal number of T-cells was made. 20 µL of protein lysate was mixed with fire-fly luciferase substrate (Promega, WI) and the luminosity measured (monolight 3010, Pharmingen).

Cell-sorting
Sorting the CD8+ fraction of T-cells was performed using anti-CD8 conjugated paramagnetic beads in the CD8+ T-cell isolation kit (Miltenyi biotech, Auburn, CA) and following the manufacturer's instructions.

Co-cultures
After transduction, cells were rested in RPMI 10% FCS 5mMol L-glutamine, without IL-2, for 5-7 days. On the day of first stimulation, the cells were washed counted and plated at $1x10^6$ per well of a 24 well plate at a ratio of 1:1 with 80 Gy irradiated LAN-1 cells. Co-cultures were performed in the absence of IL-2 and in the presence of low-dose IL-2 (20 U/ml).

Cytokine assays
Supernatant was stored at –80°C and the concentration of IFN-γ, IL-2, IL-4, IL-5, IL-10 and TNF-α in CTL culture supernatants was measured using the Human Th1/Th2 cytokine cytometric Bead Array (BD Pharmingen, San Jose, CA) and analyzed with a FACSscan flow cytometer (Becton Dickinson, CA).

Cytotoxicity assay
The cytotoxic activity of transduced and non-transduced PHA blasts and CTL was evaluated in a standard 4-hour ^{51}Cr release assay, as previously described.[35] Target cells incubated in complete medium or in 1% Triton X-100 (Sigma, MO) were used to determine spontaneous and maximum ^{51}Cr release, respectively. The mean percentage of specific lysis of triplicate wells was calculated as 100 x (experimental release - spontaneous release)/(maximal release - spontaneous release).

Statistical analysis
ANOVA was used to evaluate if a significant difference existed between differently transduced cells. Subsequently Student's t-test was used to calculate p-values when comparing two different conditions.

Results

Expression of chimeric TCR constructs in primary T-cells.
The ectodomain, or antigen-recognizing domain of the cTCR used throughout this study, is a single-chain variable Fragment (scFv), derived from GD-2 recognizing antibody 14g2a (Figure 1a). A short spacer derived from IgG1 hinge region connects this scFv to the transmembrane domain. Endodomains used were derived from CD3ζ, CD28 endodomain fused to that of CD3-ζ, endodomain of OX40 fused to that of CD3-ζ or all three endodomains fused in the order CD28, OX40, CD3-ζ. The junctions are illustrated in Figure 1c. These chimeric receptors were expressed in human T-cells using retroviral vectors. All cTCRs migrated at the predicted size as determined by Western blots probed with an anti-CD3ζ antibody (Figure 2a). Under non-reducing conditions, these TCRs exist as homodimers (data not shown). Surface expression of each cTCR on primary T-cells was confirmed by detection of the single chain variable fragment (scFv), using a polyclonal goat anti-mouse-Fab antibody. Constructs containing the CD28 trans-membrane domain had the brightest expression (mean MFI 342) while expression from those containing OX40-ζ and CD3-ζ domains alone had mean MFI of 244 and 198 respectively (Figure 2b). This expression was stable for over 6 weeks.

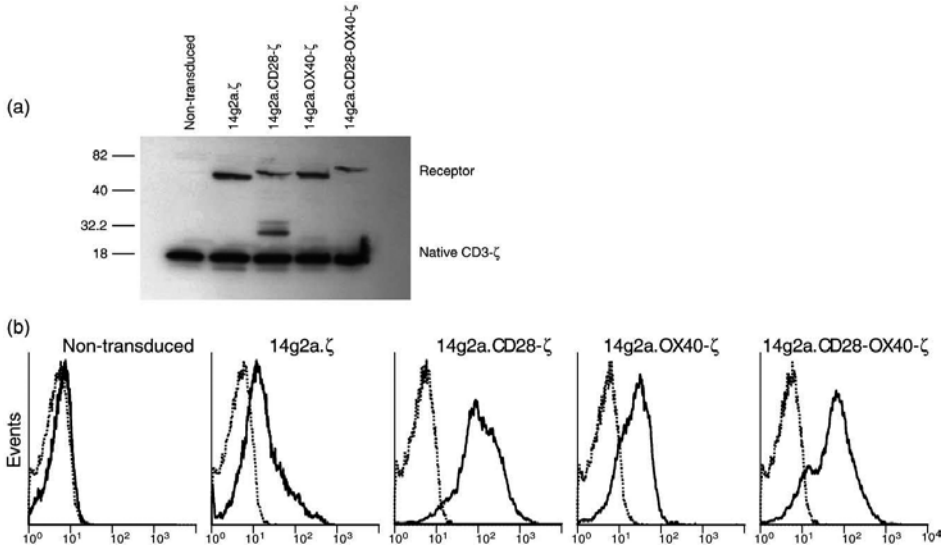

Figure 2. Expression of chimeric receptors in T-cells.
(a) *Jurkat T-cells were transduced with different constructs. Receptor-bright cells were selected using biotin-polyclonal anti-murine F(ab)' antibodies in conjunction with streptavidin-ferromagnetic beads. Western blot was performed under reducing conditions with an antibody detecting CD3ζ. Native CD3ζ is blotted for simultaneously.* **(b)** *Expression of cTCRs in primary T-cells. Peripheral blood T-cells were stimulated with PHA and IL-2 and transduced with different constructs. T-cells were stained with Cy-5 conjugated polyclonal anti-murine F(ab') and analyzed by flow-cytometry.*

OX40 as part of the TCR endodomain signals through its native mediators.
Since the cTCR sandwiches the relatively small intracellular domain of OX40 between the CD3-ζ and CD28 domains, we determined whether it retained the ability to interact with its downstream adaptor molecule TRAF2. We successfully co-immunoprecipitated TRAF2 with TCRs containing the OX40 domain (Figure 3a) in transfected 293T-cells, demonstrating sufficient access of TRAF2 to its binding site in the endodomain of the cTCRs. As OX40

triggers NFkB activity via its intermediate TRAF2, we next compared NFkB activation after stimulation of each of the cTCRs we had constructed and transduced into primary T-cells. We prepared a retroviral vector in which Luciferase expression was driven from a NFkB response element. To prevent read-through from the 5'LTR we used a vector with a non-functional 3'LTR U3 region, so that the 5' and 3' LTR were silenced following retroviral integration. Primary T-cells were first transduced with this reporter construct, and the cell cultures then split and transduced with vector encoding each receptor. Transduced T-cells were harvested on day 2 after stimulation with tumor cells, and cell lysates analyzed for luciferase activity. NFκB activity was more than an order of magnitude higher in T-cells transduced with 14g2a.CD28-OX40-ζ compared to 14g2a.CD28-ζ transductants (Figure 3b; p<0.001). These data indicate that OX40 incorporated in an cTCR is able to bind TRAF-2 to transmit its downstream signal, and potently induces NFκB activity when a CD28 signal is transmitted simultaneously.

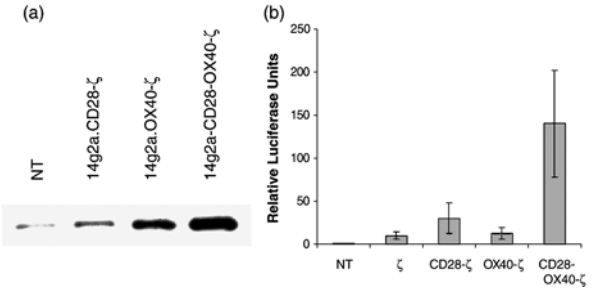

Figure 3. Down-stream signaling of chimeric receptors.
(a) *co-ip of FLAG-tagged TRAF2 with V5-tagged receptors in transfected 293T-cells.* **(b)** *NFkB-Luciferase response in primary T-cells 2 days after stimulation with LAN-1 cells. Mean and standard deviation of 3 separate experiments are shown.*

Retention of CD3ζ activation for tumor cell cytolysis by multi-domain cTCR cytoplasmic domains

We compared the initial capacity of T-cells transduced with each cTCR construct to lyse tumor cells. Receptor positive T-cells were depleted of CD56-positive cells to exclude lysis mediated by an NK cell subpopulation. T-cells transduced with all cTCR constructs killed GD2-positive neuroblastoma cells (LAN-1) whereas no lysis of the GD2-negative rhabdomyosarcoma cells (A204) was observed (Figure 4a). This killing was essentially identical regardless of the endo-domain, so that CD3ζ signaling was not compromised even when it was located deeper in the cytosol due to the presence of one or two membrane–proximal co-stimulatory domains.

14g2a-CD28-OX40-ζ construct signaling induces sustained clonal expansion.

Although killing mediated by GD2 ligation was identical regardless of the constructs used, the consequences of differential intracellular signaling mediated by the three endodomain complexes become readily apparent in experiments designed to measure cytokine secretion and long-term growth. We cultured T-cells transgenic for each construct with equal numbers of irradiated LAN-1 cells and measured the resultant T-cell clonal expansion after 7 days. There was no increase in cell numbers by non-transduced or 14g2a-ζ transduced T-cells. 14G2a-OX40-ζ transduced T-cells showed a minimal increase above baseline (1.6 fold, range 0.9-3), while the combination of CD3ζ with CD28 resulted in a 5.2 fold (range: 1.6-7.2) expansion. With the combination of OX40, CD28 and CD3ζ, however, there was a 10.7 fold (range: 4-15.5) expansion (Figure 5a; p<0.001). This hierarchy of stimulation was confirmed in transduced cells labeled with CFSE, to analyze their clonal expansion in response to GD2+ and GD2-ve target cells (Figure 5b). Only T-cells expressing 14g2a-CD28-OX40-ζ were able to fully complete their expansion program. This is also evident in photomicrographs of T-cells on day 3 post

stimulation (Figure 5c). This finding became far more pronounced after weekly re-stimulation with LAN-1 cells, when 14g2a-CD28-ζ-expressing T-cells were unable to sustain clonal expansion but that of 14G2a-CD28-OX40-ζ transduced T-cells continued beyond the third stimulation. Low dose of IL-2 (20 U/ml) extended the expansion of 14g2a-CD28-ζ transduced cells to 3 weeks, but 14g2a-CD28-OX40-ζ cells were then able to expand beyond 5 weeks.

This increased capacity for sustained proliferation correlated with a sustained capacity to kill tumor target cells. 14g2a-CD28-OX40-ζ transduced cells were still able to kill GD2-positive LAN-1 cells after 6 weeks expansion (Figure 5d), at a time when no other transductants had survived. This sustained growth is not autonomous: proliferation of 14g2a-CD28-OX40-ζ remained fully dependent on the continued presence of stimulation with specific antigen.

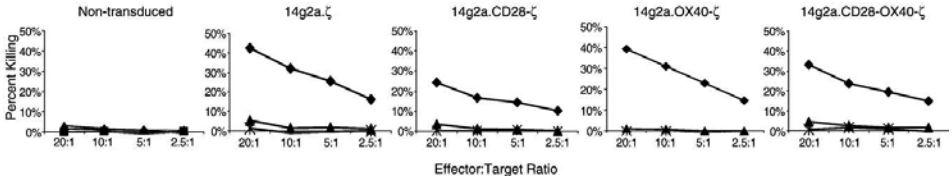

Figure 4. cTCR mediated killing of Target cells.
Chromium release assay using transduced CD56-depleted transduced peripheral blood T-cells as effectors. LAN-1 (♦) a GD-2 positive neuroblastoma cell-line is killed by all constructs but not by non-transduced T-cells. A204 (x) is a GD-2 negative rhadomyosarcoma cell line and is not killed by transduced cells.

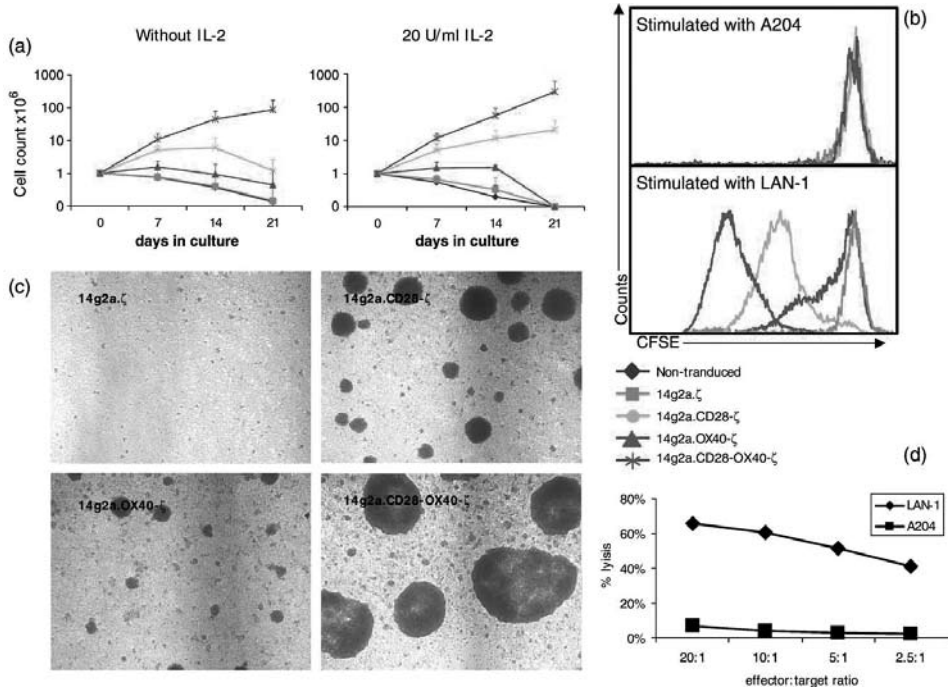

Figure 5. Proliferation of transduced T-cells.
(a) *Growth curves with weekly stimulation with LAN-1 cells without and with IL-2. Mean and standard deviation of 5 separate experiments with primary T-cells from 5 different donors are shown.* **(b)** *CFSE staining of transduced cells stimulated with LAN-1 or A204 cells.* **(c)** *Photomicrograph of cells three days after stimulation with LAN-1 cells.* **(d)** *Chromium release assay 14g2a-CD28-OX40-ζ transduced T-cells after 50 days expansion with weekly stimulation by LAN-1 cells.*

Hence, incorporation of CD28 alone is sufficient for a short-term proliferative signal, but an additional OX40 signal is required to fully develop and maintain T-cell expansion upon activation through the cTCR.

While CD8+ T-cells may not express OX40, they do express adaptor molecules (e.g. TRAF-2) required to transmit OX40 signals. CD8+ T-cells transduced with 14g2a.28-OX40-ζ should be able to proliferate in response to GD2 positive targets independently of CD4+ cells. We demonstrated this, by isolating CD8+ transduced T-cells with magnetic beads, and comparing their expansion with unsorted transduced cells (supplementary data figure 1).

14g2a-CD28-OX40-ζ transduced T-cells produce IL-2 upon activation

A key mechanism of cTCR mediated survival and proliferation is likely the result of cTCR-regulated production of the T-cell autocrine/paracrine growth factor IL-2. We compared the pattern of cytokine release induced by the different constructs by measuring the concentration of IFN-γ, IL-2, TNF-α, IL-4, IL-5 and IL-10 at 72 hours after antigenic stimulation (Figure 6). Cells transduced with the 14g2a.CD28-OX40-ζ construct secreted approximately 10-fold more IL-2 compared to cells transduced with 14g2a.CD28-ζ ($p < 0.01$). TNF-α was 5-fold increased in cells expressing the 14g2a.CD28-OX40-ζ construct ($p < 0.01$). None of the cTCR constructs tested resulted in secretion of the Th2 cytokines IL-10 or IL-4 release greater than 100 pg/ml.

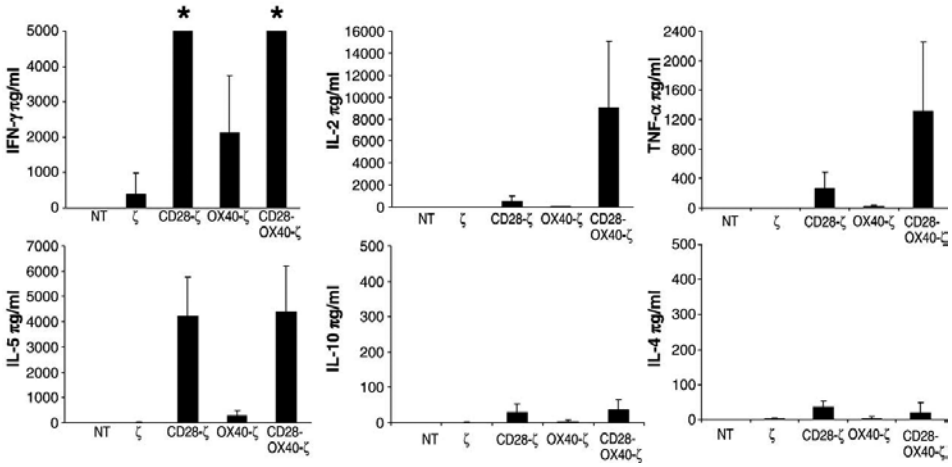

Figure 6. Cytokine Release Pattern from Transduced T-cells.

*Concentration of Th1 and Th2 cytokines in the cell supernatant was measured 3 days after stimulation with either LAN-1 cells or A204 cells. Mean and standard deviation of 5 separate experiments with primary T-cells from 5 different donors are shown. * = >5000 pg/ml.*

Discussion

We have generated antigen-specific receptors that signal through artificial endodomains composed of combinations of intracellular elements of CD3ζ, CD28 and OX40. These constructs were compared with our original cTCR 14g2a-ζ[26], that contains the antigen binding domain of the anti-GD2a antibody 14g2a and the intracellular segment of CD3-ζ. Constructs 14g2a-CD28-ζ, 14g2a-OX40-ζ and 14g2a-CD28-OX40-ζ were stably expressed by primary T-cells after retroviral transduction. All constructs triggered effective killing of GD-2 expressing target cells but only the combination of OX40 and CD28 and CD3-ζ signals *in cis* resulted in maximal NFκB activation associated with increased and prolonged proliferation and augmented cytokine release. Importantly, 14g2a-CD28-OX40-ζ transduced T-cells maintained their proliferative capacity and cytolytic function after multiple encounters with tumor cells. Supplying these three signals from one receptor overcomes the major defect of our current GD-2 targeting cTCR, which may result in improved persistence of transduced T-cells , and better function in the tumor microenvironment.

Although successful in some animal models[27-29], cTCRs containing solely the CD3ζ endodomains, may not be capable of mounting an effective anti-tumor response, particularly when targeting tumors lacking co-stimulatory molecules. For example, T-cells from mice transgenic for a CD3ζ-based receptor fail to proliferate in response to cTCR ligation[11], while in humans, T-cells expressing HIV-*env* specific CD4-ζ receptors did not appear to proliferate, and had no easily discernible clinical benefit.[6-8] Two approaches to circumvent this limitation have been proposed. First, antigen-specific T-cells can be selected and expanded and then transduced with cTCRs to generate bi-specific T-cells. After administration, repeated exposure of the native T-cell receptor to antigen and co-stimulator molecules on APC will supply the necessary signals to expand and maintain activity against the target antigens recognized by the cTCR[24,30]. Alternatively, the signaling domain of the chimeric receptor itself can be altered. Hence, the addition of a CD28 endodomain allows primary T-cells to release IL-2 and to proliferate in response to chimeric receptor engagement.[13,14] cTCRs with a CD28 component have been tested [31], and demonstrate improved function[32].

However, while CD28 activation may initiate clonal expansion and IL-2 release from naïve T-cells, it is now clear that additional signals cooperate in sustaining long-term T-cell growth, activity and survival: in the absence of additional signals, most T-cells rapidly undergo apoptosis[12]. We sought to improve signaling further by incorporation of another domain – OX40, and show *in vitro* data of the consequences. OX40 appears to be one of the principle additional co-stimulatory molecules. In the absence of OX40 , only a small number of antigen-reactive T-cells accumulate in response to antigen[33], and mice that are OX40-deficient or are receiving neutralizing antibodies to OX40L show lower susceptibility to T-cell mediated pathology, including EAE and GVHD.[34-36] Since tumor "rejection" likely needs the expansion and persistence of tumor reactive T-cells, we reasoned that the incorporation of an OX40 signaling domain in a chimeric receptor molecule might favor the development of these properties[37], an expectation confirmed by our data. Although both OX40 and CD28 activate NFκB this occurs via different mediators (Vav and P13K for CD28 and TRAF2 for OX40) and distinct additional signals may be transmitted (eg Akt and AP1) independently of FκB.[38,39] The combination of CD28 and OX40 signaling domains within a single receptor led to proliferation that appeared to mimic the physiologic expansion of naive T-cells following exposure to antigen on APC.

We constructed 14g2a-CD28-OX40-ζ receptor with endodomain components linked in the following order: CD28 followed by OX40, followed by CD3ζ. Previous reports have documented that constructs in which CD28 is moved from its normal membrane-proximal location are non-functional.[13] Hence we did not test constructs with the orientation OX40-CD28-ζ or OX40-ζ-CD28. Constructs with the orientation CD28-ζ-OX40 could not be stably expressed even when we introduced different length spacers between the carboxy-terminal of ζ and the OX40 endodomain (data not shown). The level of surface expression of chimeric TCR appeared to be dictated by the domain spanning the cell membrane. Transduction with cTCRs with CD28 transmembrane domain (14g2a-CD28-ζ and 14g2a.CD28-OX40-ζ) results in the brightest expression, with construct anchored by the OX40 transmembrane domain (14g2a-OX40-ζ) having intermediate expression and CD3-ζ transmembrane domain (14g2a-ζ) having the dimmest. We did not use spacers to link endodomains since these may increase the immunogenicity of the expressed constructs, leading to immune destruction of the transduced T-cells [40]. The efficacy of 14g2a-CD28-OX40-ζ endodomain suggests that any steric hindrance is limited in effect. Although it might in principle be possible to provide the endodomains as separate sequences in *trans*, they would have to be conveyed in separate vectors necessitating multiple transductions of T-cells, and thereby greatly increasing the risk of an unwanted consequence of pro-viral integration.

There are clear concerns with combining a transgene transmitting a proliferative signal and retroviral integration. Two patients with X-linked severe combined immunodeficiency who received hemopoietic stem cells engineered to constitutively express the common gamma-chain receptor developed LMO-2 overexpression due to retroviral integration, and T-cell acute lymphoblastic leukemia followed.[41] However, in our study the transduced cells are mature, memory T lymphocytes, which have an absolute dependence on antigen stimulation and IL-2 supplementation. This continued dependence on external signals to maintain cell survival alleviates concerns about the use of such transduced T-cells, particularly in the intended population of patients with advanced cancer. Certainly, prolonged follow up of patients receiving retrovirally gene modified T-cells has revealed no evidence of lymphoproliferation. Use of self-inactivating retroviral vectors may increase safety. Additionally, toxicity may also result from chimeric T-cell destruction of normal tissue also expressing the target antigen. Co-expression of the cTCR with a suitable suicide gene may help manage this possibility.[42]

Our modification to the cTCR receptor endodomain provides T-cells with panoply of required signals that would otherwise be lacking on tumor cells that supports helper independent clonal expansion. The resulting improvement in proliferation, function and survival may have considerable clinical utility.

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