



Published in final edited form as:

Cytotherapy. 2014 August ; 16(8): 1121–1131. doi:10.1016/j.jcyt.2014.02.012.

T cells redirected to IL13R α 2 with IL13 mutein-CARs have antiglioma activity but also recognize IL13R α 1

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Abstract

Outcomes for patients with glioblastoma remain poor despite aggressive multimodal therapy. Immunotherapy with genetically modified T cells expressing chimeric antigen receptors (CARs) targeting IL13R[.alpha]2, HER2, EGFRvIII, or EphA2 has shown promise for the treatment of glioma in preclinical models. Based on IL13R α 2-targeted immunotoxins that contain IL13 molecules with one or two amino acid substitutions (IL13 muteins) to confer specificity to IL13R α 2, investigators have constructed CARs with IL13 muteins as antigen binding domains. While the specificity of IL13 muteins in the context of immunotoxins is well characterized, limited information is available for CAR T cells. We constructed four 2nd generation CARs with IL13 muteins with one or two amino acid substitutions. T cells expressing all four CARs recognized IL13R α 1 or IL13R α 2 recombinant protein in contrast to control protein (IL4R) as judged by IFN α production. IL13R α 2 protein induced significantly more IL2, indicating that IL13 mutein-CAR T cells have a higher affinity to IL13R α 2 than IL13R α 1. In cytotoxicity assays, CAR T cells killed IL13R α 1- and/or IL13R α 2-positive cells in contrast to IL13R α 1- and IL13R α 2-negative controls. While we observed no significant differences between IL13 mutein CAR T cells *in vitro*, only T cells expressing IL13 mutein CARs with an E13K amino acid substitution had antitumor activity *in vivo* that resulted in a survival advantage of treated animals. Our study

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Conflict of interest: The Center for Cell and Gene Therapy has a Research Collaboration with Celgene and bluebird bio. SK, KKHC, ZY, NA, and SG have patent applications in the field of T-cell and gene modified T-cell therapy for cancer.

highlights that the specificity/avidity of ligands is context-dependent and that evaluating CAR T cells in preclinical animal model is critical to assess their potential benefit.

Keywords

Cancer; CAR; GBM; IL13R α 1; IL13R α 2; Immunotherapy; T cells

INTRODUCTION

The outcome for glioblastoma (GBM) remains poor and immunotherapy with vaccines or GBM-specific T cells is one attractive strategy to improve survival, since it does not rely on the cytotoxic mechanisms employed by conventional therapies such as chemotherapy and radiation [1-4]. Genetic modification with chimeric antigen receptors (CARs) allows for the rapid generation of tumor-specific T cells [5]. For GBM-targeted T-cell therapy several antigens are actively being pursued including interleukin 13 receptor alpha 2 (IL13R α 2), human epidermal growth factor receptor 2 (HER2), epidermal growth factor variant III (EGFRvIII), and erythropoietin-producing hepatocellular carcinoma A2 (EphA2) [6-11]. The majority of clinical studies with CAR T cells so far have focused on targeting CD19-positive hematological malignancies, and while dramatic clinical responses were observed, several patients also developed life-threatening cytokine storms [12-17]. Other reported side effects of CAR T-cell therapies include a death of one patient, who received 10¹⁰ HER2-CAR T cells after a lymphodepleting conditioning regimen, and local inflammatory responses at non-malignant tissue sites, which expressed the targeted antigen [18, 19].

Hence, one important aspect of preclinical testing of CAR T cells is to evaluate their specificity. CARs consist of an ectodomain, which is most commonly derived from a single chain variable fragment (scFv), a transmembrane domain, and an endodomain. The endodomain contains either one (1st generation), two (2nd generation), or three (3rd generation) signaling domains derived from CD3 ζ and/or co-stimulatory molecules [5]. Current IL13R α 2-specific CARs do not contain scFvs, but an IL13 mutein with one or two amino acid substitutions to preferentially redirect T cells to IL13R α 2 [8, 11]. While one study showed that T cells expressing a 1st generation CAR with an IL13 mutein, which contained an E13Y amino acid substitution, did not recognize IL13R α 1-positive cells [8], another study indicated cross-reactivity to IL13R α 1 of T cells expressing a 2nd generation CAR containing an IL13 mutein with E13K and R109K amino acid substitutions [11].

Here we report the construction of four 2nd generation CARs containing IL13 muteins with one (E13K or E13Y) or two amino acid substitution (E13K.K105R; E13Y.K105R). We show that all four CARs recognize IL13R α 1 and IL13R α 2 *ex vivo*, and that only CARs with an E13K or E13K.K105R amino acid substitutions have antitumor activity *in vivo* that is associated with a survival advantage of treated animals.

MATERIAL AND METHODS

Blood donors and cell lines

Blood samples were obtained from healthy subjects on a protocol approved by the Institutional Review Board of Baylor College of Medicine. The cell lines U373, U87, T98G, A431, 293T and Raji were purchased from the American Type Culture Collection (ATCC; Manassas, VA). SNT16 cells were kindly provided by Dr. Norio Shimizu (Tokyo Medical and Dental University, Tokyo, Japan). The generation of U373 cells expressing an enhanced green fluorescent protein firefly luciferase fusion gene (U373.eGFP.ffLuc) was previously reported [7]. To generate Raji cells expressing IL13R α 1 or IL13R α 2 we cloned cDNAs encoding IL13R α 1 or IL13R α 2 (Origene, Rockville, MD) into pCDH-CMVCS-EF1-GFP +puro (System Bioscience, Mountainview, CA). Cloning was verified by sequencing (Seqwright, Houston, TX). Raji cells were transduced with VSVG-pseudotyped lentiviral vectors to generate Raji-GFP, Raji-IL13R α 1, and Raji-IL13R α 2. Cell lines were grown in RPMI or DMEM (Thermo Scientific HyClone, Waltham, MA; Lonza, Basel, Switzerland) with 10% fetal calf serum (FCS; HyClone, Logan, UT) and 2 mM GlutaMAX-I (Invitrogen, Carlsbad, CA).

Generation of IL13-mutein CARs

Codon-optimized mini genes flanked by 5' NcoI and 3' BamHI sites were synthesized by GeneArt (Invitrogen, Carlsbad, CA) containing the immunoglobulin heavy-chain leader peptide [20] and IL13 muteins with one (E13K; E13Y) or two amino acid substitutions (E13K.K105R; E13Y.K105R). IL13 muteins were subcloned into an SFG retroviral vector containing the human IgG1-CH2CH3 domain, a CD28 transmembrane domain, and costimulatory domains derived from CD28 and the CD3 ζ -chain [21, 22]. Cloning was verified by sequencing (Seqwright, Houston, TX). The construction of the control CAR specific for murine and human fibroblast activation protein (mhFAP) has been described elsewhere [23].

Retrovirus production and transduction of T cells

RD114-pseudotyped retroviral particles were generated as previously described [6]. The protocol to transduce T cells with retroviral particles has been described in detail [7]. To activate T cells, non-tissue culture treated 24 well plates were coated with 0.5 mL OKT3 (1 μ g/mL) and CD28 (1 μ g/ml) monoclonal antibodies (BD Biosciences, Mountain View, CA) for 24 hours. On day 1, the antibody solution was removed, and wells were washed with complete media before plating 1x10⁶ peripheral blood mononuclear cells (PBMCs) per well. On day 2, recombinant human interleukin-2 (IL2; Proleukin; Chiron, Emeryville, CA) was added at a final concentration of 100 units/mL, and a separate non-tissue culture treated 24 well plate was coated with 1 mL of RetroNectin® (7 μ g/mL; Clontech, Mountainview, CA). On day 3, the RetroNectin® solution was removed and wells were washed with complete media. Each well was coated twice with 0.5 mL of retroviral supernatant for 30 minutes before adding 1.5 mL retroviral supernatant, 0.5 mL of activated PBMCs (2.5x10⁵ cells) and IL2 (final concentration of 50 units/mL). Forty eight to 72 h post transduction cells were transferred to a new 24-well pate and expanded in the presence of 50 to 100

units/mL of IL2 for 10 to 15 days before use. Non-transduced (NT) T cells, used as controls, were activated with OKT3/CD28 and expanded in parallel with 50-100 U/mL IL2.

Flow cytometry

A FACSCalibur instrument (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson, San Jose, CA) were used for all flow cytometric analyses, analyzing >10,000 events; in all cases, negative controls included isotype antibodies. Cells were washed once with PBS containing 1% fetal bovine serum (FACS buffer) before addition of antibodies. After 30 min of incubation at 4°C in the dark, the cells were washed once and fixed in 0.5% paraformaldehyde/FACS buffer before analysis. T cells were analyzed with fluorescein-conjugated monoclonal antibodies (Becton Dickinson, San Jose, CA) directed against CD3, CD4, CD8, CD45RA, CD45RO, CD56, CD62L and CCR7 surface proteins. To determine surface expression of CARs on T cells a CH2CH3 Cy5 antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used. To determine IL13R α 1 and IL13R α 2 expression of tumor cells polyclonal goat antibodies specific for IL13R α 1 and IL13R α 2 (R&D Systems, Minneapolis, MN) were used as primary antibodies and rabbit anti-goat Alexa (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody.

Co-culture assays

Recombinant protein co-culture assay—Non-tissue culture 24-well plates were precoated with recombinant human IL13R α 1-, IL13R α 2-, or IL4R-Fc chimera, (R&D Systems, Minneapolis, MN) at a final concentration of 15.6 to 500 ng/well. Plates were washed once using RPMI, and IL13 mutein-CAR and NT T cells were plated. After 24 hours (h) of culture supernatant was harvested and IFN γ and IL2 release was measured by ELISA per the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Cell culture co-culture assay—IL13 mutein-CAR T cells were cocultured with genetically modified Raji cells expressing human IL13R α 1, IL13R α 2 or GFP at a 1:4 effector to target (E:T) ratio in a 48-well plate. NT and mhFAP-CAR T cells served as controls. After 24h of incubation, culture supernatants were harvested, and the presence of IFN γ and IL2 was determined by ELISA as per the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Cytotoxicity assays

Standard chromium (^{51}Cr) release assays were performed as previously described [24]. Briefly, ^{51}Cr -labeled target cells were mixed with decreasing numbers of effector cells at E:T ratios of 40:1, 20:1, 10:1, and 5:1. Target cells incubated in complete medium alone or in 1% Triton X-100 were used to determine spontaneous and maximum ^{51}Cr release. After 4 h, released radioactivity was measured in a gamma counter (Cobra Quantum; Perkin-Elmer). The mean percentage of specific lysis of triplicate wells was calculated according to the following formula: (test release – spontaneous release)/ (maximal release – spontaneous release) \times 100.

Quantitative real time PCR

RNA was isolated from different tumor cell lines using RNeasy Mini Kit (Qiagen, Valencia, CA). Relative quantification of IL-13R α 1, IL-13R α 2, and GAPDH mRNA expression was done using TaqMan® One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Branchburg, NJ) using the primer/probes for human or murine IL13R α 1, IL13R α 2, and GAPDH (all from Applied Biosystems).

Orthotopic xenograft SCID mouse model

All animal experiments followed a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. For animal experiments ICR-SCID mice (IcrTac:ICR-Prkdc^{scid}; Taconic, Hudson, NY) were used and we used our previously described the surgical procedure [7]. Briefly, U373.eGFP.ffLuc cells (1×10^5 in 2.0 μ L) were injected 3 mm deep to the bregma, corresponding to the center of the right caudate nucleus over 5 minutes. Seven days after tumor cell injection, animals were treated with 2×10^6 NT or IL13 mutein-CAR T cells from the same donor in 2 μ L to the same tumor coordinates.

Bioluminescence imaging

Isoflurane anesthetized animals were imaged using the IVIS® system (IVIS, Xenogen Corp., Alameda, CA) as previously described [7]. The photons emitted from the luciferase-expressing tumor cells were quantified using Living Image software (Caliper Life Sciences, Hopkinton, MA). A pseudo-color image representing light intensity (blue least intense and red most intense) was generated and superimposed over the grayscale reference image. Mice were euthanized when the tumor radiance was greater than 1×10^9 on two occasions or when they met euthanasia criteria (neurological deficits, weight loss, signs of distress) in accordance with the Center for Comparative Medicine at Baylor College of Medicine.

Statistical analysis

All *in vitro* experiments were performed in triplicate, GraphPad Prism 5 software (GraphPad software, Inc., La Jolla, CA) was used for statistical analysis. Measurement data were presented as mean \pm standard deviation (SD). The differences between means were tested by appropriate tests including t-test or non-parametric Mann Whitney U test. The significance level used was $P < 0.05$. For the mouse experiments, fold changes in tumor radiance from baseline at each time point were calculated. Survival determined from the time of tumor cell injection was analyzed by the Kaplan-Meier method.

RESULTS

Generation of IL13 mutein-CAR T cells

We constructed four SFG retroviral vectors encoding IL13 mutein-CARs. While the IL13 mutein-CARs had identical IgG1-CH2CH3 spacers, CD28 transmembrane domains, and signaling domains derived from CD28 and the TCR- ζ chain, their extracellular binding domain contained IL13-muteins with increased affinity to IL13R α 2. Two had single amino acid substitutions at position 13 (E13K or E13Y; IL13Y-CAR, IL13K-CAR), and two had

an additional amino acid substitution at position 105 (K105R; IL13YR-CAR, IL13KR-CAR) (Fig. 1A). CD3/CD28-activated T cells from healthy donors were transduced with RD114-pseudotyped retroviral particles encoding the four different IL13 mutein-CARs. Five to 6 days post transduction FACS analysis was performed to determine CAR expression; 80.2 to 85.6% (n=7-14, Fig. 1B,C) of the T cells expressed IL13 mutein-CARs with no significant differences between all four constructs. There was no difference in T-cell phenotype, and transduced T cells included CD4-positive and CD8-positive subpopulations (mean 34.8±3.73%; mean 65.9±2.4%), and central (CD3+/CD62L+: 47.1±8.6%) as well and effector memory T cells (CD3+/CD62L-/CCR7-: 33.0±8.5%) (Supplementary Fig. 1).

IL13 mutein-CAR T cells recognize IL13R α 1 and IL13R α 2

To evaluate the specificity of T cells expressing the different IL13 mutein-CARs we initially took advantage of recombinant proteins encoding IL13R α 1, IL13R α 2, and IL4R. IL13 mutein-CAR or non-transduced (NT) T cells were incubated on IL13R α 1- or IL13R α 2-coated plates with IL4R- or non-coated plates serving as controls. T cells expressing all four CAR constructs produced significant levels of IFN γ (p<0.001) when stimulated with recombinant IL13R α 1 or IL13R α 2 proteins in comparison to IL4R stimulated T cells (Fig. 2A). However, only stimulation with IL13R α 2 resulted in significant IL2 production (p<0.001) (Fig. 2B). No significant IFN γ or IL2 production was observed in co-cultures with NT-T cells. These studies were performed with 24-well plates, which were coated with 500 μ g of recombinant proteins per well. To evaluate if there was a difference in IFN[.gamma] secretion with lower protein concentrations, IL13R α 1 or IL13R α 2 recombinant proteins were serially diluted. Starting at 125[.proportional]g IL13R α 2/well IL13Y- and IL13K-CAR T cells secreted significant amounts of IFN[.gamma], while 250[.proportional]g IL13R α 1/well was needed to induce IFN[.gamma] secretion. At 62.5 μ g/well neither protein induced significant IFN[.gamma] secretion (Supplementary Fig. 2).

IL13 mutein-CAR T cells recognize and kill IL13R α 2-positive cells

We screened a panel of cell lines for the expression of IL13R α 1 and IL13R α 2 using qRT-PCR and FACS analysis (3 glioma cell lines: U87, U373, T98G; 2 lymphoma cell lines: SNT16, Raji; the epidermoid carcinoma cell line A431; 293T cells and NT T cells). U87, U373, T98G expressed IL13R α 1 and IL13R α 2; SNT16, A431, and 293T expressed only IL13R α 1, and Raji and NT T cells were negative for both (Fig. 3A-C).

Since none of the cell lines only expressed IL13R α 2, we genetically modified Raji cells with a lentiviral vector encoding IL13R α 2 and GFP (Raji-IL13R α 2; Fig. 3C). To assess IL13R α 1 and IL13R α 2 recognition by IL13 mutein-CAR T cells in the same cell background, we generated Raji cells expressing IL13R α 1 and GFP (Raji-IL13R α 1) and as controls Raji cells expressing GFP (Raji-GFP) (Fig. 3B). Stimulation of T cells, expressing IL13 mutein-CARs with E13Y or E13K amino acid substitutions (IL13Y- or IL13K-CARs), with Raji-IL13R α 1 or Raji-IL13R α 2 cells resulted in significant production of IFN γ (p<0.001) and IL2 (p<0.001) in comparison to stimulations with Raji or Raji-GFP cells. However, Raji-IL13R α 2 cells induced significant higher levels of IFN[.gamma] (p=0.0016) and IL2 (p=0.0018) in comparison to Raji-IL13R α 1 cells (Fig. 4A,B). No significant IFN γ or IL2

production was observed from NT T cells or T cells expressing a CAR specific for an unrelated antigen (mhFAP).

IL13Y- and IL13K-CAR T cells also killed Raji-IL13R α 1 and Raji-IL13R α 2 cells in contrast to Raji and Raji-GFP cells (Fig. 5). NT T cells and mhFAP-CAR T cells had no cytolytic activity, demonstrating that cell killing depends on the cell surface expression of IL13R α 1 or IL13R α 2 on target cells and IL13 mutein-CARs on T cells. We confirmed for all four IL13 mutein-CAR constructs that T cells expressing these CARs kill cells that express IL13R α 1 and IL13R α 2 (U373) and cell lines (A431 or 293T) that only express IL13R α 1 (Supplementary Fig. 3). These results indicate that T cells expressing IL13 mutein-CARs recognize IL13R α 1- and IL13R α 2-positive target cells as judged by cytokine production and cytolytic activity.

Treatment of glioma xenografts with T cells expressing IL13 mutein-CARs with E13K amino acid substitutions results in improved overall survival

Since the recognition of IL13R α 1 will most likely limit the application of IL13 mutein-CAR T cells to local administration, we used the orthotopic U373 glioma xenograft model in which T cells are directly injected into tumors (20). 1×10^5 U373.eGFP.ffLuc cells were injected stereotactically into brains of SCID mice on day 0. On day 7, 2×10^6 IL13 mutein-CAR T cells were injected intracranially into the previous stereotactic tumor coordinates (n=11 per CAR construct). Mice treated with NT T cells (n=9) and untreated mice (n=10) served as controls. Response to therapy was defined as a greater than 75% reduction in bioluminescence signal on day 7 post T-cell injection. Three of 11 mice treated with IL13Y-CAR T cells, 3 of 11 mice treated with YR-CAR T cells, 4 of 11 mice treated with IL13K-CAR, and 7 of 11 mice treated with IL13KR-CAR T cells had a response. NT-T cells had no antitumor activity (Fig. 6A). While IL13K- or IL13KR-specific T-cell therapy resulted in a significant increase in overall survival in comparison to injection of NT-T cells (NT-T cells vs IL13K-CAR T cells, p=0.009; NT-T cells vs IL13KR-CAR T cells, p<0.001), IL13Y- or IL13YR-CAR T-cell therapy did not (NT-T cells vs IL13Y-CAR T cells, p=0.322; NT-T cells vs IL13KR-CAR T cells, p=0.253; Fig. 6B).

DISCUSSION

Here we describe the construction of CARs with IL13-muteins as ectodomains and demonstrate that these CARs not only recognize IL13R α 2 but also IL13R α 1. While we observed no functional difference between IL13 mutein-CARs *in vitro*, only CARs with an E13K amino acid substitution had antitumor activity in an orthotopic xenograft SCID mouse model of GBM resulting in a survival advantage of treated animals.

IL13R α 2 is a promising immunotherapy target for GBM [25]. The receptor has been targeted with immunotoxins, vaccines, and antigen-specific T cells with encouraging results [8, 11, 26-28]. To redirect immunotoxin to IL13R α 2, investigators have taken advantage of IL13 muteins that have increased binding affinity to IL13R α 2 [29, 30]. IL13 contains 4 α -helices (A, B, C, D), and amino acid substitutions at position 13 (E13Y or E13K) within α -helix A greatly decreases the ability of IL13 to bind to IL13R α 1. In addition, amino acid substitutions in α -helix D (K105R, R109K) increase the affinity of IL13 to IL13R α 2 [30,

31]. Pseudomonas-based IL13 mutein immunotoxins have shown preferential killing of IL13R α 2-positive cells, and based on these findings 2 groups of investigators have generated IL13-mutein-based CARs [8, 11]. CD8-positive T-cell clones expressing a 1st generation CAR with an E13Y IL13 mutein only recognized IL13R α 2-positive target cells, but these T-cell clones did not produce IL2 upon stimulation. *In vivo* these cells had anti-glioma activity, however the targeted glioma cell line was genetically modified to express IL2 [8]. Kong *et al.* reported the construction of a 2nd generation CAR with an E13K.R109K IL13 mutein. T cells expressing this CAR recognized IL13R α 1-positive cells in co-culture assays and had antitumor activity in the U251 (U373) xenograft model with a long-term, disease-free survival rate of ~30% [11].

Our study now extends these findings and clarifies the specificity of CARs containing IL13 muteins. We constructed 4 CARs that contained 2 or 4 amino acid substitutions. Two CARs contained E13Y or E13K amino acid substitutions in α -helix A and 2 CARs had an additional amino acid substitution in α -helix D (K105R). We used K105R instead of R109K since the resulting IL13 mutein has a higher affinity towards IL13R α 2 [30]. We demonstrate that T cells expressing IL13 mutein-CARs recognize IL13R α 1 and IL13R α 2 recombinant proteins and kill Raji cells that express either IL13R α 1 or IL13R α 2. The killing of IL13R α 1-positive target cells was unexpected since the recognition of IL13R α 1 by IL13 mutein-based immunotoxins is greatly reduced [29, 30, 32]. Our results suggest that the overall avidity of IL13 mutein CARs arrayed on the cell surface of T cells is greater towards IL13R α 1 than IL13 mutein-based immunotoxins, which only contain a single binding site. Nevertheless, IL13R α 2 recombinant protein and IL13R α 2-expressing Raji cells induced significantly higher levels of IL2 by IL13 mutein-CAR T cells than their IL13R α 1 counterparts. Since effector function of CAR T cells depends in part on the affinity of the CAR [33], our results suggest that IL13 mutein-CAR T cells have a higher affinity towards IL13R α 2 than IL13R α 1.

Our finding that IL13 mutein-CAR T cells recognize IL13R α 1 should not preclude their local administration in humans, due to the clinical experience with the local, convection-enhanced delivery of a pseudomonas-immunotoxin into GBMs, which recognize IL13R α 1 and IL13R α 2 (Cintredekin besudotox) [28]. No dose-limiting toxicity was observed, and patients with optimally positioned catheters had a median survival of 55.6 weeks, which is encouraging in the setting of progressive high grade glioma [28]. In addition, we observed no side effects post injection of IL13 mutein-CAR T cells, which is especially encouraging since our CARs recognize murine IL13R α 1 and IL13R α 2 (Supplementary Fig. 4). However, infused IL13 mutein-CAR T cells persisted for less than one week, and additional safety studies are needed with IL13 mutein-CAR T cells, that expand and persist for several weeks in a xenograft or immune competent mouse model. Another strategy to minimize potential side effects is the use of T cells that express a CAR containing an IL13R α 2-specific scFv ectodomain. Two groups have reported the isolation of IL13R α 2-specific scFvs [34, 35] and in the future these scFvs should be evaluated in the context of CAR T-cell therapy.

In vivo, only the injection of T cells, expressing IL13 mutein-CARs with an E13K amino acid substitution, was associated with improved survival. This was surprising since we did not observe any significant difference between E13Y- and E13K-mutein CAR T cells *in*

vitro. A recent study suggest that structural components of CARs, which are not involved in signaling, influence the *in vivo* function of CAR T cells [33]. Accordingly, amino acids with different side chains (K: positive side chain; Y: hydrophobic side chain) in the antigen-binding domain of a CAR could potentially impact CAR T-cell function *in vivo*. The long-term survival rate of ~ 20% was similar to the reported rate by Kong *et al.*, who used a 2nd generation E13K.R109K IL13 mutein-CAR targeting the same glioma cell line *in vivo* [11]. To evaluate how long T cells persist *in vivo*, T cells were ‘double transduced’ with retroviral vectors encoding the IL13KR CAR or eGFP.ffLuc. CAR T cells persisted for less than one week at the tumor site, as judged by bioluminescence imaging (Supplementary Fig. 5), indicating that limited T-cell persistence at the tumor site is most likely responsible for tumor progression. Since we could not detect bioluminescence signal outside the glioma site, T cells did not migrate away from gliomas, but most likely underwent apoptosis. Inclusion of additional costimulatory domains besides CD28 might improve persistence of infused T cells [22, 36]. In addition, targeting multiple tumor antigens might improve antitumor effects. For example, we have shown that targeting glioma with IL13 mutein- and HER2-CAR T cells results in enhanced antitumor effects in the U373 xenograft model [37].

In conclusion, we have generated IL13 mutein-based CARs and show that these CARs recognize not only IL13R α 2 but also IL13R α 1. T cells expressing IL13 mutein-CARs with E13K amino acid substitutions had antitumor activity *in vivo* resulting in a survival advantage of treated animals. Our study highlights that the specificity/avidity of ligands is context-dependent and provides the rationale to develop IL13R α 2-specific CARs with an IL13R α 2-specific scFv as an antigen recognition domain to prevent cross reactivity to IL13R α 1. In addition, our results indicate that evaluating CAR T cells in preclinical animal model is critical to assess their potential benefit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by The Dana Foundation (SG and NA), The Clayton Foundation for Research (SG and NA), and the James S McDonnell Foundation (SG). KKHC is a Melnick scholar and was supported by NIH grants 5T32HL092332 and 5T32GM007330.

ABBREVIATIONS

CAR	chimeric antigen receptor
EGFRvIII	epidermal growth factor variant III
EphA2	erythropoietin-producing hepatocellular carcinoma A2
FACS	fluorescence activated cell sorting
GBM	glioblastoma
GFP	green fluorescent protein

HER2	human epidermal growth factor receptor 2
IFN	interferon
IL	interleukin
IL13Rα1	interleukin 13 receptor alpha 1
IL13Rα2	interleukin 13 receptor alpha 2
scFv	single chain variable fragment
NT	non-transduced
PBMC	peripheral blood mononuclear cells

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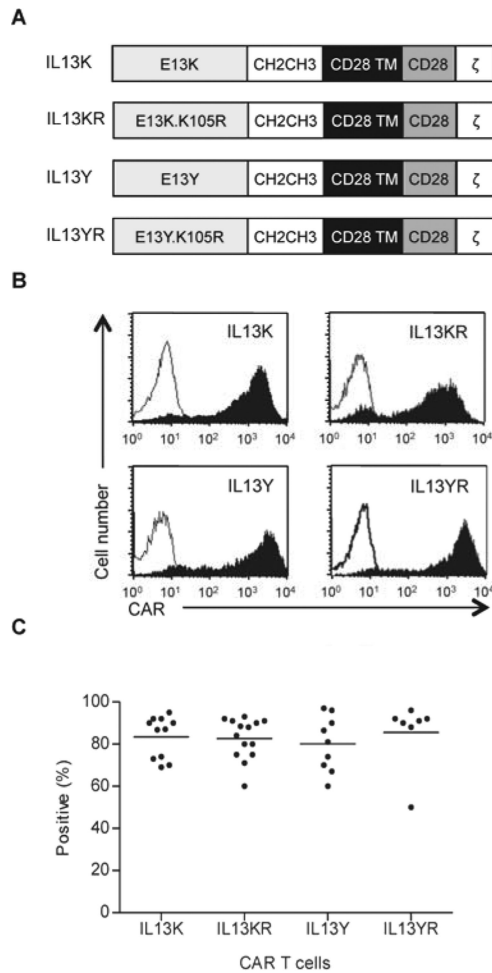


Figure 1. Generation of IL13 mutein-CAR T cells

(A) Scheme of IL13 mutein-CARs. All four constructs consisted of an IgG1-CH2CH3 domain, a CD28 transmembrane (TM) domain, and costimulatory domains derived from CD28 and the CD3 ζ-chain. The binding domain contained different IL13 muteins with one (E13K or E13Y) or two amino acid substitution (E13K.K105R; E13Y.K105R). (C,B) CAR expression was confirmed using FACS analysis. Representative plots (B) and summary data (C) is shown (mean 80.2% – 85.6%, n=7-14 per CAR construct).

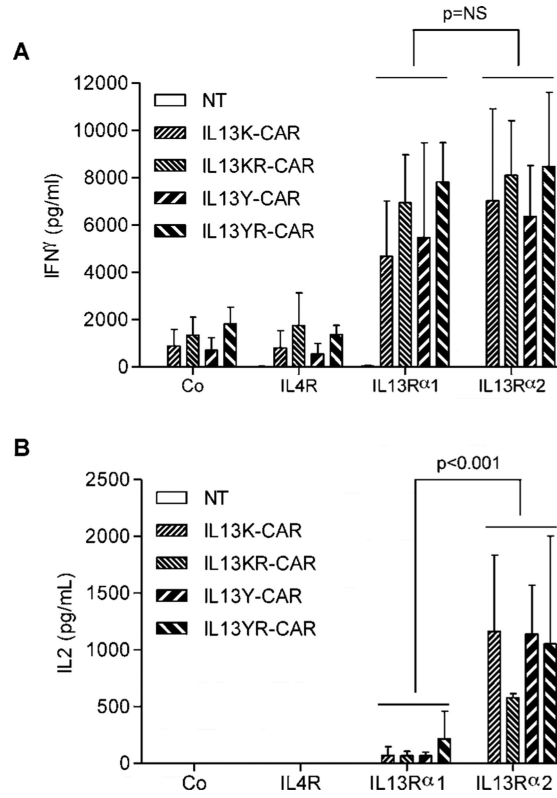


Figure 2. IL13 mutein-CAR T cells release cytokines after stimulation with recombinant IL13R α 1 and IL13R α 2 proteins
 IL13 mutein-CAR or non-transduced (NT) T cells were stimulated with recombinant IL13R α 1, IL13R α 2, or IL4R proteins. After 24h IFN γ (A) or IL2 (B) was measured by ELISA (n=3). T cells expressing all four CAR constructs expressed significant levels of IFN γ (p<0.001) when stimulated with recombinant IL13R α 2 or IL13R α 1 proteins in comparison to IL4R stimulated T cells. Only stimulation with recombinant IL13R α 2 protein induced significant levels of IL2 (p<0.001).

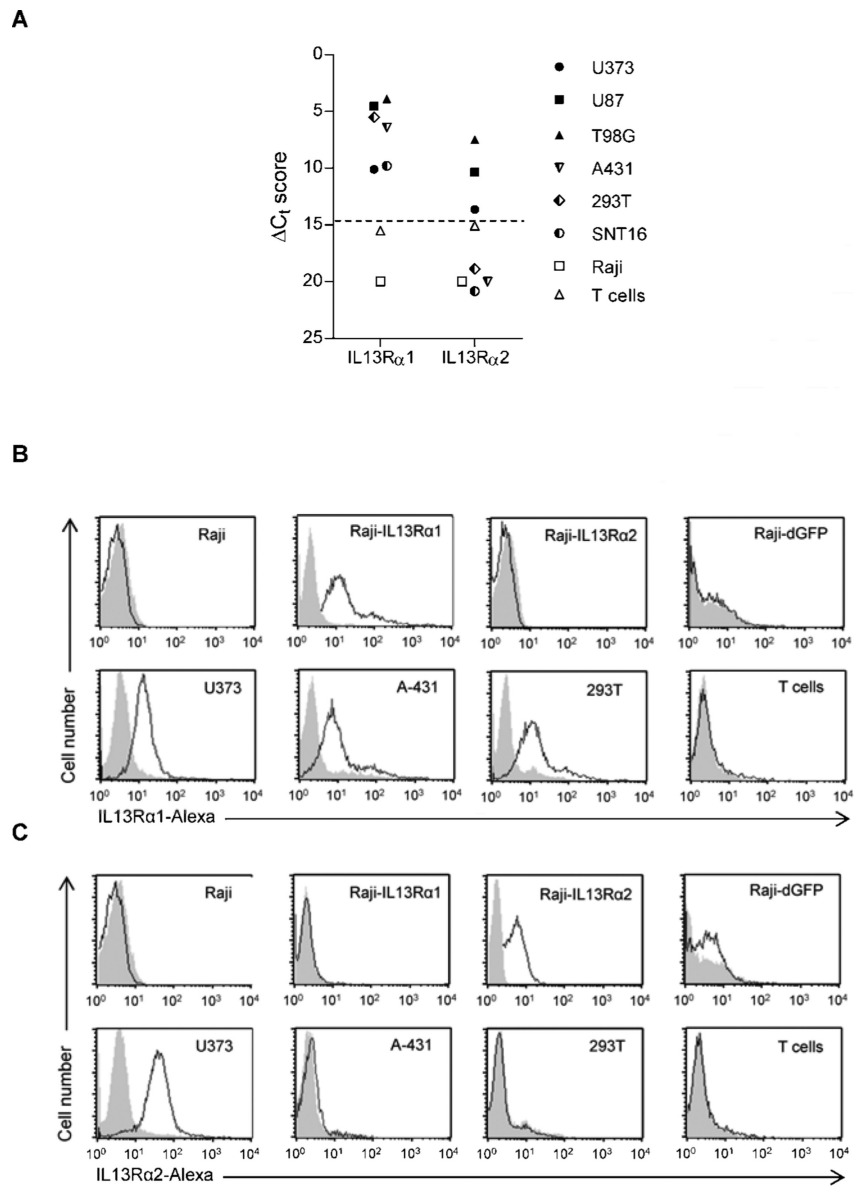


Figure 3. Expression of IL13R α 1 and IL13R α 2 in cell lines
 (A) qRT-PCR for IL13R α 1 or IL13R α 2. FACS analysis for IL13R α 1 (B) or IL13R α 2 (C).

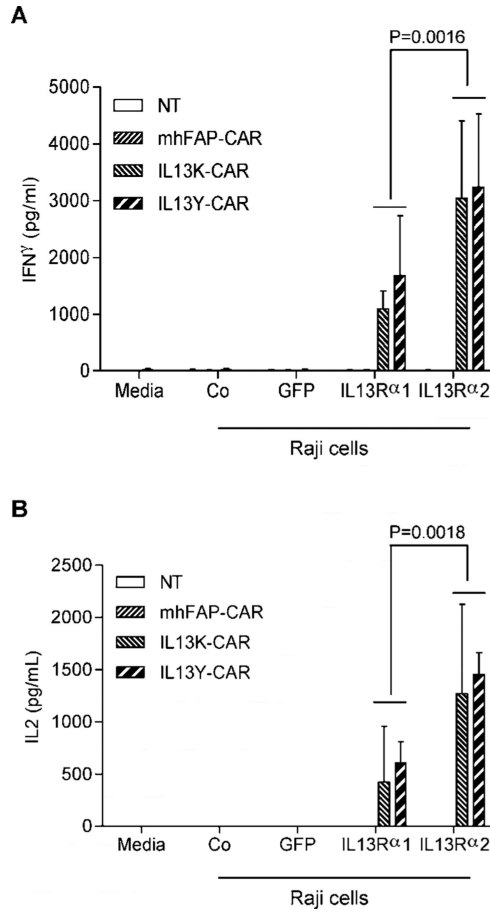


Figure 4. IL13 mutein-CAR T cells recognize IL13R α 1- and IL13R α 2-positive cells (A,B) IL13 mutein-CAR T cells (IL13Y-CAR, IL13K-CAR) were cocultured with genetically modified Raji cells expressing IL13R α 1, IL13R α 2 or GFP, or unmodified Raji cells (Co) at a 1:4 E:T ratio. NT and mhFAP-CAR T cells served as controls. After 24h IFN γ (A) or IL2 (B) was measured by ELISA (n=3). Only stimulation of IL13 mutein-CAR T cells with Raji-IL13R α 1 or Raji-IL13R α 2 cells resulted in significant production of IFN γ (p<0.001) and of IL2 (p<0.001). Raji-IL13R α 2 cells induced significant higher levels of IFN γ (p=0.0016) and IL2 (p=0.0018) in comparison to Raji-IL13R α 1 cells.

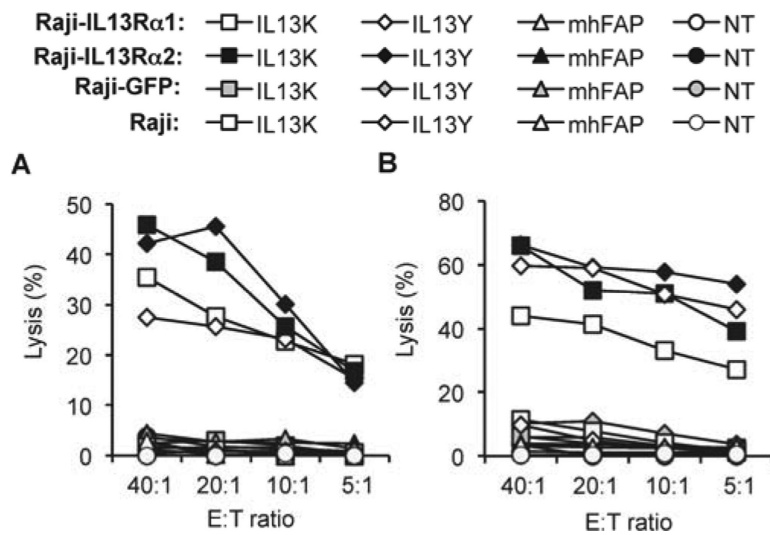
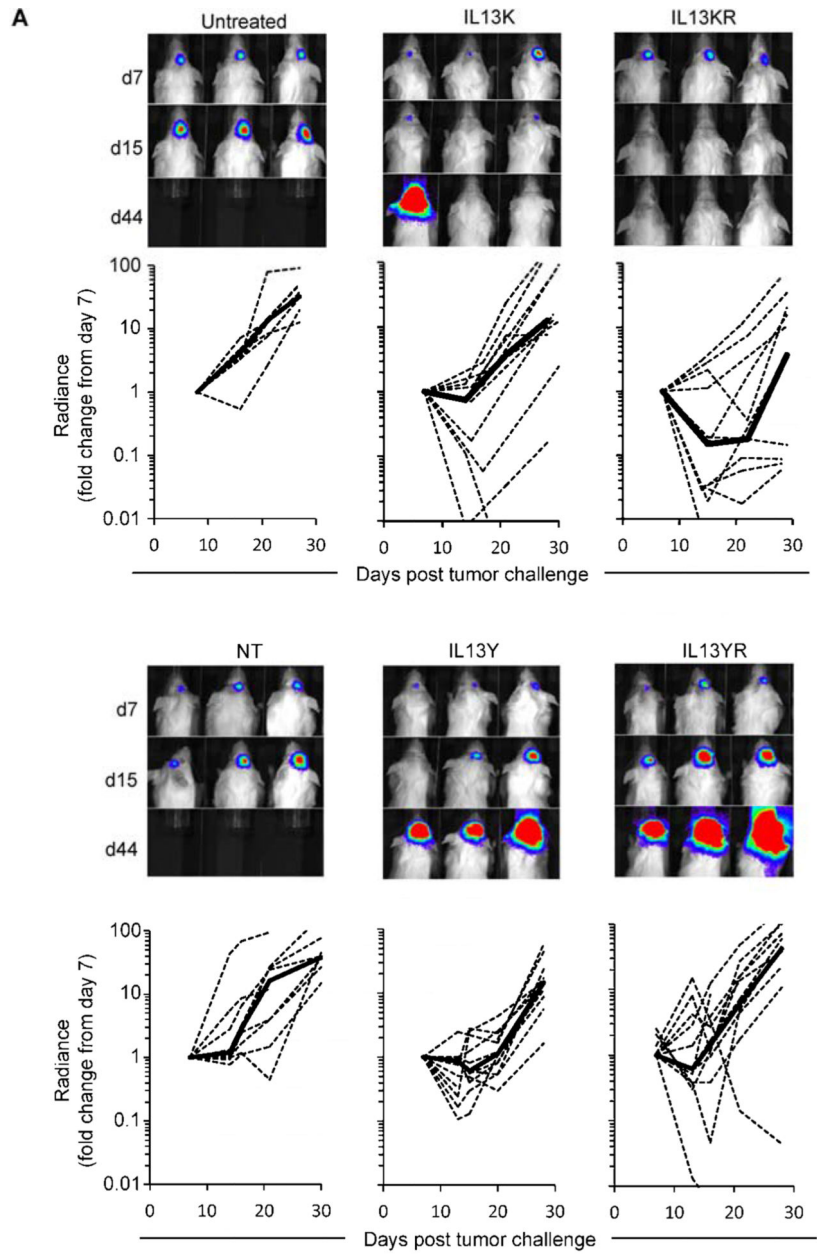


Figure 5. IL13 mutein-CAR T cells kill IL13R α 1- and IL13R α 2-positive cells
(A,B) IL13 mutein-CAR T cells (IL13Y-CAR, IL13K-CAR) killed Raji-IL13R α 1 and Raji-IL13R α 2 cells in contrast to Raji and Raji-GFP cells in a standard 4h cytotoxicity assay. Controls (NT T cells or mhFAP-CAR T cells) exhibited no cytolytic activity (results for 2 donors are shown).



B

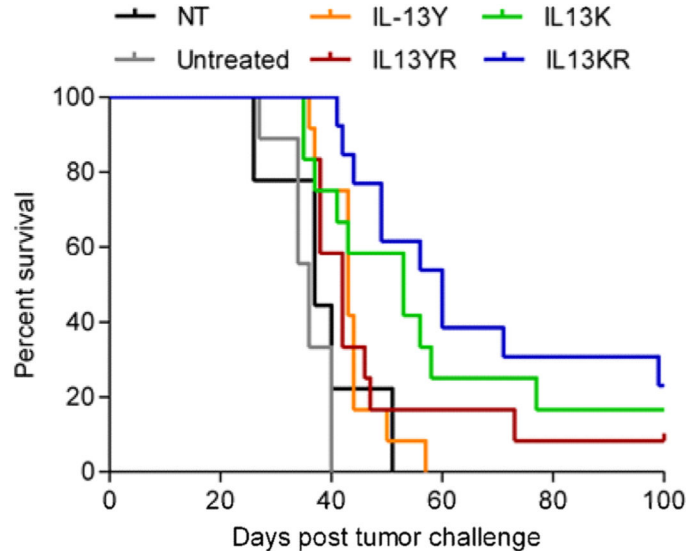


Figure 6. Treatment of glioma xenograft with T cells expressing IL13 mutein-CARs with E13K amino acid substitutions results in improved overall survival
 U373 glioma bearing mice were treated on day 7 with IL13K- (n=11), IL13Y- (n=11), IL13KR- (n=11), or IL13YR-CAR (n=11) T cells. Untreated mice (n=10) or mice treated with NT T cells (n=9) served as controls. (A) Representative images for each group and quantitative bioluminescence (radiance = photons/sec/cm²/sr) imaging data for all mice are shown (dotted lines: individual mice; solid lines: median). (B) Kaplan-Meier survival analysis (NT-T cells vs IL13K-CAR T cells, p=0.009; NT-T cells vs IL13KR-CAR T cells, p<0.001; NT-T cells vs IL13Y-CAR T cells, p=0.322; NT-T cells vs IL13KR-CAR T cells, p=0.253).