Cancer Immunol Immunother (2002) 51: 417–423
DOI 10.1007/s00262-002-0301-7

ORIGINAL ARTICLE

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Comparison of the TCR ζ-chain with the FcR γ-chain in chimeric TCR constructs for T cell activation and apoptosis

Received: 13 April 2002 / Accepted: 1 May 2002 / Published online: 23 July 2002

Abstract A promising strategy for cancer treatment is adoptive gene therapy/immunotherapy by genetically modifying T cells with a chimeric T cell receptor (cTCR). When transduced T cells (T-bodies) specifically bind to tumor antigens through cTCR, they will become cytotoxic T lymphocytes (CTL) and lyse the tumor cells in a non-major histocompatibility complex (MHC)-restricted manner. Both the FcR γ-chain and the TCR ζ-chain have been used to construct such cTCR, and both have shown specific cytolytic functions against tumor cells. However, most researchers believe that the ζ-chain generates stronger cytolytic activities against tumor than the γ-chain and therefore would be a better candidate for cTCR construction. On the other hand, because of the lack of costimulation signaling in such constructs, the T-body might cause activation-induced T cell death (AICD) when bound to tumor antigens. Therefore, one can argue that the γ-chain might generate less AICD than the ζ-chain because the γ-chain has only one immunoreceptor tyrosine-based activation motif (ITAM), and the cytolytic activities can be therefore recycled. Two cTCR, GAHγ and GAHζ, were constructed and evaluated for cytokine production, specific cytolytic function and AICD in T-bodies after exposure to tumor cells. Using EGP-2-positive LS174T colorectal carcinoma cells as targets, there was no substantial difference observed between a γ-chain or ζ-chain as the T-body signaling moiety in terms of specific cytolytic functions and induced cytokine production. This paper also demonstrates that, in the absence of a costimulation system, tumor antigen may not trigger apoptosis of T cells transduced with a cTCR carrying either an FcR γ-chain or a TCR ζ-chain. These observations challenge current ideas about the role of ITAM in T cell activation.

Keywords Adoptive cancer immunotherapy · Chimeric T cell receptor · FcR γ-chain · T-body · TCR ζ-chain

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Introduction

A promising cancer gene therapy approach is to transduce T cells with a chimeric T cell receptor (cTCR) so that transduced T cells can directly target and destroy tumor cells in a non-major histocompatibility complex (MHC)-restricted manner [2, 3, 5, 8, 14, 21, 23]. A T-body [8] is a T cell transduced with a cTCR comprised of a single chain fragment of the variable regions (scFv) of an antibody against a tumor-associated antigen (TAA). This scFv is attached to the intracellular portion of a T cell signaling chain, such as the Ig FcRI γ-chain (FcRγ) [6, 7, 24, 33, 34] or the T cell receptor ζ-chain (TCRζ) [7, 11, 12, 18, 21]. A hinge or spacer is often inserted between the scFv and signaling chain for more efficient binding of the scFv to the TAA [24].

The TAA epithelial glycoprotein-2 (EGP-2) is overexpressed in a wide variety of human solid tumors, including pancreatic and colorectal carcinomas [4]. EGP-2 is also called EpCAM, CO17–1A and KSA [4]. EGP-2 has been used as a tumor marker to diagnose and define the stages of colorectal cancer progression [15, 22]. Adjuvant therapy with a monoclonal antibody (mAb) directed against EGP-2 has been demonstrated to prolong remission and extend survival of post-operative colorectal cancer patients [26, 27].

We have constructed a cTCR molecule that includes an scFv based on the murine anti-EGP-2 mAb GA733.2 attached to an extracellular hinge that is connected to the FcR-γ cytoplasmic domain [24]. T cells were activated in vitro with immobilized anti-CD3 antibodies and transduced with the chimeric gene carried by a retroviral vector derived from the Moloney murine leukemia virus. T cells transduced with this cTCR recognize and specifically destroy EGP-2-positive tumor cells, which is evidenced by cytotoxicity assays and increased cytokine production [6, 24].

The FcR γ-chain is structurally homologous to the ζ-chain. Studies have shown that the genes for these two proteins have evolved from a common ancestor by duplication [16]. In an in vitro model using neutrophils and natural killer (NK) cells, the ζ-chain triggers significantly more antigen-specific cytolytic function than the γ-chain [28], and is therefore often preferred in cTCR construction [21]. It is, however, not known if the expression of the ζ-chain results in more activation-induced T cell death (AICD) than the γ-chain following tumor antigen stimulation. The three immunoreceptor tyrosine-based activation motifs (ITAM) present in the ζ-chain, compared to the single ITAM in the γ-chain, might mediate greater AICD. In the present study, we compared these two signaling chains for their ability to induce cytokine production, specific cytolytic functions and AICD in transduced T cells following tumor cell exposure.

The clinical efficacy of T-bodies could depend upon the ability of these cells to recycle their lytic activity against multiple tumor targets. Since tumor cells may cause Fas/FasL-mediated apoptosis upon specific MHC class I-restricted recognition [10, 38], a major concern is that the tumor antigen may induce AICD or anergy by activating the T-body [13]. It is not clear whether non-MHC-restricted tumor cell recognition by T cells that carry tumor-specific cTCR without costimulation will induce apoptosis of transduced T cells upon tumor cell exposure. Therefore in the present work we also studied activation-induced T-body apoptosis and FasL expression following tumor cell exposure.

When using T-bodies in clinical trials, it is very important to determine the fate of the T-body following single or repeated tumor cell exposure. We have observed that specific tumor targets actually stimulated GAHγ T-body activation after a second tumor cell exposure [25]. The data presented in this report should provide additional support for using T-bodies with either the ζ-chain or the γ-chain in cancer immunotherapy clinical trials.

Materials and methods

Cell lines

LS174T (EGP-2 positive), H716 (NCI H716, EGP-2 negative), MvLu1 and PG13 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). The MvLu1 cell line was used for titering retrovirus and was cultured in MEM (Gibco, Gaithersburg, Md.) containing 10% fetal bovine serum (FBS; Hyclone, Logan, Utah). PG13 is a MMLV packaging cell line cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% heat-inactivated FBS and 2 mM L-glutamine (Gibco). LS174T and H716 cells are EGP-2 positive and negative cell lines, respectively, and were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, Md.) with 10% FBS and 2 mM L-glutamine and grown at 37°C with 5% CO_2.

Retroviral vectors

Construction of the recombinant retroviral vectors pSAM and pSAM-GAHγ has been described previously [24]. The ζ-signaling chain coding region was generated by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and primers 5'-CTGCTGGATCCCAAACTCTGCTACC-3' (sense) and 5'-TACTCGAGCTGTTAGCGAGGGGGC-3' (antisense), digested with *Bam*HI and *Xho*I. The *Bam*HI-*Xho*I γ-fragment of pSAM–GAHγ was then removed and replaced with the *Bam*HI-*Xho*I ζ-fragment, which, analogous to the γ-construct, encodes the transmembrane and cytoplasmic residues of the TCRζ-chain (Fig. 1). Correct ζ-sequence and reading frame were confirmed by DNA sequencing in both directions (UWM Dept of Biological Sciences, DNA Sequencing Service, Milwaukee, Wis.).

Production of recombinant retroviruses

High-titer retrovirus stocks were generated based on a previously described protocol [24] with some modifications. Briefly, the selected high titer PG13 clone was cultured in DMEM containing 10% FBS. When the cells reached 100% confluence, the DMEM was replaced with X-vivo 15 serum-free medium (BioWhittaker) and cultured at 32°C with 5% CO_2. Viral supernatant was collected every 24 h, filtered through a 0.2-μm pore-size filter and stored at –80°C. The virus titers were evaluated on MvLu1 target cells selected with G418 (800 μg/ml) [24]; viral titers in all studies ranged from 4×10^5 to 1×10^6 colony-forming units/ml (CFU/ml).

Preparation of activated T cells and gene transduction

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway). To activate T

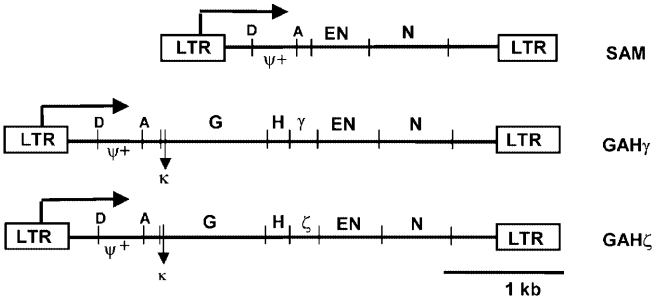

Fig. 1. Depictions of three recombinant retroviral vectors used in the present studies. The SAM vector contains long terminal repeats (*LTR*) derived from the Moloney murine leukemia virus and upstream splice donor (*D*) and splice acceptor (*A*) sites. ψ^+ indicates the packaging signal. The GAHγ vector has an insert composed of the leader sequence of the SC15κ light chain (κ), the scFv region (*G*) of murine GA733.2 mAb, the hinge (*H*) derived from human CD8α Ig, and the transmembrane/cytoplasmic domain of human FcR γ-chain. Downstream are the internal ribosome entry site (*EN*) and the neo^r gene (*N*). In the GAHζ vector, the γ-chain was replaced with the transmembrane/cytoplasmic domain of the human TCR ζ-chain

cells, $CD3^+$ T cells were cultured in 24-well plates that were pre-coated with an anti-CD3 mAb (OKT3, Ortho Pharmaceuticals, Raritan, N.J.; 1 μg/ml per well). The cultures were set up at a concentration of 2×10^6 $CD3^+$ T cells/2 ml per well in X-vivo 15 medium containing 5% human serum (BioWhittaker), and 100 IU/ml recombinant human IL-2 (Chiron, Emeryville, Calif.). After 48 h of culture, the activated T cells (ATC) were transduced with the recombinant retrovirus as follows: ATC (5×10^5) in 0.5 ml medium were placed in each well of a 24-well non-tissue-culture-treated plate (Becton Dickinson, Franklin Lakes, N.J.) that was pre-coated with RetroNectin (RN; Takara Shuzo, Japan). For RN precoating, the plate was treated with 2 μg/ml per well of RN for 24 h at 4°C and washed twice with 2 ml of PBS. The retrovirus supernatant (1.5–2.3 ml) was added to the ATC at a multiplicity of infection (MOI) of 2, and supplemented with 100 IU/ml of IL-2 and 8 μg/ml of protamine sulfate. The cells and virus were centrifuged at 650 *g* for 20 min at 30°C followed by overnight incubation at 37°C. The next day, the cells were pelleted and the transduction process was repeated in the same RN-coated dish. Cultures were subsequently maintained at 1×10^6 cells/ml in X-vivo 15 plus 5% human serum supplemented with 100 IU/ml of IL-2 and fed by replacing half of the culture volume with fresh medium plus 200 IU/ml of IL-2 every 2 days. The transduced T cells were selected for with G418 (400 μg/ml) for 7 days.

Analysis of transduction efficiency

Retroviral vector transduction efficiencies were determined by using G418 selection and by comparative PCR analysis using neo^r-positive reference standards, as previously described [24] with one modification: quantitated increasing amounts of linearized neo gene-containing plasmid DNA were mixed with the lysate from 2,000 T cells to simulate different percentages of transduced cells. These samples were used as standards instead of using neo-transduced NIH 3T3 cell lysates (Fig. 2). For the G418 selection, equal amounts of transduced T cells were cultured in two wells, one with G418 (400 μg/ml) and one without G418. On day 7 post-transduction, the percentage transduction efficiencies was calculated by the fold expansion of the $G418^+$ group divided by the fold expansion of the $G418^-$ group using the following formula:

$$\frac{\text{fold expansion of the G418}^+\text{ group}}{\text{fold expansion of the G418}^-\text{ group}} \times 100$$

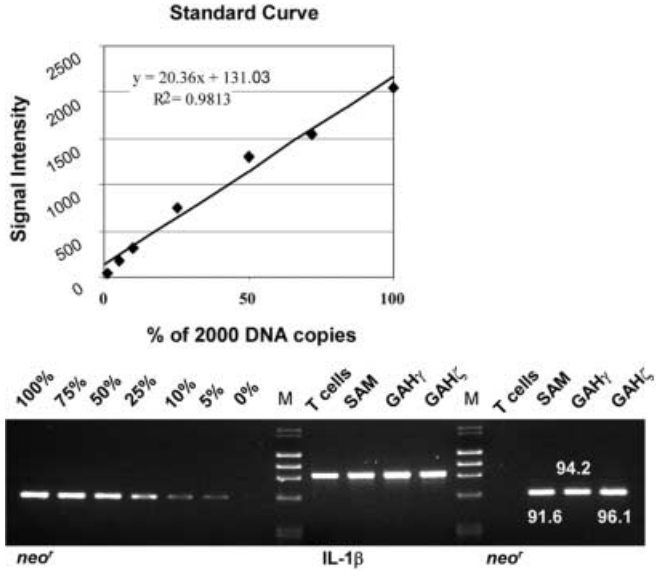

Fig. 2. Semi-quantitative PCR analysis of transduction efficiencies. Primers specific for the neo^r gene (PCR product of 610 bp) were used for amplifying the transduced gene. Primers for the IL-1β gene were used as a normalizing control for cell-equivalent amounts of lysate added to each PCR reaction. The PCR products were separated by agarose gel electrophoresis and analyzed using a Gel-Doc 2000 system (Bio-Rad, Hercules, Calif.). In this assay, the transduction efficiency in *SAM* was 91.6%; in *GAH*γ 94.2%, and in *GAH*ζ, 96.1%. *M* indicates molecular size markers

Cytotoxicity assays

The cytotoxicity of day 14 T-bodies against tumor targets was measured using a ^{51}Cr-release assay. LS174T target cells were plated in triplicate wells at 4×10^4 cells per well in a flat-bottomed 96-well tissue culture dish. Following overnight incubation at 37°C, the cells were labeled with 2 μCi/well of ^{51}Cr ($Na_2{}^{51}CrO_4$, 37 MBq/ml; Amersham Life Science, Arlington Heights, Ill.) in 100 μl RPMI/10% FBS medium for 2 h at 37°C. The adherent cells were then gently washed three times with warmed medium. For non-adherent H716 cells, 1×10^6 cells were labeled with ^{51}Cr for 1 h, washed three times, and plated in triplicate at 2×10^4 cells per well in a V-bottomed 96-well dish. The T-bodies were added to target cells in 200 μl of 100% fresh X-vivo 15 containing IL-2 (100 IU/ml) and 5% human serum at the appropriate effector:target (E:T) ratios. Four-hour assays were carried out as described previously [24].

Cytokine production assays

T-bodies selected in G418 were transferred to 100% fresh medium containing IL-2 (100 IU/ml), and applied to tumor targets at an E:T ratio of 5:1 (Fig. 3). After a 24-h incubation period, cells were centrifuged at 450 *g* for 5 min. Supernatants were analyzed for human interferon-γ (IFN-γ) production using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minn.). Results are expressed as mean values of triplicate determinations (pg cytokine/ml per 10^6 T-bodies).

Phenotype analysis

Monoclonal antibodies conjugated with different fluorochromes including fluorescein isothiocyanate, phycoerythrin, allophycocyanin and peridinin chlorophyll protein (Becton Dickinson, San Jose,

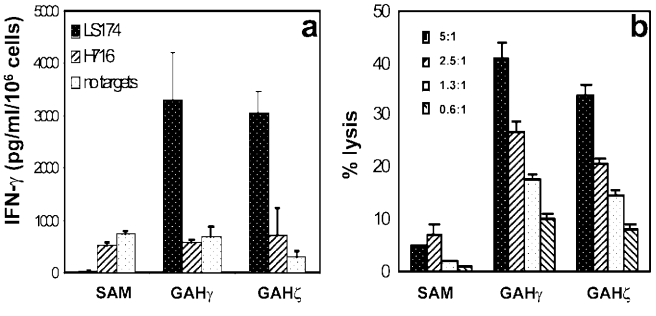

Fig. 3A, B. Comparison of cytokine production and cytolytic activity of GAHγ and GAHζ T-bodies. In these experiments day 14 T-bodies were used. **A** IFN-γ production on EGP-2-negative targets (H716) and EGP-2-positive targets (LS174T) at an E:T ratio of 5:1 for 24 h at 37°C. Supernatants (50 μl) were collected for ELISA in triplicate. **B** T-body cytolytic assays using LS174T cells as targets at the indicated E:T ratios, carried out in triplicate. Lysis of ^{51}Cr-labeled LS174T cells was determined after incubation for 4 h at 37°C. The SD of triplicate determinations are indicated in both panels. Experiments were performed three times using three different donors; a representative experiment has been shown

Calif.) directed against various cell surface markers were used to stain the T-bodies. Cells were evaluated for the expression of CD3, CD4, CD8, CD14, CD19, CD25, CD69, CD95, and CD95L. Apoptosis was measured using an annexin V–PE apoptosis kit I (Pharmingen, San Diego, Calif.). As a positive control for apoptosis, cells were pre-incubated in 120 μM camptothecin (Sigma, St. Louis, Mo.) for 4 h. Staining patterns were analyzed by flow cytometry on a FACSCalibur flow cytometer using CellQuest (Becton Dickinson, San Jose, CA) and WinMidi software (Joseph Trotter, Scripps, La Jolla, Calif.). Positive expression was indicated by the percentage of cells showing positive for a specific marker above isotype-negative staining.

Results

The cTCR bearing the FcR γ-chain or the TCR ζ-chain confer similar levels of specific cytotoxicity and IFN-γ production

T cells were transduced with three constructs, SAM, GAHγ and GAHζ, to compare the anti-tumor responses conferred by the two signaling chains. Fig. 3 shows representative data on IFN-γ production and cytolytic activity of transduced T cells from one of the three healthy donors that were evaluated. Panel A of Fig. 3 shows the IFN-γ production by T-bodies which were cultured for 14 days and then exposed to tumor targets at an E:T ratio of 5:1 for 24 h. Both GAHγ and GAHζ T-bodies showed increased IFN-γ production of about 3- to 5-fold upon exposure to the EGP-2-positive LS174T cells compared to exposure to the EGP-2-negative H716 cells or no tumor cell exposure. T cells transduced with the SAM vector served as a control. These results demonstrate that both GAHγ and GAHζ T-bodies produce high levels of IFN-γ when exposed to EGP-2-positive LS174T cells, but not when exposed to EGP-2 negative H716 cells, and that there was no distinguishing difference between GAHγ and GAHζ T-bodies in terms of IFN-γ production. All three T-body groups were 90% to 100% transduced based on the semi-quantitative PCR assays and were G418-selected.

Panel B of Fig. 3 shows data from a 4-h cytolytic function assay using ^{51}Cr-labeled LS174T cells as targets. There was no clear difference between the GAHγ and GAHζ T-bodies in terms of their lytic function from one donor to another (data not shown). The percentage lysis of EGP-2$^-$ H716 cells was $< 10\%$ at an E:T ratio of 5:1 (data not shown) in both GAHγ and GAHζ groups.

There was no increased apoptosis or CD95L overexpression detected in T-bodies following tumor cell exposure.

We studied whether apoptosis occurred in T-bodies following tumor cell exposure and whether the GAHζ group generated more apoptosis that the GAHγ group. Three constructs, SAM, GAHγ and GAHζ, were used for these experiments. Fig. 4 shows the percentage apoptotic cells of these T-bodies in the presence or absence of tumor cells from four individuals. The control "tumor–" group was never exposed to tumor cells and the "tumor+" group was exposed to tumor cells for 24 h prior to the assay. For a positive control, non-transduced T cells were pre-incubated with 120 μM camptothecin for 4 h before staining with annexin V, and untreated T cells served as a negative control. Fig. 4 shows that tumor cell exposure did not induce T-body apoptosis even though 3- to 5-fold higher IFN-γ production was observed at 24 h (Fig. 3) and 100% tumor lytic activity was evident at the same time and at the same E:T ratio (data not shown). Interestingly, GAHζ T-bodies, with three ITAM in the signaling chain, did not undergo more apoptosis than the GAHγ T-bodies that had one signaling chain ITAM.

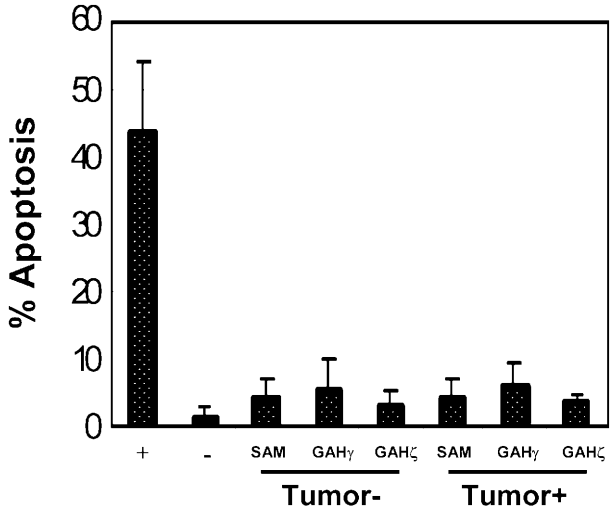

Fig. 4. Specific apoptosis of GAHγ and GAHζ T-bodies following tumor cell exposure to LS174T cells at an E:T ratio of 5:1 for 24 h at 37°C. As a positive control (+), T cells were treated with 120 μM camptothecin (CT) for 4 h at 37°C; T cells incubated in the absence of CT and tumor served as a negative control (–). SD are indicated for results concerning four individuals

To provide further evidence that the exposure of both GAHγ and GAHζ T-bodies to tumor does not cause apoptosis, CD95L (FasL) expression was determined by flow cytometry with an anti-CD95L antibody. Expression of CD95L plays a critical role in TCR-mediated AICD [1, 19]. Fig. 5 shows T cells activated with immobilized anti-CD3 antibody as a positive control for CD95L (FasL) expression. Shown are data from one of two similar experiments using T cells from two healthy donors. The results indicate that tumor cell exposure did not stimulate CD95L expression (SD ≤ 5%); in either GAHγ or GAHζ T-bodies while over 20% of the cells expressed CD95L when T-bodies were simulated with immobilized anti-CD3. Fas expression in T-bodies was also examined at the same time. There was no increased Fas expression detected following tumor cell exposure (data not shown). It is not clear why there was less FasL upregulation in anti-CD3 treated non-chimeric control T cells than in T-bodies (Fig. 5). One explanation could be that T cells had never left their "conditioning medium" that may contain certain elements that prevent FasL expression and T cell apoptosis.

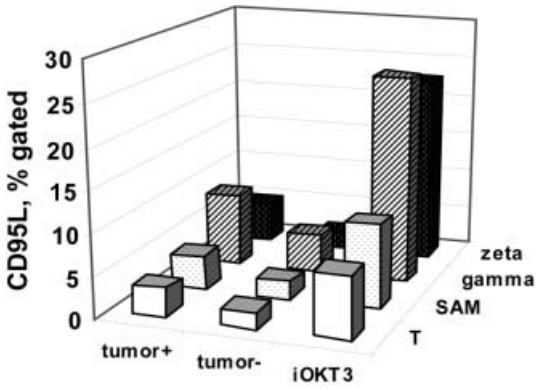

Fig. 5. FasL (CD95L) expression following tumor cell exposure. T-bodies were exposed to tumor cells at an E:T ratio of 5:1 on day 14 for 24 h (tumor+) or not exposed as a negative control (tumor–). The gamma is GAHγ and the zeta is GAHζ T-bodies, T-bodies were plated on anti-CD3 mAb (iOKT3) coated plates for 24 h at 37°C. Only CD3-positive T cells were gated for evaluation of CD95L expression. Shown is one representative experiment of two using two different donors

We also observed increased CD25 and CD69 expression and proliferation of T-bodies in three donors (data not shown), which further suggested that tumor binding might promote T-body activation.

Discussion

Compromised immunity against tumors, including defects in T-cell signaling, can result in the growth of tumors. Adoptive immunotherapy with T-bodies, could overcome the limitations associated with insufficient numbers or ineffective responses of endogenous tumor-specific T cells [37]. Clinical studies have shown that the presence of T-bodies can be detected at 1 year after infusion [20]. However, using the FcR γ-chain or TCR ζ-chain for a cTCR construct has been a controversial topic. Our prediction was that the γ-chain might be a better candidate for signaling T cell activation than the ζ-chain because T cell activation through the TCR ζ-chain might result in AICD and prevent the recycling of T-bodies in future clinical applications, even though the ζ-chain may produce more T cell activation due to the three ITAM which could be more effective in generating cytotoxic activity. Surprisingly, both the γ-chain and the ζ-chain generated similar amounts of T cell activation in our cytotoxicity assay and in IFN-γ ELISA. We were not able to measure the surface expression of either cTCR by flow cytometry. In theory, after G418 drug selection, 100% of the T cells used for these experiments should carry the transduced gene and should express the cTCR. It is also possible that the gene expression levels in these two T-body groups may vary.

Our previous data [24, 25] and the results of others have shown that T-bodies can produce specific cytotoxic function upon binding to a tumor target. The fate of T-bodies after killing tumor remains one of the major concerns for future clinical applications, because reactivation of the T-body (or repeated tumor cell exposure) through the cTCR without the involvement of costimulation via CD28 might result in AICD or anergy mediated by the ITAM of the TCR ζ-chain [9, 13]. Therefore, a logical speculation is that the γ-chain may produce less – at least three-fold less – AICD. It was very interesting to observe in our experiments that neither of these two signaling chains induced any measurable AICD. In AICD assays, both GAHγ and GAHγ T-bodies did not undergo apoptosis after killing LS174T tumor cells, even though LS174T cells express high levels of FasL [30]. Previous studies have also shown that, without the presence of co-stimulation, cytotoxic T cells could be generated by exposure to tumor antigen which did not undergo apoptosis [31]. A possible explanation for the apoptosis resistance of the T-body following tumor cell exposure involves the concept of activation thresholds [17, 32]. The same signals promoting T cell activation and differentiation into effector and memory cells are also responsible for AICD. Different thresholds of TCR signaling generated by different stimuli might be involved in deciding the life or death of these T cells. Based on the hypothesis of Matshui et al. [17], there are two thresholds of T cell activation. When moderately stimulated, T cells can be activated to the low (1st) threshold that permits the expression of components that up-regulate the immune response. A strong and concurrent stimulation through two or more receptors can result in T cells being activated to the high (2nd) threshold, which elicits the apoptosis of activated T cells by the induction of FasL.

The TCR number, not the nature of the triggering ligand, plays a critical role in the threshold of T cell activation [29, 32]. Our GAHγ cTCR expression in T cells was very low, not detectable by flow cytometry with an anti-Id-antibody against GA733.2-scFv, as has also been reported by Daly et al. [6] using the same scFv. However, it can be detected by a tumor-specific lytic assay using high-EGP-2-expressing LS174T tumor targets. We hypothesize that, due to the low number of cTCR on the T cell surface, overexpressed EGP-2 antigen on tumor cells may not be able to generate the 2nd threshold of activation and therefore may not cause T-body apoptosis. However, our results did not rule out the possibility that, due to very low cTCR expression, possible differences in the activities of these two cTCR could not be detected.

To study the functional balance between T-body expression and TAA density, Weijtens et al. [34, 35] found that the low expression of their cTCR, which was also undetectable by flow cytometry, was efficient in allowing the triggering of the T-bodies' cytolytic function by high density TAA, but not by low density TAA. These results may benefit the clinical application of our GAH T-bodies because there is a low amount of EGP-2 expression on normal epithelial tissues [4], and they may be potential T-body targets. The low level of cTCR expression in our GAHγ and GAHζ T-bodies may serve to

further reduce the likelihood that they would attack normal epithelial tissues expressing low EGP-2 levels, targeting only high-EGP-2-expressing tumor cells.

The ζ-chain alone is sufficient for primary T cell activation and functional maturation, and is capable of activating both memory and naive T cells, as well as promoting T cell differentiation into cytotoxic T cells (CTL), T helper cells 1 (TH1) and TH2 cells [11]. The ζ-chain of the TCR is primarily associated with T cells and the FcRI γ-chain is primarily associated with neutrophils and macrophages [28]. The ζ-chain is more effective than the γ-chain at triggering NK- and neutrophil-mediated cytolysis in vitro [28]. Because there are three ITAM in the TCR ζ-chain while there is only one ITAM in the FcRI γ-chain [36], many researchers have preferred to use the ζ-chain for T-body construction and have suggested that the ζ-chain generates stronger cytolytic function than the γ-chain [18, 21]. According to previously published work, TCR/FcRI-γ T cells are capable of undergoing AICD, but its induction requires 10 times as much anti-CD3 mAb as that required for AICD of wild-type T cells [29]. To study the possible AICD and cytolytic function induced by ITAM, we compared the TCR ζ-chain and FcRI γ-chain. We observed that both chains triggered significant specific cytotoxic activities, but we did not observe any clear difference in terms of their activation strength or cytolytic efficiency. The level of apoptosis did not differ greatly between antigen-stimulated T cells bearing either of the two cTCR; this was further supported by preliminary semi-quantitative RT–PCR experiments which suggested that *bcl*–X_L expression levels in the two T-bodies were not significantly different after stimulation (data not shown). The GAHζ T-bodies did not produce three-fold higher lytic function or apoptosis in comparison to the GAHγ T-body, as has been observed by others in NK cells and neutrophils [28]. Both T-bodies displayed similar levels of lytic activity, and neither underwent apoptosis following tumor cell exposure. Our results may support the hypothesis that AICD of mature T cells is not dependent upon the number of ITAM, but is probably dependent on the level of surface TCR, as has been hypothesized by others [29].

LS174T cells are FasL-expressing targets [30]. It has been reported that the co-cultivation of FasL-expressing cells with Jurkat T cells results in Fas–FasL-mediated T cell apoptosis [30]. We did not observe any measurable increase of FasL expression in the T-bodies following tumor exposure (Fig. 5). These observations support our previous hypothesis that tumor re-exposure actually promotes T-body activation, resulting in increased recycled cytolytic function, cytokine production and T-body proliferation [25].

Acknowledgements This work was supported by the Vince Lombardi Trust Fund, the Northwestern Mutual Foundation and St. Luke's Medical Center. We thank J. Treisman for providing the original pSAM-GAγ-EN vector; K. Trevor and N. Garlie for scientific discussions, and M. Wing for technical support in cloning the ζ-chain.

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