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A chimeric antigen receptor for TRAIL-receptor 1 induces apoptosis in various types of tumor cells

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ABSTRACT

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and its associated receptors (TRAIL-R/TR) are attractive targets for cancer therapy because TRAIL induces apoptosis in tumor cells through TR while having little cytotoxicity on normal cells. Therefore, many agonistic monoclonal antibodies (mAbs) specific for TR have been produced, and these induce apoptosis in multiple tumor cell types. However, some TR-expressing tumor cells are resistant to TR-specific mAb-induced apoptosis. In this study, we constructed a chimeric antigen receptor (CAR) of a TRAIL-receptor 1 (TR1)-specific single chain variable fragment (scFv) antibody (TR1-scFv-CAR) and expressed it on a Jurkat T cell line, the KHYG-1 NK cell line, and human peripheral blood lymphocytes (PBLs). We found that the TR1-scFv-CAR-expressing Jurkat cells killed target cells via TR1-mediated apoptosis, whereas TR1-scFv-CAR-expressing KHYG-1 cells and PBLs killed target cells not only via TR1-mediated apoptosis but also via CAR signal-induced cytolysis, resulting in cytotoxicity on a broader range if target cells than with TR1-scFv-CAR-expressing Jurkat cells. The results suggest that TR1-scFv-CAR could be a new candidate for cancer gene therapy.

1. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its associated receptors (TRAIL-R/TR) are attractive targets for cancer therapy because TRAIL induces apoptosis in tumor cells through TR while having little cytotoxicity on normal cells *in vitro* or *in vivo* [1–3]. TRAIL-receptor 1 (TR1/DR4) and TRAIL-R2 (TR2/DR5) contain a cytoplasmic death domain that triggers apoptosis upon TRAIL-binding, whereas TRAIL-R3 (TR3/DcR1) and TRAIL-R4 (TR4/DcR2) lack such a functional death domain [2]. The binding of TRAIL to TR1 or TR2 results in conformational changes in the receptors, which trigger an apoptotic signal [2]. In this context, many agonistic monoclonal antibodies (mAbs) specific for TR1 or TR2 have been produced to induce apoptotic cell death in multiple tumor cell types but not in normal cells. However, not all TR-expressing cells are sensitive to the tumoricidal activities of TR1- and TR2-specific mAbs [4,5]. Thus, agents that induce apoptosis in a broader range of TR-expressing tumors are desirable. Recently, we have developed ten fully human antibodies specific for human TR1 using a single lymphocyte analysis method [6,7]. The antibodies efficiently induced apoptosis in tumor cell lines in the presence of secondary antibody or recombinant TRAIL; however, they did not show significant tumoricidal activities alone. The results indicated that cross-linking and trimerization of TRs are a pre-requisite for bringing intracellular death domains into close proximity to induce apoptosis.

In addition to antibody therapeutics, adoptive transfer of genetically engineered (chimeric antigen receptor (CAR)-expressing) T cells is a promising immunotherapy [8,9]. CAR consists of the tumor antigen binding domain of a single-chain variable fragment (scFv) antibody fused to intracellular signaling domains capable of activating T cells upon antigen stimulation, a concept first reported by Eshhar and his colleagues in 1989 [10]. The design of CARs has evolved over the decades since their first description, with the goal of enhancing T cell signaling functions. In the first generation of CARs, the intracellular signaling domain was based on the CD3ζ chain and conferred upon the engineered T cells the ability to secrete cytokines and mediate the lysis of target cells. The second generation of CARs incorporated additional intracellular domains,

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usually from T cell co-stimulatory molecules such as CD28, resulting in enhanced cell proliferation upon contact with target antigens in addition to cytokine release and cell lysis. The third generation incorporated other signaling domains (i.e., 4-1BB or OX40) to improve effector cell function and survival [9].

In this study, we produced a CAR of a TR1-specific scFv antibody (TR1-scFv-CAR) with the aim of inducing efficient trimerization of TR1 with cell surface TR1-scFv-CAR, leading to the induction of tumor cell apoptosis. Furthermore, the cytoplasmic domain of the CAR is expected to induce signals of cytolytic activity in killer cells upon CAR-crosslinking with TR1 on the target cell surface. We expressed TR1-scFv-CAR on a Jurkat human T cell line, the KHYG-1 human NK cell line and peripheral blood lymphocytes (PBLs) and performed killing assays with several tumor cell lines. We found that TR1-scFv-CAR-expressing Jurkat cells killed target cells via TR1-mediated apoptosis, whereas the TR1-scFv-CAR-expressing NK cell line and PBLs killed target cells via CAR signal-induced cytolytic activity in addition to TR1-induced apoptosis.

2. Materials and methods

2.1. Vector construction

The TR1-scFv-CAR-expression vectors were constructed as previously described [11–13]. To construct the cytoplasmic domain of CAR, CD137 (4-1BB) cDNA was first isolated from human PBLs; human CD3ζ, CD8α and CD28 cDNAs were purchased from OriGene Technologies (Rockville, MD, USA). The CD137 and CD3ζ cytoplasmic domains were amplified by PCR and fused together (4-1BBcyto-CD3ζ). The CD8α hinge domain and CD28 transmembrane and cytoplasmic domains were amplified by PCR and fused together (CD8hinge-CD28tm-cyto). The cytoplasmic domain of CAR was then constructed by linking CD8hinge-CD28tm-cyto with 4-1BBcyto-CD3ζcyto by overlap PCR and inserting the product into the pMX vector (kindly provided by Dr. Kitamura, University of Tokyo) (pMX-CAR). The cloning of the TR1-specific antibody was described previously [6]. The TR1-specific antibody, TR404, heavy and light chain variable domains were amplified and linked with the common GS-linker (TR404-scFv). Then, the leader sequence of the human κ chain was linked with TR404-scFv by overlap PCR and cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) (L-TR404-scFv). Finally, L-TR404-scFv was linked to the CAR domain using the Gibson Assembly Cloning Kit (New England Biolabs, Tokyo, Japan) (TR404-scFv-CAR) and cloned into the pMX-IRES-GFP vector (kindly provided by Dr. Kitamura) (pMX-TR404-scFv-CAR). As a control, a CD8-CAR that consists of the CD8α extracellular domain and the cytoplasmic domain of CAR was constructed by amplifying and fusing fragments with PCR and the Gibson Assembly Cloning Kit, respectively.

2.2. Cell culture

Cells were maintained in either RPMI 1640 medium (for non-adherent cells) (Wako Pure Chemical Industries Ltd., Osaka, Japan) or Dulbecco's Modified Eagle Medium (for adherent cells) supplemented with 10% fetal bovine serum (Biowest, Nuaillè, France), 100 μg/ml streptomycin, and 100 U/ml penicillin. KHYG-1 cells were purchased from the Human Science Research Resources Bank (JCRB0156; Tokyo, Japan) and were cultured in RPMI 1640 medium containing 100 U/mL of recombinant human interleukin-2 (rhIL-2, Peprotech). JurkatΔβ cells were purchased from ATCC (Manassas, VA) and maintained in RPMI 1640 medium. CHO-S cells (Invitrogen, Carlsbad, CA) were cultured in FreeStyle CHO Expression Medium (Invitrogen) in the presence of 8% CO_2 in air with continual agitation in a Bio-Shaker (Titec, Saitama, Japan). TR404 antibodies were prepared as described previously [14].

2.3. Retroviral transduction

The pMX-TR404-scFv-CAR vector was transfected into Phoenix-A cells (kindly provided by Dr. G. Nolan, Stanford University) with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). The culture supernatant was collected 72 h after transfection. Transduction efficiency was monitored by eGFP expression.

2.4. Preparation of TR1-scFv-CAR-transduced cells

To establish stable expression of TR1-scFv-CAR in JurkatΔβ and KHYG-1 cells, the cells were retrovirally transduced with TR1-scFv-CAR. Briefly, wells in a 24-well plate were coated with 50 μg/ml retronectin (Takara Bio, Shiga, Japan), and TR1-specific scFv-CAR-encoding retrovirus was spin-loaded into the wells by centrifugation for 2 h at 1900*g* at 32 °C according to the manufacturer's instructions. In total, 5×10^5 cells in 1 ml of medium were added to the retrovirus-loaded well and spun down at 1000*g* at 32 °C for 10 min, followed by overnight incubation at 37 °C in 5% CO_2. GFP^+ cells were enriched by sorting with a FACSAria II (BD Biosciences, San Jose, CA). PBLs transduced with TR1-scFv-CAR were prepared as previously reported [15]. Briefly, 5×10^5 PBLs were stimulated *in vitro* with CD3/CD28 Dynabeads (Invitrogen) and 100 U/ml rhIL-2. On day 2, the stimulated PBLs were washed, and 5×10^5 cells in 1 ml of medium were added and spun down to the retrovirus-loaded well as described above. On day 3, the PBLs were transferred onto newly prepared retrovirus-coated plates as on day 2 and cultured with 100 U/ml rhIL-2. On day 10, the TR1-scFv-CAR-transduced PBLs were evaluated for the expression of the appropriate CAR by flow cytometry.

2.5. Flow cytometry

To examine TR1-scFv-CAR-expression on the cell surface, cells were incubated with 1 μg/ml recombinant TR1-Fc (R&D Systems, Minneapolis, MN) for 20 min on ice. The cells were then washed and stained with R-phycoerythrin (PE)-conjugated human IgG-specific antibody (Sigma) for 20 min on ice. After being washed, the cells were analyzed with a FACSCanto flow cytometer (BD Biosciences). PE-conjugated human CD137 (4-1BB)-specific antibody (BioLegend Inc., San Diego, CA) was used to analyze cell activation.

2.6. Killing activity assay

The cytotoxicity of the TR1-scFv-CAR-transduced cells was analyzed by the ^{51}Cr (PerkinElmer, Boston, MA) release assay. Briefly, target cells were loaded with ^{51}Cr for 60 min at 37 °C. Then, the target cells and TR1-scFv-CAR-transduced cells (effector cells) were plated in 96-well plates at the indicated effector-to-target (E/T) ratios and incubated for 4 h at 37 °C in humidified air containing 5% CO_2. After incubation, the supernatants were transferred to wells of LumaPlate (PerkinElmer) and dried. The fluorescence (F) was measured using a TopCount (Perkin Elmer). The percentage of cytotoxicity was calculated using the following formula: % lysis = (F experiment − F spontaneous)/(F maximal − F spontaneous) × 100. Assays were performed in triplicate.

2.7. Enzyme-linked immunosorbent assay (ELISA) of Granzyme B-secretion

In total, 1×10^5 TR1-scFv-CAR-transduced cells were co-cultured with 1×10^5 Colo205 cells. After incubation for 24 h, the supernatants were collected, and the amount of Granzyme B in

the supernatant was measured by ELISA (Mabtech AB, Nacka Strand, Sweden) according to the manufacturer's instructions.

3. Results

3.1. TR1-scFV-CAR construction and its cell surface expression

To examine whether the presence of TR1-scFv-CAR on cells can efficiently induce apoptosis in target tumor cells, we produced TR1-scFv-CAR constructs from the TR404 TR1-specific antibody, which was described previously [6,14] (Fig. 1A). We also produced CD8-CAR constructs as a negative control. We retrovirally transduced these CARs into JurkatΔβ human T cells, which did not express a TCR on the cell surface. Flow cytometry revealed that recombinant soluble human TR1 bound to the TR404-scFv-CAR-transduced JurkatΔβ (TR404-scFv-CAR JurkatΔβ) cells, showing that TR404-scFv-CAR was expressed on the surface of JurkatΔβ cells (Fig. 1B).

3.2. Effect of TR1-scFv-CAR on the JurkatΔβ T cell line

To evaluate the cytotoxic effect of TR404-scFv-CAR on JurkatΔβ cells, we used four target cell lines (Colo205, Daudi, K562, and MCF7). As shown in Fig. 2A, TR404 antibody bound almost equally to the four cell lines, showing that these cell lines expressed similar amounts of TR1 on their cell surfaces. JurkatΔβ cells did not express TR1 on the surface, in agreement with a previous report [16] (data not shown). When TR404-scFv-CAR JurkatΔβ cells were co-cultured with the target cells, 9–12% of Colo205 cells were killed at an E/T ratio of 13–50, but killing of the other three cell lines was not detected (Fig. 2B).

Because Jurkat cells display a helper T cell phenotype, we anticipated that TR404-scFv-CAR JurkatΔβ cells would kill Colo205 cells via TR1-mediated apoptosis but not via cell-mediated cytolytic activity. To examine the cytotoxic mechanisms of TR404-scFv-CAR on JurkatΔβ cells, we first analyzed whether TR404-scFv-CAR-mediated signals activated the JurkatΔβ cells. As shown in Fig. 2C, the CD137 activation marker was up-regulated in TR404-scFv-CAR JurkatΔβ cells after co-culturing with Colo205 cells. In contrast, CD137 up-regulation was not observed on CD8-CAR JurkatΔβ cells, indicating that TR404-scFv-CAR-induced activation provides a signal to effector cells upon binding to TR1 on the target cells. Next, we examined the induction of cytolytic activity by analyzing the secretion of Granzyme B, which is increased upon the induction of cell-mediated cytotoxicity [17–19]. As shown in Fig. 2D, Granzyme B-secretion was barely detectable when TR404-scFv-CAR JurkatΔβ cells were stimulated with Colo205 cells. These results suggest that TR404-scFv-CAR JurkatΔβ cells killed Colo205 cells by inducing apoptosis through TR1 on Colo205 cells, but not through cell-mediated cytolysis.

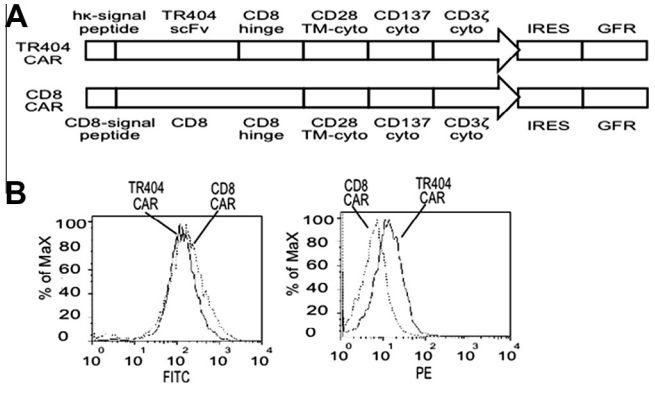

Fig. 1. Construction and expression of TR1-scFV-CAR. (A) Schematic diagram of the TR404-scFv-CAR, containing the CD8 hinge region (CD8-hinge), CD28 transmembrane and intracellular signaling domains (CD28 TM-cyto), the CD137 signaling domain (CD137-cyto) and the CD3ζ signaling domain (CD3ζ-cyto). The internal ribosomal entry site (IRES) and eGFP transduction marker are also linked in the expression vectors. The CD8-CAR contains the extracellular domain of human CD8 in place of TR1-scFv. (B) Expression of TR404-scFv-CAR (solid line) or CD8-CAR (dashed line) on JurkatΔβ cells. GFP expression (left) and binding of recombinant human TR1-Fc (right) were analyzed by flow cytometry.

3.3. Effect of TR1-scFv-CAR on the KHYG-1 human NK cell line

We then examined the effect of TR404-scFv-CAR expressed on the KHYG-1 human NK cell line. We retrovirally transduced KHYG-1 cells with the CARs and enriched for eGFP$^+$ cells (Fig. 3A). TR404-scFv-CAR KHYG-1 cells bound to recombinant soluble human TR1, but CD8-CAR KHYG-1 cells did not. The cytotoxicity of TR404-scFv-CAR KHYG-1 cells against Colo205 cells increased from 30% to 33% as the E/T ratio increased from 13 to 50, whereas CD8-CAR KHYG-1 cells did not show this cytotoxicity (Fig. 3B). TR404-scFv-CAR KHYG-1 cells, but not CD8-CAR KHYG-1 cells, also showed cytotoxicity against Daudi and MCF7 cells. In contrast, not only TR404-scFv-CAR KHYG-1 cells but also CD8-CAR KHYG-1 cells showed a corresponding cytotoxicity against K562 cells that did not express MHC class I molecules and were NK cell-sensitive [20], indicating that K562 cells were killed by KHYG-1 cells through the NK cell activity of the latter.

We next examined if TR404-scFv-CAR-mediated signals activated TR404-scFv-CAR KHYG-1 cells. When TR404-scFv-CAR KHYG-1 cells were co-cultured with Colo205 cells, CD137 was up-regulated on the KHYG-1 cells, but not on the CD8-CAR KHYG-1 cells (Fig. 3C), indicating that crosslinking of TR404-scFv-CAR on KHYG-1 cells with TR1 on the target cells induced KHYG-1 cell activation. We then examined whether activated TR404-scFv-CAR KHYG-1 cells secreted Granzyme B. When TR404-scFv-CAR KHYG-1 cells were cultured in the presence of Colo205 cells, they secreted substantial amounts of Granzyme B, whereas CD8-CAR KHYG-1 cells did not (Fig. 3D). Taken together, these data imply that TR404-scFv-CAR KHYG-1 cells exert their cytotoxic activity toward the target cells via two pathways: first, by induction of target cell apoptosis by aggregating TR1 on the target cells, and second, by induction of perforin/Granzyme B-mediated cytolytic activity of KHYG-1 cells through TR404-scFv-CAR-mediated signaling. Consequently, TR404-scFv-CAR KHYG-1 cells exerted their cytotoxic activity against a broader range of tumor cells, when compared with TR404-scFv-CAR JurkatΔβ cells.

3.4. Effect of TR1-scFv-CAR on human PBLs

To explore the use of the TR404-scFv-CAR in cancer immunotherapy, we examined whether TR404-scFv-CAR-transduced PBLs exhibit cytotoxic activity against various target cells. We prepared PBLs from a healthy donor, retrovirally transduced these cells with TR404-scFv-CAR, and examined their cytotoxicity against the target cells. Flow cytometry revealed that 26% of TR404-scFv-CAR-transduced PBLs bound to recombinant soluble human TR1. The co-expression levels of eGFP and CAR revealed similar transduction efficiencies of the TR404-scFv-CAR and CD8-CAR retroviral vectors (Fig. 4A). We then determined the killing activity of TR404-scFv-CAR PBLs against the cell lines (Fig. 4B). Killing activity of TR404-scFv-CAR PBLs against Colo205 cells increased from 9% to 17% as the E/T ratio increased from 13 to 50, whereas CD8-CAR PBLs did not show the cytotoxicity. TR404-scFv-CAR PBLs also showed cytotoxicity to K562 cells as well as MCF7 cells, whereas CD8-CAR-transduced PBLs did not. Although background cytotoxicity was high (9–26%) against Daudi cells, TR404-scFv-CAR PBLs still

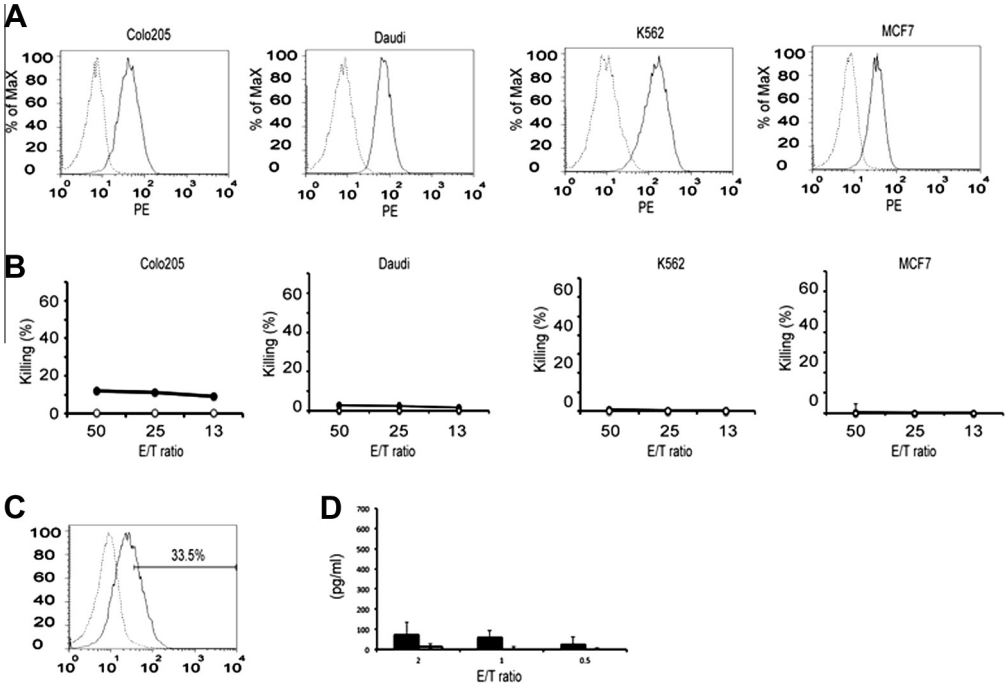

Fig. 2. Cytotoxic effect of TR404-scFv-CAR on JurkatΔβ cells. (A) TR1-expression on Colo205, Daudi, K562 and MCF7 cell lines. Cells were stained with TR404 TR1-specific antibody and R-PE-conjugated human IgG-specific antibody (solid line) or with R-PE-conjugated human IgG-specific antibody alone (dashed line), and analyzed by flow cytometry. (B) ^{51}Cr release assay of the TR404-scFv-CAR (closed circle)- or CD8-CAR (open circle)-expressing JurkatΔβ cells. (C) CD137-up-regulation. TR404-scFv-CAR-expressing (solid line) or CD8-CAR-expressing JurkatΔβ cells (dashed line) were cultured with Colo205 cells and CD137-expression was analyzed by flow cytometry. (D) Granzyme B-secretion by TR404-scFv-CAR-expressing JurkatΔβ cells. TR404-scFv-CAR-expressing (closed column) or CD8-CAR-expressing JurkatΔβ cells (open column) were cultured with Colo205 cells at the indicated E/T ratio for 24 h. Granzyme B in the culture supernatants was determined by ELISA in triplicate. The data are shown as the mean ± SD.

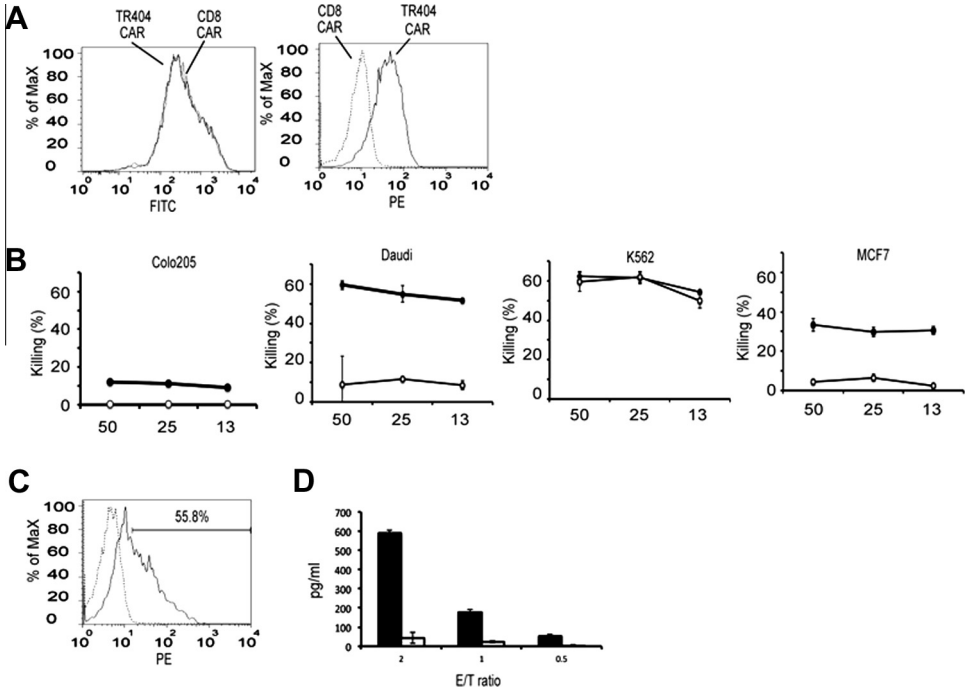

Fig. 3. Cytotoxic effect of TR404-scFv-CAR on an NK cell line. (A) Expression of TR404-scFv-CAR or CD8-CAR on KHYG-1 cells. KHYG-1 cells were retrovirally transduced with TR404-scFv-CAR (closed line) or CD8-CAR (dashed line), and GFP-expression (left) and the binding of recombinant human TR1-Fc (right) were analyzed by flow cytometry. (B) The cytotoxicity of the TR404-scFv-CAR (closed circle)- or CD8-CAR (open circle)-expressing KHYG-1 cells (^{51}Cr release assay). (C) CD137-expression. TR404-scFv-CAR-expressing (solid line) or CD8-CAR-expressing KHYG-1 cells (dashed line) were cultured with Colo205 cells and CD137-expression was analyzed by flow cytometry. (D) Granzyme B-secretion. TR404-scFv-CAR-expressing (closed column) or CD8-CAR-expressing KHYG-1 cells (open column) were cultured with Colo205 cells at the indicated E/T ratio for 24 h. Granzyme B in the culture supernatants was determined by ELISA in triplicate and the data are shown as the mean ± SD.

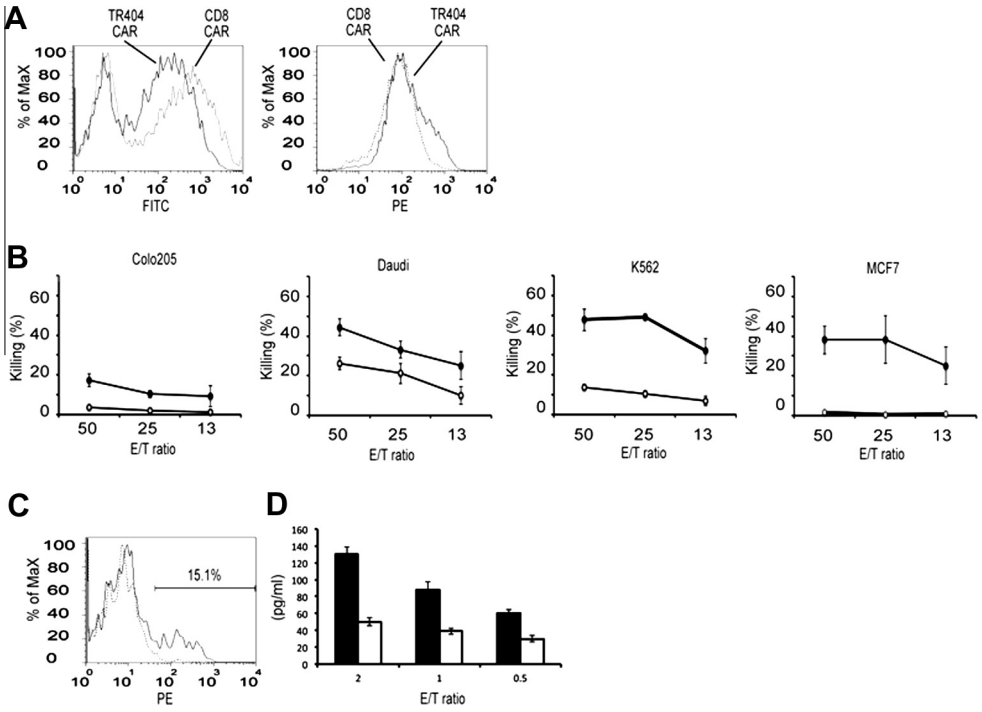

Fig. 4. Cytotoxic effect of TR404-scFv-CAR on human peripheral blood lymphocytes. (A) Expression of TR404-scFv-CAR or CD8-CAR on PBLs. PBLs were retrovirally transduced with TR-CAR or CD8-CAR, and GFP-expression (left) and binding of recombinant human TR1-Fc (right) were analyzed by flow cytometry. (B) The cytotoxicity of the TR404-scFv-CAR-expressing PBLs (^{51}Cr release assay). (C) CD137-expression. TR404-scFv-CAR-expressing (solid line) or CD8-CAR-expressing PBLs (dashed line) were cultured with Colo205 cells and CD137-expression was analyzed by flow cytometry. (D) Granzyme B-secretion. TR404-scFv-CAR-expressing (closed bars) or CD8-CAR-expressing PBLs (open bars) were cultured with Colo205 cells at the indicated E/T ratio for 24 h. Granzyme B in the culture supernatants was determined by ELISA in triplicate and the data are shown as the mean ± SD.

showed increased cytotoxicity (up to 25–44%) against these target cells.

We next examined whether TR404-scFv-CAR signals activated PBLs by crosslinking the CAR via TR1 on the target cells. CD137 expression on TR404-scFv-CAR PBLs was up-regulated when the cells were co-cultured with Colo205 cells; however, that of CD8-CAR PBLs was not (Fig. 4C). Granzyme B-secretion from TR404-scFv-CAR PBLs was also induced by co-culturing with the target cells (Fig. 4D). These data show that TR404-scFv-CAR PBLs exert their cytotoxic activity toward the target cells in a similar manner to TR404-scFv-CAR KHYG-1 cells: via induction of cell apoptosis by aggregating TR1 and by perforin/Granzyme B-mediated cytolytic activity.

4. Discussion

This study was performed to examine whether TR1-specific scFv-CAR-expressing effector cells can efficiently kill tumor cells by using dual mechanisms: induction of apoptotic signals from TR1 on the target cells and CAR-induced cytolytic activity of the effector cells. In the present study, to construct the CAR, we used TR404 TR1-specific human IgG that did not compete with TRAIL for binding to TR1 [6]. JurkatΔβ cells, which have a helper T cell phenotype, that expressed TR404-scFv-CAR showed killing activity toward Colo205 cells in the absence of Granzyme B-secretion, whereas KHYG-1 NK cells and PBLs expressing TR404-scFv-CAR showed killing activity with concomitant secretion of Granzyme B. These results indicate that TR404-scFv-CAR induced the cytolytic activity of KHYG-1 cells and PBLs and that the JurkatΔβ cells killed target cells by inducing apoptosis through the crosslinking of TR1 on target cells with TR404-scFv-CAR. As previously described [6], the TR404 antibody required crosslinking secondary antibodies to induce apoptosis in target cells, which makes it difficult to use for antibody therapeutics. The secondary crosslinking antibodies were not required for inducing target cell apoptosis by TR404-scFv-CAR expressed on the effector cells, and thus TR404-scFv-CAR eliminates the therapeutic disadvantage of the TR404 antibody.

We previously showed that the TR404 antibody kills not only Colo205 cells but also Daudi and K562 cells in the presence of secondary crosslinking antibodies [6]. This finding led to the following question: why did TR404-scFv-CAR JurkatΔβ cells fail to kill Daudi and K562 cells in this study? One reason may be that the cell surface anchoring of TR404-scFv is not sufficient to induce crosslinking of TR1 on target cells and subsequent apoptosis. In fact, Bridgeman et al. reported that a CAR containing the ζ transmembrane domain facilitated CAR dimerization and strengthened its effect on cytokine-secretion [21]. We used the CD28 transmembrane domain to produce the CAR. We are now investigating whether a TR1-scFv-CAR containing the ζ transmembrane domain exerts an effect on TR1-mediated apoptosis.

We considered the best types of effector cells for expression of TR404-scFv-CAR. NK cells are possible candidates [22,23], and TR404-scFv-CAR-expressing NK cell line killed target cells in TR404-scFv-CAR-specific manner [22]. However, the adoption of primary NK cells is largely limited by difficulties of ex vivo cell expansion and by the variation in NK cell activity among patients [23]. Another possible effector cell is a T cell. In the study, we stimulated PBLs with CD3- and CD28-specific antibodies in the presence of IL-2. Consequently, more than 90% of PBLs were $CD3^+$ T cells, and approximately half of them were $CD8^+$ cells (data not shown). TR404-scFv-CAR-transduced PBLs efficiently killed all of the examined cell lines. However, unlike the NK cell line, activated PBLs killed K562 cells in a TR404-scFv-CAR dependent manner, indicating that the main effector cells were T cells, not NK cells.

In summary, we found that combining the scFv of a TR1-specific antibody with a CAR induced apoptosis in the target tumor cells via TR1 and CAR-induced cytolysis, resulting in efficient killing of various tumor cell lines. Our data suggest that TR1-scFv-CAR may be a new candidate for gene therapy in cancer patients.

Acknowledgments

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