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Bispecific Antibody-Bound T Cells as a Novel Anticancer Immunotherapy

Jaewon Cho[1,†], Nara Tae[2,†], Jae-Hee Ahn[1], Sun-Young Chang[3], Hyun-Jeong Ko[1,2,*] and Dae Hee Kim[1,2,*]

[1]*Department of Pharmacy, Kangwon National University, Chuncheon 24341,*
[2]*Kangwon Institute of Inclusive Technology, Kangwon National University, Chuncheon 24341,*
[3]*College of Pharmacy, Ajou University, Suwon 16499, Republic of Korea*

Abstract

Chimeric antigen receptor T (CAR-T) cell therapy is one of the promising anticancer treatments. It shows a high overall response rate with complete response to blood cancer. However, there is a limitation to solid tumor treatment. Additionally, this currently approved therapy exhibits side effects such as cytokine release syndrome and neurotoxicity. Alternatively, bispecific antibody is an innovative therapeutic tool that simultaneously engages specific immune cells to disease-related target cells. Since programmed death ligand 1 (PD-L1) is an immune checkpoint molecule highly expressed in some cancer cells, in the current study, we generated αCD3xαPD-L1 bispecific antibody (BiTE) which can engage T cells to PD-L1$^+$ cancer cells. We observed that the BiTE-bound OT-1 T cells effectively killed cancer cells *in vitro* and *in vivo*. They substantially increased the recruitment of effector memory CD8$^+$ T cells having CD8$^+$CD44$^+$CD62L^{low} phenotype in tumor. Interestingly, we also observed that BiTE-bound polyclonal T cells showed highly efficacious tumor killing activity *in vivo* in comparison with the direct intravenous treatment of bispecific antibody, suggesting that PD-L1-directed migration and engagement of activated T cells might increase cancer cell killing. Additionally, BiTE-bound CAR-T cells which targets human Her-2/neu exhibited enhanced killing effect on Her-2-expressing cancer cells *in vivo*, suggesting that this could be a novel therapeutic regimen. Collectively, our results suggested that engaging activated T cells with cancer cells using αCD3xαPD-L1 BiTE could be an innovative next generation anticancer therapy which exerts simultaneous inhibitory functions on PD-L1 as well as increasing the infiltration of activated T cells having effector memory phenotype in tumor site.

Key Words: PD-L1, CAR-T, Bispecific antibody, Bispecific T cell engager, Anticancer immunotherapy

INTRODUCTION

Cancer is the second leading cause of death globally. To treat various types of cancers, several treatments including ‘chemotherapy’, to destroy rapidly growing cancer cells, ‘surgical procedure’, to remove the cancer tissues physically, ‘radiation therapy’, to deliver high doses of radiation on cancer cells, and ‘immunotherapy’, to invigorate our body’s anti-cancer immunity have been developed (Waldman *et al.*, 2020). However, it is still difficult to achieve complete cure for cancer.

In recent years, ‘chimeric antigen receptor T (CAR-T) cell’ therapy has been one of the most actively studied treatments for a variety of cancers. In CAR-T cell therapy, T cells from a patient are harvested and genetically engineered to express a chimeric antigen receptor which can recognize specific tumor-associated antigens such as CD19 and B-cell maturation antigen (BCMA). Then these genetically engineered T cells are infused back into the patient (Teoh and Chng, 2021). In clinical studies involving CAR-T cell therapies, a 72-83% overall response rate against refractory or relapsed B cell acute lymphoblastic leukemia (Zheng *et al.*, 2018) was reported. However, there are some limitations of CAR-T therapy for solid tumors which have originated from immunosuppressive tumor microenvironment (TME). The TME includes heterogeneous population of suppressive immune cells, such as regulatory T cells, tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC), which are preferentially recruited to the TME and inhibit the activation of CAR-T cells

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***Corresponding Authors**
E-mail: kimdh1@gmail.com (Kim DH), hjko@kangwon.ac.kr (Ko HJ)
Tel: +82-33-250-8086 (Kim DH), +82-33-250-6923 (Ko HJ)
Fax: +82-33-250-8088 (Kim DH), +82-33-259-5631 (Ko HJ)

[†]The first two authors contributed equally to this work.

(Newick *et al*., 2017). Cancer cells can also directly inhibit CAR-T cells' function through the induction of the inhibitory immune checkpoint receptor expression on T cells such as Cytotoxic T Lymphocyte Antigen-4 (CTLA-4), Programmed cell Death-1 (PD-1), Lymphocyte Activating-3 (LAG-3), and T-cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3) (Waldman *et al*., 2020). These inhibitory conditions can aggravate the hypofunction of CAR-T cells and restrict their persistence within the tumor, which limit the efficacy of immune therapies using CAR-T cells.

Programmed death-ligand 1 (PD-L1, a transmembrane protein also referred to as CD274 or B7 homolog 1) is an immune checkpoint molecule that causes the generation of inhibitory signals by binding with PD-1 on the counterpart cells (Qin *et al*., 2019). Expression of PD-L1 has been detected in several types of tumor cells including hepatocellular carcinoma, breast cancer, malignant melanoma, leukemia, and lung cancer, which is regarded as a sign of immune-suppressive environment in such cancers. Engagement of PD-L1 on cancer cells with PD-1 on activated T cells results in the suppression of immune surveillance via immune checkpoint signaling. High PD-L1 expression is also reported in multiple myeloma and/or its tumor infiltrating immune cells. Blocking the PD-1/PD-L1 interaction via anti-PD-L1 antibody treatment also showed anti-cancer potential in previous studies (Ahn *et al*., 2021).

In search for novel and efficient anti-cancer immunotherapies, bispecific antibodies with anti-PD-L1 binding component and diverse binding partners are under development in several studies. They include PD-L1xCD3, PD-L1x4-1BB (CD137) and PD-L1xLAG-3, etc. (Horn *et al*., 2017; Geuijen *et al*., 2021; Jeong *et al*., 2021; Jiang *et al*., 2021), for which few oncology programs are in phase 2 clinical studies. Generally, bispecific antibody has been considered as an innovative next generation anti-cancer therapy that exerts simultaneous inhibitory functions on dual target antigens or engage specific immune cells to disease-related target cells (You *et al*., 2021). Based on the ability of some bispecific antibody (e.g., αCD3xαPD-L1) for combining the target and immune cells, its T-cell-engaging activity has been compared to the function of CAR-T cells.

Therefore, in our current study, we generated αCD3xαPD-L1 bispecific antibody (BiTE) and tested the T cells and CAR-T cells tethered with BiTE for the cancer-targeting specificity and anti-cancer efficacy, respectively. We observed that such bispecific antibody-bound T cells (BiTE:T cells) showed a higher tumor killing activity *in vivo* in comparison with the direct intravenous treatment with bispecific antibody alone. In addition, when bispecific antibody was coupled with CAR-T cells, their combination further enhanced cancer-killing efficacies which was evident from the *in vivo* model studies, suggesting that bispecific antibody-bound CAR-T cell can be a novel therapeutic regimen.

MATERIALS AND METHODS

Cell lines

Murine colon adenocarcinoma cell line CT26 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Ovalbumin (OVA)-transfected B16/F10 melanoma (MO5) was kindly provided by Dr. Kenneth Rock (University of Massachusetts, Worcester, MA, USA) and human HER-2/neu-expressing CT26 colon carcinoma (Her-2/CT26) was developed by transduction of CT26 using a retroviral vector system (Chung *et al*., 2006; Ko *et al*., 2007). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (all from Invitrogen, Carlsbad, CA, USA) and incubated at 37°C in a 5% CO_2 incubator. The expression of PD-L1 was confirmed with phycoerythrin (PE) conjugated anti-mouse PD-L1 antibody from BD Bioscience (San Jose, CA, USA). IM-9 is a human multiple myeloma cell line and was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The IM-9 cell line was maintained in RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Cell lines were subcultured every 2 or 3 days.

Vector structure and construction protocol for CAR-T cells

The single chain fragment variable (scFv) sequence encoding 'Her-2/neu (ErbB2)'-targeting ML39 was synthesized based on the cDNA sequence from the patent (PCT/US2007/024287). It was used to generate ErbB2-targeting CAR construct, which contains the mouse CD8α signal sequence followed by the ML39 scFv linked to the hinge domain of the CD8α molecule and intracellular signaling domains of the CD28 and CD3zeta molecules. Mock lentivirus vector was used to construct CAR-T for the negative control. The fragments were subcloned into the plasmid MSCV-IRES-Thy1.1 DEST (pMIT) vector which was purchased from Addgene (Watertown, MA, USA). High titer replication defective lentiviral vectors were produced and concentrated before use.

In vitro T-cell transduction and culturing

Negative selection using the $CD8\alpha^+$ T Cell Isolation Kit, mouse (MACS, Bergisch Gladbach, Germany) was applied to isolate the primary mouse T cells from spleen (or blood samples) from BALB/c or C57BL/6 mice. For the preparation of activated T cells, mouse T cells were incubated with 4 μg/mL concentration of anti-CD3 antibody (Biolegend, San Diego, CA, USA) and 2 μg/mL for anti-CD28 antibody (Biolegend). T cells were cultured in RPMI 1640 medium supplemented with GlutaMAX^{TM}-I (Invitrogen) and 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 10 mM HEPES buffer, 100 μg/mL of Pen/Strep, 100 μg/mL of gentamycin, and 50 μM mercaptoethanol (all from Invitrogen). The end of stimulation was determined based on the downward shifts of the peaks from 'CellTrace^{TM} Violet' (CTV) staining (Invitrogen), which usually took 2 days after stimulation.

Construction of αCD3xαPD-L1 BiTE

All variable domains of the antibodies have been previously patented or published. Variable domains of anti-PD-L1 antibody were obtained by phage display as previously described (Choi *et al*., 2020). Variable domains of the anti-CD3 (145-2C11) antibody (Alegre *et al*., 1995) were codon-optimized and synthesized commercially (Macrogen, Seoul, Korea). A $(G_4S_1)_3$ linker was used to fuse V_L-V_H (anti-PD-L1) or V_H-V_L (anti-CD3) and a G_4S_1 linker was used to fuse anti-PD-L1 (V_L-V_H) scFv fragment and anti-CD3 (V_H-V_L) scFv fragment by overlap-extension PCR. Subsequently, PCR fragments were subcloned into pCEP4 vector which allows the accurate in-frame translation of αCD3xαPD-L1 BiTE.

Production and purification of αCD3xαPD-L1 BiTE

Expression construct was made with the DNA of αCD3xαPD-L1 BiTE followed by a His6 tag. DNA was transiently transfected into FreeStyle™ 293-F Cells (Gibco) by FectoPRO (Polyplus, Illkirch-Graffenstaden, France) following the manufacturer's instructions. After 5 days of culture, supernatants from the transfected cells were purified by open-column chromatography using Ni-NTA agarose (Qiagen, MD, USA). Elution fractions were collected and dialyzed against PBS (pH 7.2). Protein concentration was determined using NanoDrop ND-2000 spectrophotometer (Thermo Scientific, MA, USA) based on absorption at 280 nm. Purity of protein was detected using SDS-PAGE and Coomassie brilliant blue staining.

Cytotoxicity assays

Target cells and T cells were seeded in a 96-well plate (10^4 cells/well); the cells were treated with 0.1 μg/mL BiTE and incubated for 24 h at 37°C and 5% CO_2. After the incubation, 10 μL of cell counting kit 8 solution (Cell Counting Kit-8, Dojindo Co., Kumamoto, Japan) was added to each well and incubated for an additional 2 h. The absorbance at 450 nm was measured by a SpectraMax i3 microplate reader (Molecular Devices, San Jose, CA, USA).

Animal experiments

Six weeks old female mice including BALB/c and C57BL/6 were purchased from KOATECH (Pyeongtaek, Korea). The mice were maintained under specific pathogen-free conditions in the experimental facilities at Kangwon National University (Chuncheon, Korea). All the animal experiments were performed according to the approved guidelines of the Institutional Animal Care and Use committee of Kangwon National University (KW-201007-1). To establish a mouse tumor model, seven weeks old mice were challenged with 2×10^6 MO5 cells or Her-2/CT26 cells subcutaneously. Tumor length, height, and width were measured using calipers and the tumor volume was calculated as 1/6π×length (mm)×height (mm)×width

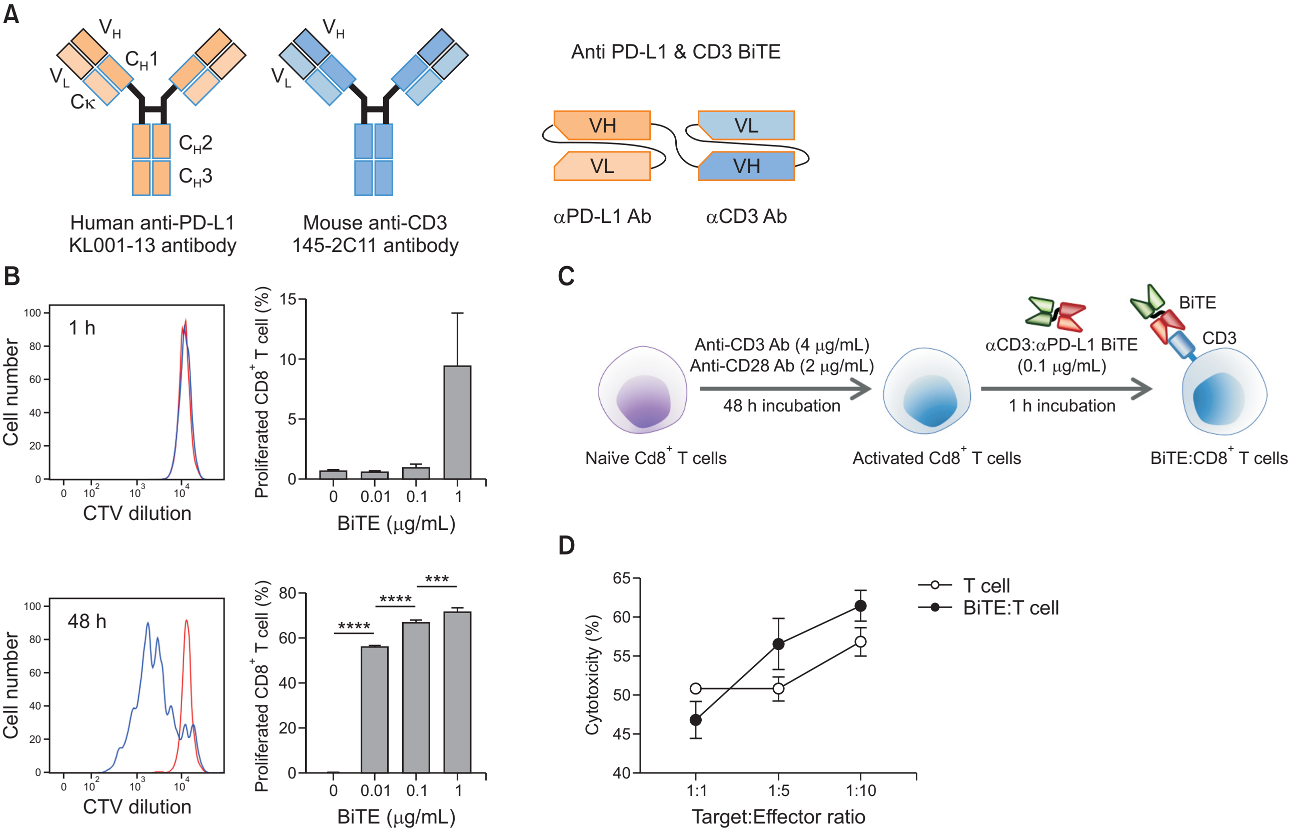

Fig. 1. Generation of bispecific T-cell engager-bound T cells (BiTE:T cells) as a novel anticancer cell therapy. (A) Bispecific T-cell engager molecules were generated with two single chain fragment variables (scFvs). mCD3xPD-L1 BiTE contains scFvs of anti-CD3ε antibody (145-2C11) and anti-PD-L1 antibody (KL001-13), which were linked with 'GGGGS' linker. (B) mCD3xPD-L1 BiTE was cultured with $CD8^+$ T cells for 1 h or 48 h in following concentration: 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL. T cells were stained with 1 μM of CellTrace™ Violet (CTV) and dilution of CTV was measured with FACS to determine T cell proliferation. Non-treated T cells were used as a negative control and indicated it as red line in figure. ***$p<0.001$, ****$p<0.0001$ (Student's t-test; n=3/group). (C) Schematic diagram of BiTE-bound T cells (BiTE:T cells) preparation. To prepare BiTE:T cells, $CD8^+$ T cells were isolated from the spleen of mice and incubated for 48 h with 4 μg/mL of αCD3 antibody and 2 μg/mL of αCD28 antibody. Then mCD3xPD-L1 BiTE (0.1 μg/mL) were incubated with the $CD8^+$ T cells for 1 h. BiTE-bound $CD8^+$ T cells (BiTE:T cells) were obtained after removing the unbound BiTEs by washing with PBS. (D) $CD8^+$ T cells from wild type mice isolated using magnetic beads. T cells or BiTE:T cells were prepared following protocol shown in 1C. T cells or BiTE:T cells were incubated with MO5 cells (1×10^4/well) for 16 h. Represented ratio is Target cell:Effector cell. Cancer killing activity of BiTE:T cells or BiTE:OT-1 cells was measured by CCK8 and CTV assay.

(mm).

Isolation and analysis of tumor-infiltrating lymphocytes

Tumor tissues were harvested and minced using sterile razor blades. The tumor pieces were digested using an enzyme mixture containing 400 U/mL collagenase type IV purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA) and 0.02 mg/mL DNase I purchased from Sigma Aldrich (St. Louis, MO, USA) in RPMI 1640 and incubated at 37°C for 45 min and passed through 70 μm cell strainers (BD Bioscience). A Percoll gradient from GE Healthcare (Chicago, IL, USA) was then used to separate cancer cells and enrich lymphocytes.

Statistical analysis

Statistical analyses were performed using Graphpad Prism 9 (GraphPad Software, LLC, San Diego, CA, USA). Unpaired two-tailed Student's t-tests were used when the data had a Gaussian distribution with similar variances. One-way analysis of variance (ANOVA) was used to compare more than two groups followed by post hoc tests (Bonferroni test). The threshold for statistical significance was $p<0.05$, with 95% confidence intervals for all of the analyses.

RESULTS

Structure of BiTE and schematic explanation of BiTE-bound CD8 T cell (BiTE:T cell) induction

A bispecific T-cell engager molecule, a form of bispecific antibody, was generated with two tandemly linked single chain Fvs (scFvs) (Fig. 1A), with the scFvs originated from anti-CD3ε antibody (145-2C11) and anti-PD-L1 antibody (KL001-13). To test the functionality of this bispecific T-cell engager molecule on the activation of T cells, we attempted to determine the minimal concentration of BiTE molecule which can induce T cell proliferation. Incubation of BiTE molecules (0.01, 0.1 μg/mL concentration) with T cells for 1 h could not achieve proliferative activation of naïve CD8 T cells. However, after 48 h of incubation, CD8 T cells acquired sufficient proliferative activation, which was shown as substantial dye dilution profile for CTV-labeled CD8 T cells (Fig. 1B). This data suggests that T cells can be incorporated with BiTE molecules without strong proliferative activation during a short period of incubation (~1 h). However, T cells can still retain their functional activation after a relatively long period of incubation (~48 h) with a certain concentration of BiTE molecules.

The T cells are usually co-incubated with the beads coated with anti-CD3/CD28 antibodies or soluble anti-CD3 antibody with IL-2 to achieve the required activation during the manufacturing process of therapeutic CAR-T cells (Li and Kurlander, 2010; Zhang *et al*., 2017). Therefore, treatment of anti-

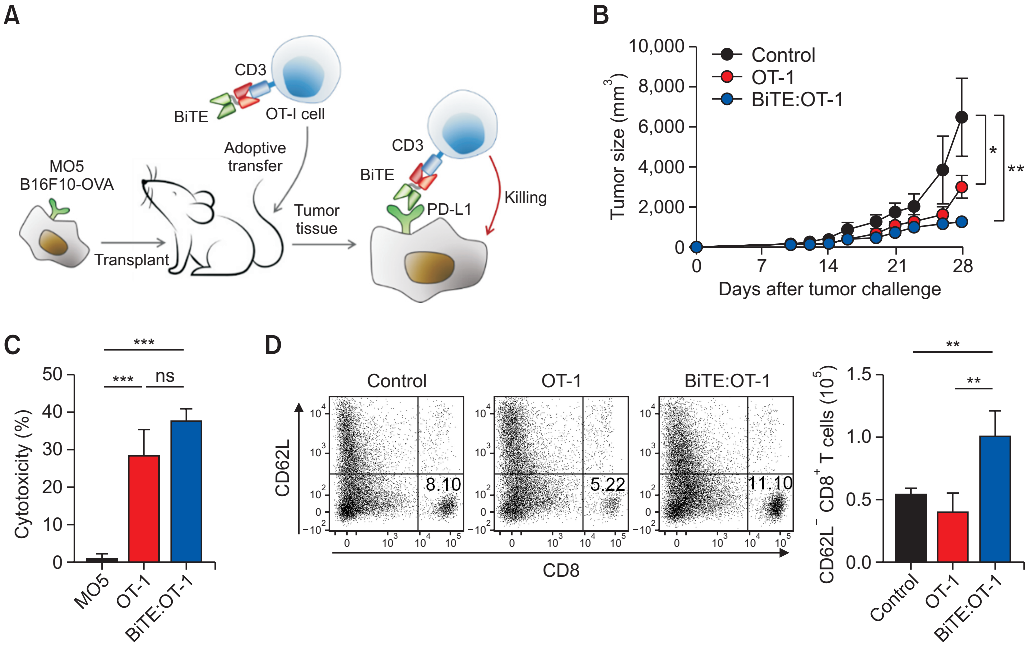

Fig. 2. *In vivo* antitumor effect of BiTE:OT-1 cells. (A) Scheme of the *in vivo* experiment for BiTE:OT-1 T cells. Groups of mice were subcutaneously injected with MO5 (2×10^6/mouse) in their left flank and the tumor size was monitored for 28 days. C57BL/6 mice, which were transplanted with MO5, were injected with OT-1 T cells or BiTE:OT-1 T cells (2×10^5/mouse) after 10 and 20 days of tumor cell injection. (B) Tumor size was measured in every 2 or 3 days. All groups of mice were sacrificed on day 28. *$p<0.05$, **$p<0.01$ (Student's t-test; n=6/group). (C) CD8$^+$ T cells from wild type mice or OVA-specific OT-1 CD8$^+$ T cells (>90% of which were V$_\alpha$2-positive) isolated from OT-1 mice using magnetic beads. BiTE:T cells or BiTE:OT-1 cells were prepared following protocol shown in 1C. BiTE:T cells or BiTE:OT-1 cells were incubated with MO5 cells for 16 h. Cancer killing activity of BiTE:T cells or BiTE:OT-1 cells were measured by CCK8 and CTV assay. ***$p<0.001$ (Student's t-test; n=3/group). (D) Tumor-infiltrating lymphocytes (TILs) were analyzed. Effector memory T cells (CD3$^+$CD8$^+$ CD62L^{low}) were analyzed by FACS. The population and number of them were shown. **$p<0.01$, ***$p<0.001$ (Student's t-test; n=4/group). These data are representative of three independent experiments.

CD3/CD28 antibodies with T cells before incubation with BiTE molecules was carried out in our experiment for the relevant T cell activation (Fig. 1C).

To verify that BiTE molecule can exert cancer killing activities through bridging between cancer cells and T cells, mouse CD3 (mCD3)xPD-L1-targeting BiTE was loaded on to polyclonal CD8 T cells that were isolated from splenocytes of C57BL/6 mice. Briefly, isolated T cells were activated using anti-CD3/CD28 antibodies for 48 h. Then, those CD8 T cells were incubated with 0.1 μg/mL of mCD3xPD-L1-targeting BiTE for 2 h. After washing out non-bound BiTE, mCD3xPD-L1 BiTE:T cells were obtained and co-cultured with MO5 cells, which highly express PD-L1 on their surface, for 16 h. The effector cells were seeded with the represented ratio (Target cell : Effector cell). The cancer killing activity of BiTE:T cells was determined by CCK8 activities of MO5 cells (Fig. 1D). mCD3xPD-L1 BiTE:T cells significantly suppressed CCK8 activity more than when MO5 co-cultured with plain T cells (Fig. 1D). These results suggest that BiTE:T cells can effectively target and kill cancer cells expressing tumor antigen on their surface *in vitro*.

Binding of BiTE on OT-1 T cell showed potent anti-tumor effect against OVA-expressing MO5 cells than plain OT-1 T cell

To ascertain whether the BiTE-loaded CD8 T cells can exert anti-cancer function as CAR-T cell does *in vivo*, mCD3/PD-L1 targeting BiTE molecule was preincubated with OT-1 T cells for 1 h to generate BiTE-bound T cells. For *in vivo* efficacy test, MO5 cells (2×10^6 cells/mouse) were subcutaneously inoculated in the left flank of C57BL/6 mouse and then after 10 days, plain OT-1 T cells or BiTE-bound OT-1 T cells (BiTE:OT-1) were transferred to MO5-grafted mice (Fig. 2A). The mice treated with BiTE:OT-1 showed higher tumor growth inhibition than the group treated with plain OT-1 T cells (Fig. 2B). We next assessed whether BiTE could shows the cytotoxicity effects *in vitro*. The cancer killing activity of BiTE:OT-1 was determined by CCK8 activities of MO5 cells. BiTE:OT-1 cells suppressed CCK8 activity more than when MO5 co-cultured with OT-1 cells, but it was not significantly differenced (Fig. 2C). However, there was significant increase in the level of tumor-infiltrating lymphocytes having effector memory phenotype (CD62L^{low} CD8$^+$) in mice treated with BiTE:OT-1 than the group with only OT-1 treatment (Fig. 2D). This suggests

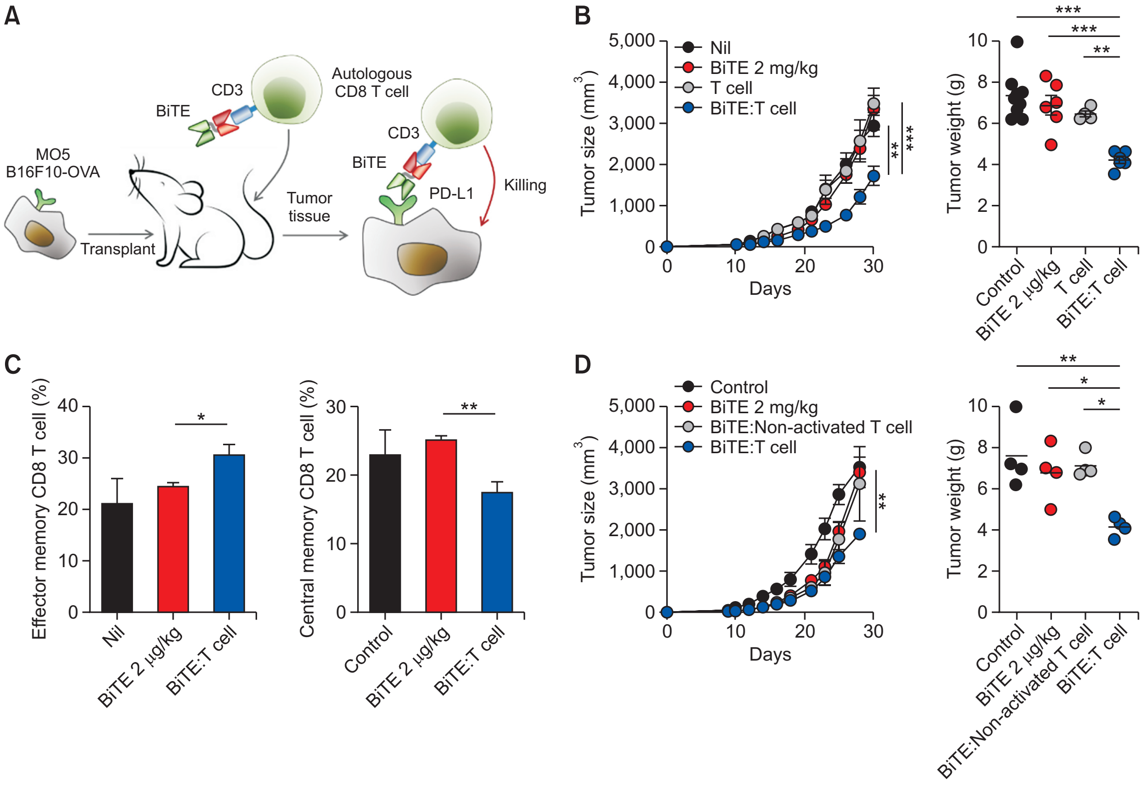

Fig. 3. Antitumor effect of BiTE:T cells constructed with activated autologous polyclonal CD8$^+$ T cells. (A) Scheme of the *in vivo* experiment for BiTE-bound autologous polyclonal T cells (BiTE:T cells). Groups of mice were subcutaneously injected with MO5 (2×10^6/mouse) in their left flank and monitored for 30 days. BiTE (2 μg/kg), autologous polyclonal T cells, or BiTE:T cells (2×10^5/mouse) were intravenously transferred to tumor-bearing mice after 10 days and 20 days of tumor injection. (B) Tumor sizes were monitored for every 2 days and the tumor weights were measured at day 30 after euthanizing the mice. **p<0.01, ***p<0.001 (Student's t-test; n=6/group). (C) CD3$^+$CD8$^+$CD44$^+$CD62L$^+$ effector memory T cells and CD3$^+$CD8$^+$CD44$^+$CD62L$^-$ central memory T cells in tumor infiltrating lymphocytes (TILs) of every group of mice were analyzed by FACS. *p<0.05, **p<0.01 (Student's t-test; n=4/group). (D) MO5 cells were grafted in the left flank of C57BL/6 and BiTE (2 μg/kg), and BiTE-bound T cells (BiTE:T) with or without activation through αCD3 and αCD28 were intravenously injected after 10 days and 20 days of tumor challenge. Tumor sizes were monitored for every 2 days or 3 days. Non-activated T cells were also cultured for the same period as the activated T cells. After euthanizing the mice, tumor weight was measured. *p<0.05, **p<0.01 (Student's t-test; n=4/group). All the data shown are representative of three independent experiments.

that OT-1 T cells bound to mCD3xPD-L1 BiTE showed substantial effect on the recruitment of effector memory $CD8^+$ T cells in a tumor.

Tumor growth was suppressed after transferring BiTE-bound polyclonal CD8 T cells

Through the experiment with B16.MO5 cells and OT-1 cells in C57BL/6 mouse model, we confirmed that mCD3/PD-L1 BiTE molecule can induce enhanced tumor-growth inhibition of OT-1 T cells compared to the matched tumor cells treated with plain OT-1 T cells. Further, we extended the utility of BiTE-bound T cells from antigen-specific OT-1 T cells to polyclonal CD8 T cells. To evaluate the anti-tumor activity of CD8 T cells bound with mCD3/PD-L1 BiTE, 2×10^5 of mCD3/PD-L1 BiTE:T cells were intravenously administered to MO5-bearing mice on the 10th day after initial tumor cell graft (Fig. 3A). Introduced mCD3xPD-L1 BiTE:T cells significantly suppressed tumor growth compared to both 2 μg/kg of BiTE intraperitoneal injection and plain T cell treatment (Fig. 3B). Additionally, analysis of T cell population in tumor showed that effector memory $CD8^+$ T cells were increased compared to the control. However, central memory $CD8^+$ T cells were decreased (Fig. 3C), suggesting an enhanced ratio of effector memory to central memory T cells. This result is consistent with the reports of previous studies suggesting that the increased effector memory to central memory T cells ratio in tumor serves as a predictive biomarker of enhanced anticancer immune response against several cancers (Liu *et al*., 2020; Principe *et al*., 2020). To assess that BiTE efficacy shows more efficient when the T cell is activated, we examine the function of BiTE:T depends of its activation. As a result, activated T cells with BiTE shows significantly suppressed tumor growth compared to both 2 μg/kg of BiTE intraperitoneal injection and non-activated T cells with BiTE (Fig. 3D). These results indicate that mCD3xPD-L1 BiTE can direct polyclonal $CD8^+$ T cells to the tumor and inhibit the tumor growth when it is bound to polyclonal $CD8^+$ T cells. This is similar to the function of CAR-T cells from the polyclonal T cells that can be directed to and kill cancer cells through surface scFv introduced by lentiviral transduction.

Binding of BiTE on CAR-T cells also potentiated anti-tumor activity

To extend the application of BiTE-bound T cells to CAR-T cell system, we generated BiTE-bound CAR-T cells (BiTE:ML39 CAR-T) by incubation of ML39 CAR-T cells expressing anti-human Her2/neu (ErbB2) scFv with 0.1 μg/mL of

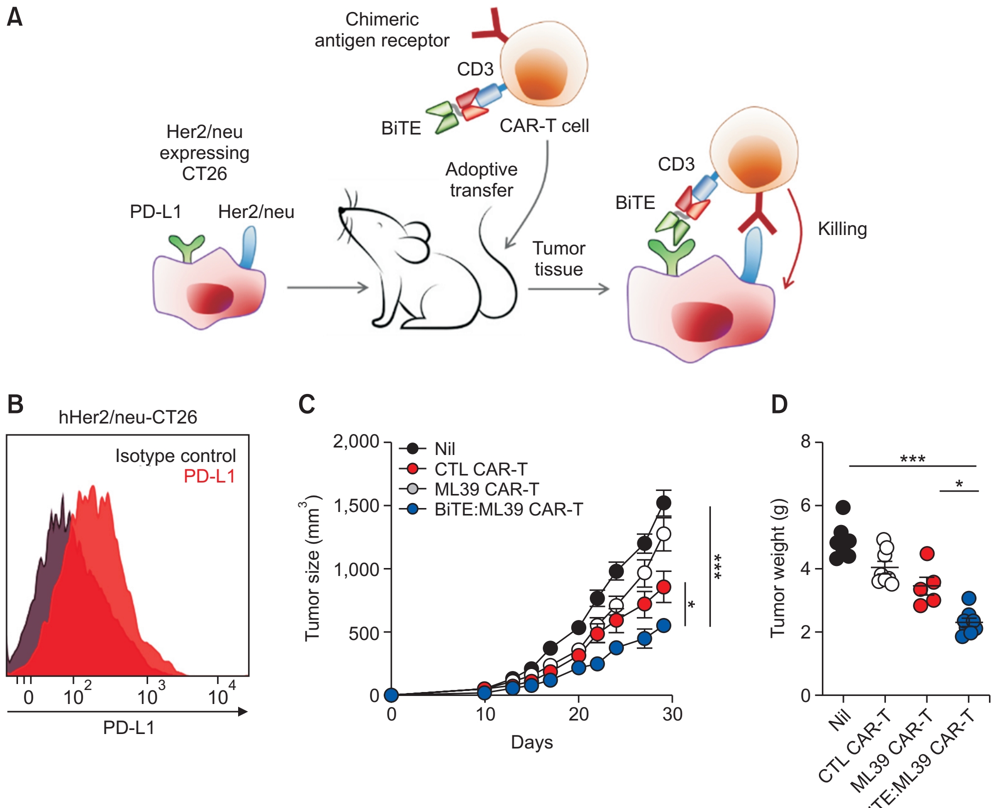

Fig. 4. Novel antitumor cell therapy of BiTE:ML39 CAR-T. (A) Diagram of the *in vivo* study for BiTE-bound ML39 CAR-T cells (BiTE:ML39 CAR-T). Groups of mice were grafted with Her-2/CT26 cells (2×10^6/mouse) into their left flank and monitored for 30 days. ML39 CAR-T cells were generated to express anti-hHer2/neu (ErbB2) for targeting the Her-2/CT26 cells. After 10 and 20 days of tumor injection, mice were injected with control CAR-T cells (CTL CAR-T), ML39 CAR-T cells, or BiTE:ML39 CAR-T cells (2×10^5/mouse). (B) Confirmation of PD-L1 expression on the surface of Her-2/CT26 cells. (C) Tumor sizes were monitored for every 2 days. *$p<0.05$, ***$p<0.001$ (Student's t-test; n=10/group). (D) After euthanizing every group of mice, the tumor was removed from their body and the tumor mass was measured. *$p<0.05$, ***$p<0.001$ (Student's t-test; n=5-8/group). These data are representative of three independent experiments.

BiTE for 1 h (Fig. 4A). To evaluate the activity of BiTE:ML39 CAR-T, we adopted human Her2/neu-expression CT26 colon cancer transplantation model (Ko *et al*., 2007). We confirmed that Her-2/CT26 cells also express PD-L1 on their surface (Fig. 4B). Intravenous adoptive transfer of BiTE:ML39 CAR-T cells (2×10^5 cells/mouse) significantly suppressed the tumor growth compared to ML39 CAR-T, control CAR-T (CTL CAR-T), and Nil group (Fig. 4C). We also confirmed that the tumor weight measured on the day of sacrifice of mice indicated that BiTE:ML39 CAR-T group showed the least tumor growth compared with the other groups (Fig. 4D). From this result, we could confirm that the mCD3xPD-L1 BiTE:CAR-T inhibits tumor growth and plays a role in the engaging T cells to cancer cells as well. Collectively, these results suggest that binding of mCD3xPD-L1 to CAR-T cells showed significant anticancer effect compared to CAR-T cells alone.

DISCUSSION

Bispecific antibody and CAR-T cell therapies have been successful therapeutic options for several hematological malignancies. These have been recent breakthroughs in the research of oncology and immune-checkpoint inhibitory agents (Edeline *et al*., 2021). Although both therapies share mechanistic similarities in T cell-mediated killing of tumor cells by redirecting autologous T cells to cancer cell surface antigens, they have distinct characteristics and clinical applications. In general, CAR-T cell therapy has shown better response in B-cell malignancies. High rates of complete responses, over 50% in Non-Hodgkin lymphoma (NHL) (Neelapu *et al*., 2017) and 90% in Acute Lymphocytic Leukemia (ALL) (Maude *et al*., 2018) and over 70% complete response and/or partial response in Chronic Lymphocytic Leukemia (CLL) (Turtle *et al*., 2017) for the CD19 targeting CAR-T cell therapy were reported. However, in case of solid tumors, only a 29% of pooled response rate was reported in contrast to the 71% response rate in hematological tumors (Yu and Hua, 2019). Further therapeutic improvements are still needed even in CAR-T cell therapy. Such low response rates for solid tumors are attributed to several factors such as the loss of target gene expression (Baird *et al*., 2021), the solid tumor's immunosuppressive microenvironment (Moon *et al*., 2014), the impaired T-cell trafficking (Kershaw *et al*., 2006), and the suboptimal phenotype of the infused CAR-T cells (Kershaw *et al*., 2013). Thus, numerous strategies to improve the clinical efficacies of CAR-T cells for solid tumors are under development. They include combinational approaches with drugs targeting different tumor antigens (Till *et al*., 2012) and the modification of chemokine receptors on CAR-T cells to enhance their migration into solid tumors (Moon *et al*., 2011).

To achieve highly efficacious anti-tumor activities and devise simpler, versatile T cell-based therapeutic agents, we have investigated bispecific antibody-bound T cells in the murine tumor models. When mCD3xPD-L1-targeting BiTE was bound to OT-1 T cells and applied to B16.MO5-bearing mice, it showed higher tumor growth inhibition than treatment with OT-1 cells alone. This shows that BiTE binding on T cells augments the tumor-killing activities of OT-1 T cells through BiTE-mediated T cell receptor (TCR) activation, additional to the intrinsic activation through direct OT-1 T cell and OVA:MHC-I interaction.

To extend the utility of BiTE-bound T cells, we also tested BiTE binding to polyclonal CD8 T cells, which are composed of T cells with diverse TCR repertoire. Introduced mCD3xPD-L1 BiTE:T cells significantly suppressed tumor growth compared to both 2 μg/mL of BiTE or plain T cell treatments. This further confirmed that BiTE-mediated TCR activation on diverse T cell population is enough to suppress the tumor growth. Enhanced effector memory T cells in tumor also indicated that mCD3x-PD-L1 BiTE can direct polyclonal CD8 T cells to tumor and inhibit the tumor growth regardless of their TCR specificities towards tumor antigens.

When BiTE-bound CAR-T cells were applied to the human Her2/neu-expressing CT26 murine colon tumor model, similar to the BiTE-bound OT-1 cell activation, BiTE-bound CAR-T cells also showed an enhanced tumor killing activity compared to that from sole CAR construct. This result reiterated the advantage of using BiTE-bound CAR-T cells against tumor cells.

The BiTE molecule, which is composed of the tumor antigen binding scFvs and anti-CD3 binding scFv, has T cell recruiting activity towards tumor sites when it is administered intravenously to the patients. One such molecule, blinatumomab, which targets B cells in acute lymphoblastic leukemia, showed strong tumor killing activities and got US FDA approval in 2014 (Franquiz and Short, 2020). However, owing to its small size and the associated short half-life in blood circulation, it needs to be administered as continuous infusion for almost 4 weeks either in hospital setting or at home during the first treatment cycle, which has serious patient compliance issues. In comparison with blinatumomab and CAR-T cell therapy, BiTE-bound T cell or CAR-T cell therapy has several advantages. As BiTE molecules are incubated with T cells and these T cells are infused back to patient's blood stream, BiTE-bound T cell strategy does not need continuous infusion similar to blinatumomab and shows better tumor killing activities. This could result in highly improved patient compliance and enhanced anti-tumor responses. Additionally, in contrast to the complicated process of CAR-T cell manufacture using lentiviral transduction of CAR construct into activated T cells from leukapheresis of patient's blood, the procedure of BiTE binding on the peripheral blood mononuclear cells (PBMC) is much simpler. BiTE-bound T cells can be manufactured using a low-cost process; however, further molecular and process optimization are needed. Furthermore, the strategy of applying BiTE-bound T cells is highly versatile. Therefore, several BiTE molecules can be incorporated into the patient's PBMC to cope with the heterogeneous nature of tumor cells. This strategy has the potential to apply combinatory BiTE molecules on patient's autologous T cells simultaneously or in sequential application of different BiTE molecules during each treatment cycle.

Interestingly enough, our experiments on BiTE-bound CAR-T cells also showed enhanced tumor killing activities *in vivo*. This could be due to the additional activation signals from BiTE-TCR complex which augmented CAR-T cells' activities or anti-PD-L1 component in BiTE molecule could have exerted inhibitory function against immune suppression from tumoral PD-L1 molecules. In addition, this result implies that the BiTE binding strategy discussed in this study could be incorporated into CAR-T cell therapy. This could enhance the therapeutic efficacy of CAR-T cells or redirect CAR-T cells using BiTEs recognizing other tumor antigens and CD3 molecule.

Because pharmacokinetics is an important aspect in the development of any therapeutic agent, it will be of great interest to investigate the pharmacokinetic properties of BiTE-bound T cells. As shown *in vivo* assay of BiTE-bound T cells and BiTE alone treatment, BiTE molecules bound to T cells appear to have sufficient circulation time to exhibit antitumor activity than when BiTE alone is administered, which imply that BiTE tethered on T cells or CAR-T cells either induce higher T cell activation or has enhanced pharmacokinetic properties. We are currently working on this subject to elucidate the detailed mechanism of the enhanced antitumor effect of BiTE-bound T cells compared to treatment with T cells or BiTE alone *in vivo*.

In summary, our study using bispecific antibody-bound T cells (BiTE:T cells) showed highly efficacious tumor killing activities *in vivo* and enhanced tumor killing efficacies of CAR-T cells by BiTE-bound CAR-T cells. This provides a novel combinatory anticancer therapeutic regimen.

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