Anti-mucin 1 chimeric antigen receptor T cells for adoptive T cell therapy of cholangiocarcinoma

Kamonlapat Supimon[1], Thanich Sangsuwannukul[1], Jatuporn Sujjitjoon[1], Nattaporn Phanthaphol[1], Thaweesak Chieochansin[1], Naravat Poungvarin[1], Sopit Wongkham[2], Mutita Junking[1], and Pa-thai Yenchitsomanus[1]

[1]Mahidol University Faculty of Medicine Siriraj Hospital
[2]Khon Kaen University Faculty of Medicine

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Abstract

Current treatments for cholangiocarcinoma (CCA) are largely unsuccessful due to late diagnosis at advanced stage, leading to high mortality rate. Consequently, improved therapeutic approaches are urgently needed. A newly potential therapy – chimeric antigen receptor (CAR) T cell therapy utilizes genetically modified T cells that specifically recognize surface antigen on cancer cells without restriction by major histocompatibility complex (MHC). Mucin 1 (MUC1) is an attractive candidate antigen due to its high expression in CCA cells, and its association with poor prognosis and survival. Since anti-MUC1-CAR T cells have not previously been tested for activity in models of CCA, we set forth to test their utility in this setting. A fourth generation anti-MUC1-CAR construct was engineered to contain anti-MUC1-single-chain variable fragment (scFv) and three co-stimulatory domains (CD28, CD137, and CD27) linked to CD3ζ. Cultures of anti-MUC1-CAR T cells consisted primarily of cytotoxic (CD8+) T cells (75.13±11.65%, $p<0.001$). Anti-MUC1-CAR T cells produced increased levels of IFN-γ when exposed to MUC1-positive KKU-100 and KKU-213A CCA cells (31.33±6.02% and 46.5±8.82%, respectively; both $p<0.05$). Moreover, anti-MUC1-CAR T cells demonstrated specific killing activity against KKU-100 (45.88±7.45%, $p<0.05$) and KKU-213A cells (66.03±3.14%, $p<0.001$) at an effector to target ratio of 5:1, but demonstrated negligible cytolytic activity against control immortal cholangiocytes (MMNK-1 cells). These activities of anti-MUC1-CAR T cells supports the development of this approach as an adoptive T cell therapeutic strategy for CCA.

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[1] Siriraj Center of Research Excellence for Cancer Immunotherapy (SiCORE-CIT), Research Department, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[2] Graduate Program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[3] Division of Molecular Medicine, Research Department, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[4] Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[5] *Department of Biochemistry, and Center for Translational Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand*

[6] *Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen, Thailand*

***Corresponding Author** Prof. Pa-thai Yenchitsomanus, PhD

Division of Molecular Medicine, Research Department Faculty of Medicine Siriraj Hospital, Mahidol University 2 Wanglang Road, Bangkoknoi, Bangkok 10700, Thailand

Tel: (+66) 2-419-2777; Fax: (+66) 2-411-0169

E-mail: pathai.yen@mahidol.edu; ptyench@gmail.com

Short title: Anti-MUC1-CAR T cells for CCA

List of abbreviations:

ALL Acute lymphoblastic leukemia

CAR T cells Chimeric antigen receptor T cells

CCA Cholangiocarcinoma

DC Dendritic cells

EGFR Epidermal growth factor receptor

HNSCC Head and neck squamous cell carcinoma

MHC Major histocompatibility complex

MUC1 Mucin 1

NSCLC Non-small cell lung cancer

OLT Orthotopic liver transplantation

PFS Progression-free survival

scFv Single chain variable fragment

TILs Tumor-infiltrating lymphocytes

TNBC Triple negative breast cancer model

Summary

Current treatments for cholangiocarcinoma (CCA) are largely unsuccessful due to late diagnosis at advanced stage, leading to high mortality rate. Consequently, improved therapeutic approaches are urgently needed. A newly potential therapy – chimeric antigen receptor (CAR) T cell therapy utilizes genetically modified T cells that specifically recognize surface antigen on cancer cells without restriction by major histocompatibility complex (MHC). Mucin 1 (MUC1) is an attractive candidate antigen due to its high expression in CCA cells, and its association with poor prognosis and survival. Since anti-MUC1-CAR T cells have not previously been tested for activity in models of CCA, we set forth to test their utility in this setting. A fourth generation anti-MUC1-CAR construct was engineered to contain anti-MUC1-single-chain variable fragment (scFv) and three co-stimulatory domains (CD28, CD137, and CD27) linked to CD3ζ. Cultures of anti-MUC1-CAR T cells consisted primarily of cytotoxic ($CD8^+$) T cells (75.13±11.65%, $p<0.001$). Anti-MUC1-CAR T cells produced increased levels of IFN-γ when exposed to MUC1-positive KKU-100 and KKU-213A CCA cells (31.33±6.02% and 46.5±8.82%, respectively; both $p<0.05$). Moreover, anti-MUC1-CAR T cells demonstrated specific killing activity against KKU-100 (45.88±7.45%, $p<0.05$) and KKU-213A cells (66.03±3.14%, $p<0.001$) at an effector to target ratio of 5:1, but demonstrated negligible cytolytic activity against control immortal cholangiocytes

(MMNK-1 cells). These activities of anti-MUC1-CAR T cells supports the development of this approach as an adoptive T cell therapeutic strategy for CCA.

Keywords: Cholangiocarcinoma; mucin 1; chimeric antigen receptor T cells; anti-MUC1-CAR T cells

Introduction

Cholangiocarcinoma (CCA) is an epithelial malignancy of the biliary tree which is extremely challenging to treat.[1] Although the incidence of CCA is rare in most Western countries[2], it is comparatively high in Chile, Bolivia, South Korea, and Thailand.[3] CCA is a major public health problem in Thailand, especially in the Northeastern region, and it is associated with liver fluke (*Opisthorchis viverrini* , OV) infection.[4] The 1-year mortality rate of Thai CCA patients was 81.68% during 2009 to 2013.[5] Standard treatments, including surgery, radiation, chemotherapy, and targeted therapy, are generally unsuccessful in the treatment of CCA patients with advanced-stage disease.[6] The 5-year survival rate of patients after surgical resection is low (23-63%), and the post-operative recurrence rate at 5 years is about 70-80%.[7] Chemotherapy is a palliative treatment modality for unresectable patients; however, response rate is very poor, and side effects frequent.[8] Targeted therapy drug, such as infigratinib, can be used in a subgroup of patients with advanced/metastatic CCA with *FGFR2*translocations.[9] Orthotopic liver transplantation (OLT) has been reported for CCA treatment; however, patients treated with OLT had a high recurrence rate, and less than 20% had long-term survival.[10] Therefore, new therapeutic approaches are urgently required.

Immunotherapies have been reported for CCA treatment.[6]Several studies used monoclonal antibodies against epidermal growth factor receptor (EGFR) to control CCA.[11] Unresectable or metastatic CCA patients treated with cetuximab plus oxaliplatin and gemcitabine (GEMOX) showed increased progression-free survival (PFS).[12,13] Patients treated with cancer lysate-pulsed dendritic cells (DCs) combined with *ex vivo* activated T cells also showed increased median PFS and overall survival (OS) compared to patients treated with surgery alone.[14] In addition, $CD4^+$ tumor-infiltrating lymphocytes (TILs) that recognize mutated erbb2 interacting protein (ERBB2IP) were expanded*ex vivo* and used for treatment of a CCA patient.[15] After treatment, the patient showed tumor regression and prolonged stabilization of disease. Although DC-based therapies and T cell transfer have yielded encouraging early results, these approaches are susceptible to MHC downregulation and therapeutic failure.[16] Adoptive T cell therapy using chimeric antigen receptor (CAR) T cells is an alternative strategy for cancer therapy with high efficacy in hematological malignancies.[17] Chimeric antigen receptors are synthetic fusions that recognize surface cancer antigens and enable engineered T cells to induce cancer cell apoptosis without MHC recognition.[18]

CAR T cells showed impressive efficacy against relapsed or refractory B-cell acute lymphoblastic leukemia (ALL), and CD19-CAR T cells have now been approved in multiple territories.[19] However, the anti-tumor effect of CAR T cells is less evident in solid tumors.[17] Generally, three generations of CAR T cells against cancers have been produced.[20] Among these, the common structure of CAR included antigen recognition, spacer, transmembrane, and intracellular domains. The intracellular domain of the first generation of CAR contained only CD3ζ, while the second and third generation CAR consisted of one and two co-stimulatory molecules, respectively, that are linked to CD3ζ. Modified CAR T cells to co-express the CAR molecule with ligands or cytokines to enhance T cell function (so called armored CAR T cells) were also used, and they showed high anti-tumor efficiency.[21] The second and third generations of CAR T cells demonstrated higher anti-tumor effects compared to the first generation.[22,23] We, therefore, decided to take advantage of the different properties of co-stimulatory molecules to create a fourth generation of CAR T cells that contain three costimulatory molecules (CD28, CD137, and CD27) linked to CD3ζ that may enhance anti-tumor efficiency against CCA.

Selection of antigens on cancer cells that are suitable for targeting by CAR T cells and efficient for induction of CAR T cell responses is essential for designing an effective immunotherapy approach that includes avoidance of side effects.[24] Among several cancer antigens, mucin 1 (MUC1), which is a type I transmembrane protein that plays role in mucous membrane protection, signal transduction, and modulation of immune system[25], is one of the best potential target antigens for CAR T cell therapy because it is overexpressed in several

cancers and its expression is related to cancer progression.[26] This potential target antigen was also found to be expressed in 50-86.5% of CCA tissues among patients from several countries.[27-30] Interestingly, the cancer-associated MUC1 is hypoglycosylated, compared to the heavily glycosylated form found in normal cells, which means that it can be specifically targeted by CAR T cells without on-target off-tumor effect.[31]

Several studies have generated and modified CAR T cells targeting MUC1, and have investigated their functional efficacies in several cancer models.[22, 23, 32-34] The use of these CAR T cells was also reported from some clinical trials.[35, 36] To date, none of these CAR T cells has been approved for clinical use. The anti-tumor efficacy of MUC1-targeting CAR T cells was studied against breast cancer by comparing the single chain variable fragment (scFv), spacer length, and generations of CAR.[22] CAR T cells targeting hypoglycosylated MUC1 were studied in hematological and pancreatic cancers, and cancer regression was reported in both models.[32] The efficacy of MUC1-targeting CAR T cells against non-small cell lung cancer (NSCLC) model was also reported.[37] Furthermore, high efficacy of hypoglycosylated MUC1-targeting CAR T cells was demonstrated in triple negative breast cancer model (TNBC).[33] Other developments include the description of MUC1-CAR T cells with IL-22 secretion in head and neck squamous cell carcinoma (HNSCC)[34], and combination of MUC1-targeting CAR T cells with an inverted cytokine receptor (IL-4/ IL-7) in breast cancer model.[23] These studies illustrated impressive anti-tumor functions of MUC1-targeting CAR T cells against several cancers. However, activity of MUC1-targeting CAR T cells in models of CCA has never been reported. This study set out to engineer anti-MUC1 CAR T cells and examine their anti-tumor effects against MUC1-expressing CCA cells. Our results provide encouragement for the application of anti-MUC1-CAR T cells in the immunotherapy of CCA.

Materials and methods

Cell lines and culture condition

MMNK-1 (cholangiocytes), KKU-055, and KKU-213A (cholangiocarcinoma), and MCF-7 (breast cancer) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), while KKU-100 cells were maintained in DMEM-F12 media containing 10% heat-inactivated fetal bovine serum (FBS) and 100 µg/ ml of streptomycin and penicillin.[38] Peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 5% heat-inactivated human AB serum (R5). Activated PBMCs were cultured in R5 containing recombinant human (rh) IL-2, IL-7, and IL-15 cytokines.

Immunohistochemistry (IHC)

Human cancer tissue sections from CCA patients were kindly provided by Khon Kaen University. The protocol of study using these tissues was approved by the Ethics Committee for Human Research, Khon Kaen University (no. HE591063). Tissues were de-paraffinized and rehydrated with stepwise decreasing concentrations of ethanol. Subsequently, antigen retrieval was performed by immersing slides in sodium citrate buffer (pH 6.0) with 0.05% Tween-20 and heating by a microwave oven at warm mode for 10 minutes. After blocking of endogenous peroxide using 0.3% hydrogen peroxide for 30 minutes, the tissues were blocked with 5% skim milk and stained with anti-MUC1 antibody (VU4H5, Santacruz Biotechnology, Inc., Dallas, TX, USA) overnight. After washing, HRP-conjugated EnVision secondary antibody (Dako, CA, USA) was added followed by 3, 3'-diaminobenzidine (DAB) for signal development. Finally, the tissues were counterstained with hematoxylin, mounted, and visualized for brown color area under a light microscope.

Construction of lentiviral encoded anti-MUC1-CAR

To generate a lentiviral (pCDH) construct encoding for an anti-MUC1-CAR, an anti-MUC1 scFv containing a $[G4S]_3$ linker derived from the HMFG2 monoclonal antibody (a gift of Dr. John Maher, King's College London)[22] was codon optimized. The synthesized cDNA (Integrated DNA Technologies, Coralville, IA, USA) was inserted between an upstream EF-1α and CD8α leader sequence and downstream CD8 hinge and transmembrane domain. The CAR endodomain consisted of a tandem fusion of the intracellular domains of CD28, CD137, CD27, and CD3ζ respectively. The DNA fragment was amplified using specific primers

containing cutting sites of the restriction enzymes *EcoR* I and*Mre* I (Supplementary Table 1). After amplification by polymerase chain reaction (PCR), the amplified product was digested with the restriction enzymes 5' *EcoR* I and 3' *Mre* I (Supplementary Figure 1a). Lentiviral vector containing CD28, CD137, CD27, and CD3ζ (pCDH-CAR) was also digested with the same restriction enzymes. The digested pCDH-CAR and amplified DNA fragment were ligated and transformed into competent E. coli cells. Transformant colonies were screened to obtain colonies containing anti-MUC1-CAR plasmid by colony PCR (Supplementary Figure 1b). The amplified anti-MUC1-CAR plasmids showing a product size of 1,867 bps were analyzed by Sanger DNA sequencing. Of the clones obtained, clone #45 showed 100% sequence consensus with the reference sequence (Supplementary Figure 1c).

To produce lentiviral particles, the anti-MUC1-CAR construct was transfected into Lenti-X^{TM} 293T cells (Takara Bio Inc., Shiga, Japan) with psPAX2 and pMD2.G plasmids. Supernatants were collected at 48 and 72 hours.

Transfection

Anti-MUC1-CAR construct was also transfected into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. After 48 hours, transfected cells were collected for anti-MUC1-CAR molecule detection by immunoblot and flow cytometry.

Peripheral blood mononuclear cell (PBMC) preparation and activation

Blood samples were collected from healthy donors who signed the informed consent document that was approved by the Siriraj Institutional Review Board (number Si 101/2020). PBMCs were collected using Corning® Lymphocyte Separation Medium (Corning Inc., Corning, New York, USA) following the manufacturer's process. PBMCs were then plated in a 100 mm culture dish to allow adherence of unwanted monocytes. After 6 hours, non-adherent cells were gently collected as a source of lymphocytes and activated using 5 μg/ml of phytohemagglutinin (PHA)-L (Roche, Basel, Switzerland) for three days.

MUC1-targeting CAR T cell production

The activated lymphocytes were mixed with 10 μg/mL of protamine sulfate (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) and lentiviral supernatants (added at a multiplicity of infection of 10) and centrifuged for 90 minutes at 1,200 g in plates coated with Retronectin® (Takara Bio). The transduced lymphocytes were maintained in culture medium containing rhIL-2 (20 ng/mL), rhIL-7 (10 ng/mL), and rhIL-15 (40 ng/mL). On day 8 after activation, anti-MUC1-CAR expression was detected on transduced cell surface by flow cytometry as indicated below. Activated lymphocytes mixed with protamine sulfate that were not transduced with lentiviral supernatants were included as untransduced control cells.

Immunoblot analysis

MMNK-1, KKU-055, KKU-100, KKU-213A, and MCF-7 cells were collected and lysed using 8 M urea lysis buffer. Protein concentration was measured using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Total protein lysates were run through SDS-PAGE, transferred to a nitrocellulose membrane, and then blocked with 5% BSA. MUC1 protein expression was detected using anti-MUC1 antibody (VU4H5). Band intensity was determined using ImageJ software[39] and normalized with β-actin. CAR expression in transfected HEK293T cells was detected using anti-CD3ζ (Santa Cruz Biotechnology).

Immunofluorescence assay (IFA)

MMNK-1, KKU-055, KKU-100, KKU-213A, and MCF-7 cells were plated and allowed to expand on coverslips. After cell attachment, the cells were fixed with 4% paraformaldehyde and blocked with 1% BSA. The cells were stained overnight with anti-MUC1 antibody (EP1024Y; Abcam, Cambridge, UK). MUC1 expression imaging was performed on a Zeiss LSM 800 confocal microscope (Zeiss Microscopy, Jena, Germany).

To test cytolytic activity, stably wasabi-luciferase-expressing KKU-213A cells were co-cultured with anti-MUC1-CAR T cells at effector to target (E:T) ratios of 5:1, 2.5:1, and 1:1. After 18 hours of co-culture,

the CAR T cells were removed and the remaining target cells were captured under fluorescence microscope. Reduction of the green fluorescence signal from the conditions of target cells with CAR T cells and target cells with UTD cells was compared.

Flow cytometry

MUC1 expression on membrane of cancer cells was freshly stained with anti-MUC1 antibody (EP1024Y) diluted to 1:50 in 2% FBS in PBS. Antibodies, including anti-CD3-FITC, CD4-APC, CD8-APC, CD19-APC, CD16-APC, and CD56-PE (ImmunoTools, Friesoythe, Germany), were used to investigate PBMC, PHA-activated lymphocytes, and anti-MUC1-CAR T cell phenotypes.

To detect anti-MUC1-CAR expression on transduced lymphocytes, the transduced cells were harvested, blocked with 1% BSA, and stained with biotin-conjugated protein-L (Thermo Fisher Scientific) for 45 minutes. Alexa Fluor 488 dye-conjugated streptavidin was finally added. The data were acquired on a BD FACSVerse or BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software version 10.

IFN-γ production study

Anti-MUC1-CAR T cells were co-cultured with MMNK-1, KKU-100, and KKU213 cells at an E:T ratio of 5:1. Brefeldin A was added after one hour of co-culture and further incubated for 5 hours. The CAR T cells were harvested, stained with anti-CD3-FITC antibody, and fixed with 1% formaldehyde. The cells were permeabilized and stained with anti-IFN-γ-PE antibody (ImmunoTools) diluted in 0.1% saponin for 30 minutes. After incubation, IFN-γ production in CAR T cells was measured by flow cytometry.

Luciferase assay

Anti-MUC1-CAR T cells were co-cultured with stably wasabi-luciferase-expressing target cells at E:T ratios of 1:1, 2.5:1, and 5:1. After 18 hours, the CAR T cells were removed, and the target cells were washed with PBS. The remaining target cells were detected for luciferase activity using a Pierce Firefly Luciferase Glow Assay Kit (Thermo Fisher Scientific). Luminescence was immediately determined using a luminometer. The percentage of killing was calculated using this formula [% specific killing = 100 - ((luciferase activity from well with effector and target cells) / (luciferase activity from well with target cells) x 100)].

Statistical analysis

Data are representative of three or more independent experiments. The data were statistically analyzed using GraphPad Prism version 7 (GraphPad Software, Inc., San Diego, CA, USA, www.graphpad.com). The results are shown as mean ± standard deviation (SD) or ± standard error of the mean (SEM). Significant differences between compared data were determined by unpaired t -test ($^{*}p$ <0.05,$^{**}p$ <0.01,$^{***}p$ <0.001).

Results

MUC1 was expressed in CCA tissues

To examine MUC1 protein expression, four CCA tissue samples from Thai patients with CCA were analyzed by immunohistochemistry (IHC) method. The results showed that MUC1 was weakly expressed in adjacent normal bile duct cells (Figure 1a), but was highly expressed in all four CCA tissues (Figure 1c-f). MUC1 was found to localize in both the cytoplasm and membrane of the cancerous cells. In the tissue sample where the normal and cancerous tissues were adjacent, MUC1 was not detected in the nearby hepatocytes, but it was detected in the invading cancerous CCA cells (Figure 1b).

MUC1 was expressed in CCA cell lines

MUC1 expression in MMNK-1, KKU-055, KKU-100, KKU-213A, and MCF-7 cell lines was investigated. The result showed that MUC1 was expressed in all cell lines, as studied by immunoblot analysis (Figure 2a). MUC1 was previously reported to be highly expressed in MCF-7 cells[40], which were used as positive control cells. Protein intensity was calculated, normalized with β-actin, and illustrated as bar graphs (Figure 2b).

By IFA, cell surface expression of MUC1 was observed in all cancerous cell lines, including KKU-213A, MCF-7, KKU-055, and KKU-100. In contrast, MUC1 expression was mostly located in the cytoplasm of MMNK-1 immortalized (non-transformed) cholangiocytes, while cell surface expression by these cells was very low (Figure 2c). To test cell surface expression of MUC1, cells were stained without permeabilization of the cells and examined by flow cytometry. The results showed that MUC1 was strongly expressed on the surface of KKU-213A and MCF-7, intermediately expressed in KKU-055 and KKU-100, and weakly expressed in MMNK-1 cells (Figure 2d and 2e). The cell surface expression of MUC1 on CCA cells indicates that it is a potential target for CAR T cell immunotherapy.

Anti-MUC1-CAR T cells generated were mainly $CD8^+$ T cells

A schematic drawing of anti-MUC1-CAR construct is shown in Figure 3a. To generate the anti-MUC1-CAR construct, the sequence of anti-MUC1 scFv linked with spacer sequence was codon optimized and double-stranded DNA fragment was synthesized by Integrated DNA Technologies (Coralville, IA, USA). The DNA fragments were amplified and ligated into the pCDH-CAR plasmid. After cloning, anti-MUC1-CAR expression from the anti-MUC1-CAR plasmid was evaluated in transfected HEK293T cells. The results showed that the CAR protein could be expressed from clone #45 of the anti-MUC1-CAR plasmid on HEK293T cell surface when it was compared to untransfected cells (Supplementary Figure 2a), and the CAR protein containing CD3ζ could also be detected by immunoblot analysis (Supplementary Figure 2b).

To generate anti-MUC1-CAR T cells, PHA-activated lymphocytes were transduced with lentivirus-containing supernatant. Cell expansion after three days of PHA-activation is shown in Supplementary Figure 3a. Five to seven days after transduction, anti-MUC1-CAR expression was detected on transduced (anti-MUC1-CAR T) cell surface by flow cytometry. The transduced T cells showed 40-60% anti-MUC1-CAR expression (50.14±7.334%) compared to untransduced (UTD) T cells (p <0.0001) (Figure 3b and 3c). To determine cell phenotypes, the cells were collected in three periods, including before activation (unactivated PBMCs), after activation (PHA-activated lymphocytes), and after transduction (anti-MUC1-CAR T cells), and then they were analyzed by flow cytometry. Cytotoxic T ($CD3^+$ $CD8^+$) cells were significantly increased in PHA-activated lymphocytes (p =0.0133) and in anti-MUC1-CAR T cells (p =0.0009). In contrast, helper T ($CD3^+$ $CD4^+$), B ($CD3^-$ $CD19^+$), and NK ($CD3^-$ $CD16^+$ $CD56^+$) cells were decreased after PHA activation (Figure 3d). The representative data and gating strategy of cell phenotypes are shown in Supplementary Figure 3b. Thus, anti-MUC1-CAR T cells were successfully generated with high transduction efficiency, and the majority were $CD8^+$ T cells.

Anti-MUC1-CAR T cells increased IFN-γ production in response to exposure to MUC1-expressing CCA cells

To investigate IFN-γ production by anti-MUC1-CAR T cells in response to MUC1-positive cancer cells, MMNK-1, KKU-100, and KKU-213A cells were co-cultured with CAR T cells at an E:T ratio of 5:1. Anti-MUC1-CAR T cells without tumor exposure and untransduced T cells (UTD T) with tumor exposure were used as negative controls. After six hours of co-culture, the anti-MUC1-CAR T cells and UTD control T cells were evaluated for IFN-γ production ($CD3^+$IFN-γ^+) by intracellular staining and flow cytometry. Increased production of IFN-γ by anti-MUC1-CAR T cells was noted after exposure to KKU-100 (31.33±6.02%) and KKU-213A cells (46.5±8.822%), but it was produced at a lower level when anti-MUC1-CAR T cells were exposed to MMNK-1 cells (18.8±1.212%) (Figure 4a and 4b). The highest IFN-γ cytokine production was found in anti-MUC1-CAR T cells exposed to KKU-213A, followed by KKU-100 and MMNK-1 cells (Figure 4b). Thus, the level of IFN-γ produced by anti-MUC1-CAR T cells was related to MUC1 expression on the cancer cell membrane.

Cytotoxic function of anti-MUC1-CAR T cells on MUC1-expressing CCA cells

To examine the cytotoxic function of anti-MUC1-CAR T cells, the CAR T cells were co-cultured with MMNK-1, KKU-100, and KKU-213A cells, which showed different cell surface expression of MUC1: 4.02±0.994%, 15.14±1.526%, and 58.8±12.59%, respectively (Figure 2d and 2e). These non-transformed cholangiocytes (MMNK-1) and CCA cancer cells (KKU-100 and KKU-213A) were genetically engineered to

express green fluorescent protein and luciferase. The CAR T cells were co-cultured with these target cells at E:T ratios of 1:1, 2.5:1, and 5:1 for 18 hours. After exposure to the CAR T cells, KKU-100 and KKU-213A cells were lysed in a dose-dependent manner, but MMNK-1 cells showed low cytolysis (Figure 5a).

The cytotoxic function of anti-MUC1-CAR T cells on MMNK-1, KKU-100, and KKU-213A cells was also examined by luciferase assay, undertaken at different E:T ratios. Compared to UTD T cells, the CAR T cells showed a cytotoxic effect on KKU-100 and KKU-213A, but not on MMNK-1 cells. The cytotoxic effects of the CAR T cells on KKU-100 and KKU-213A cells at an E:T ratio of 1:1 were 34.82±13.18% (p =0.0078) and 34.34±14.62% (p =0.0213), respectively. At E:T ratios of 2.5:1 and 5:1, the percentages of KKU-100 lysis were slightly increased (41.49±9.38%,p =0.0160, and 45.88±7.45%, p =0.0237, respectively). The percentage of KKU-213A lysis after exposure to the CAR T cells at E:T ratios of 2.5:1 and 5:1 was significantly increased in a dose-dependent manner (54.16±13.56%, p =0.0018, and 66.03±3.14%,p =0.0003, respectively). In contrast, the CAR T cells showed low cytotoxic effect on MMNK-1 cells (1.87±1.75%, 13.96±9.99%, and 11.6±9.21% at E:T ratios of 1:1, 2.5:1, and 5:1, respectively) (Figure 5b).

Moreover, the cytotoxic function of the CAR T cells on MMNK-1 and KKU-213A cells after co-culturing for 24 hours was examined by crystal violet staining method. In correlation with fluorescence detection and luciferase assay (Figure 5a and 5b), the CAR T cells showed cytotoxic effect on KKU-213A, but had no effect on MMNK-1 cells (Supplementary Figure 4).

Discussion

Cholangiocarcinoma (CCA) is a bile duct epithelial malignancy that causes high mortality worldwide, and it has a high prevalence in the Northeastern population of Thailand.[41,42] Current treatments, including surgery, radiation, chemotherapy, targeted therapy, and liver transplantation, are not effective in advanced and metastatic CCA, resulting in high recurrence rate and low long-term survival.[10, 43, 44] Treatment of CCA patients with immunotherapy showed improvement in therapeutic outcome.[6] However, some limitations were also observed. The anti-cancer functions of DC-based and also T cell-infiltrating lymphocytes (TILs) therapy could be obstructed by downregulation of MHC molecule, and upregulation of immune checkpoint ligand on cancer cells, which are characterized as immune escape mechanisms.[45,46] In this study, we aimed to develop adoptive T cell therapy using CAR T cells, a highly effective modality for cancer treatment, and to investigate their cytotoxic function against CCA. CAR T cells recognize a specific TAA on cancer cells. Thus, selection of an appropriate antigen with properties that are well characterized and that are known to affect T cell response with low or no adverse side effects is essential for generation of CAR T cells. Mucin 1 (MUC1) was the specific TAA that was selected for CAR T cell targeting in the present study since it was reported as a second most attractive target antigen for cancer immunotherapy.[47]MUC1 is overexpressed in 77% of Thai CCA patients (consistent with the representative examples shown in this study), and its expression related to disease progression.[28] Interestingly, the cancer-associated MUC1 showed hypoglycosylation compared to heavy glycosylation in normal tissues, which provided benefit for cancer-specific targeting by CAR T cells.

In this study, we confirmed that MUC1 is a potential cancer antigen for CCA. MUC1 expression was investigated in four CCA tissues by IHC, and it was found that MUC1 was overexpressed both in the cytoplasm and on the membrane of CCA cells (Figure 1). Expression patterns of MUC1 in CCA tissues were similar to that of previous study[28], which reported the detection of MUC1 in Thai CCA patients from Khon Kaen Province. The researchers in that previous study observed MUC1 expression in 77% (67/87) of CCA tissues. Strong expression of MUC1 was also reported in 86.5% (32/37) of CCA tissues in Chinese patients[29], and in 65.8% (56/85) of CCA tissues in Korean patients.[30] The expression of MUC1 was low in adjacent normal bile duct epithelial cells. Similar to a previous report[29], we could not detect MUC1 expression in adjacent normal hepatocytes (Figure 1b). However, MUC1 was found to express in hepatocellular carcinoma tissues.[48]Therefore, these results suggested MUC1 as a potential target antigen for immunotherapy using CAR T cells.

Importantly, there has been no report of MUC1 expression in CCA cell lines. We, therefore, decided to investigate MUC1 expression in non-cancer and CCA cell lines, including MMNK-1, KKU-055, KKU-100,

and KKU-213A, compared to MUC1 expression in breast cancer MCF-7 cells, which were previously reported to have high expression[40] (Figure 2). MUC1 was highly expressed both in the cytoplasm and on cell surface of KKU-213A cells, while intermediate cell surface expression was found in KKU-055 and KKU-100 cells. In contrast, MUC1 was weakly expressed on the cell surface of MMNK-1 cells (Figure 2c). Thus, these cell lines with different levels of MUC1 expression were valuable for testing cytotoxicity by CAR T cells.

Specific co-stimulatory molecules are known to regulate anti-tumor function, proliferation, cytokine production, and survival of CAR T cells. In the present study, we generated lentiviral pCDH vector containing scFv derived from the reported antibody specific to MUC1[22] that was linked to CAR cassette containing CD28, CD137, CD27, and CD3ζ (anti-MUC1-CAR) (Figure 3a). CD28 is expressed both in resting and activated T cells. It was reported to promote T cell proliferation, IL-2 and Th1 cytokine production, and activation-induced cell death (AICD) resistance of CAR T cells.[49] CAR T cells containing the CD28 molecule could improve anti-cancer functions and persistence both *in vitro*and *in vivo* compared to first-generation CAR and 41BB-based CAR T cells.[50, 51] Moreover, it was shown to enhance CAR T cell activity, expansion, and longer persistence in lymphoma patients.[52] CD137 (or 4-1BB) is expressed on resting $CD8^+$ T cells and upregulated on both activated $CD4^+$ and $CD8^+$ T cells.[53] CD137 activation enhances IL-2 and IFN-γ production in $CD8^+$ T cells, while it induces IL-2 and IL-4 production in $CD4^+$ T cells. It preferentially promotes $CD8^+$ T cell proliferation.[54] In CAR T cells, the addition of CD137 was reported to promote cell proliferation, granzyme B expression, IFN-γ and TNF-α production, and apoptotic resistance *in vitro* .[55] The CD137 could also improve anti-tumor effect and persistence of CAR T cells *in vivo* .[56] Finally, CD27 incorporation was expected to enhance apoptotic resistance of CAR T cells. CD27-based CAR T cells showed enhancement of CAR T cell persistence compared to CD28-based CAR T cells.[57] Therefore, incorporation of these 3 co-stimulatory molecules could promote anti-tumor activities, proliferation, and survival of our CAR T cells. Expression of our anti-MUC1-CAR was confirmed in HEK293T cells (Supplementary Figure 2) and lentivirus transduced T cells (Figure 3b and 3c), at high transduction efficiency. The phenotypes of cells collected before activation (unactivated PBMCs), after activation (PHA-activated lymphocytes), and after transduction (anti-MUC1-CAR T) showed increased cytotoxic T ($CD3^+CD8^+$) cells (Figure 3d). Expansion of $CD8^+$ T cells might be caused by IL-2, IL-7, and IL-15 cytokines in culture condition.[58] Another study showed that significant expansion of antigen-specific $CD8^+$ T cells was observed after antigen stimulation and addition of IL-2 or IL-15 cytokine.[59] Phytohemagglutinin (PHA) used in the cell activation step was also reported to promote $CD8^+$T cell expansion.[60] Furthermore, CD27 and CD137 molecules in the CAR T cells preferentially supported $CD8^+$ T cell proliferation.[54, 61]

In addition, we investigated IFN-γ production of anti-MUC1-CAR T cells in response to exposure to MUC1-expressing CCA cells. Interferon-γ cytokine is mainly secreted from T and NK cells and contributes importantly to cancer cell clearance.[62] Increased production of IFN-γ was reported in patients with non-small-cell lung cancer (NSCLC) and urothelial cancer, which was related to enhanced progression-free survival (PFS) and overall response rates in these patients.[63] Previous studies reported a high level of IFN-γ production in anti-MUC1-CAR T cells in other cancer models.[22, 32, 33] In this study, intracellular IFN-γ cytokine production of anti-MUC1-CAR T cells was examined. The intracellular IFN-γ staining of CAR T cells against cancers has been widely used in several studies.[64, 65] The upregulation of IFN-γ was detected only in response to MUC1-positive cancer cell exposure (Figure 4). This result was observed in an antigen-dependent manner, in which anti-MUC1-CAR T cells produced high, lower, and lowest levels of IFN-γ cytokine in response to highly MUC1-expressing KKU-213A cells, intermediately MUC-1-expressing KKU-100 cells, and lowest MUC1-expressing MMNK-1 cells, respectively (Figure 4b). This suggests that IFN-γ contributes to cancer cell lysis in this particular CCA cells.

We then examined the anti-cancer effect of anti-MUC1-CAR T cells. Co-culturing of anti-MUC1-CAR T cells with CCA cells showed significant cancer cell lysis, which correlated with cell surface expression of MUC1; however, this effect was not observed with non-transformed MMNK-1 cells (Figure 2c-e and 5). Although KKU-213A cells showed approximately 58% of MUC1 surface expression, anti-MUC1-CAR T cells could induce 66.03±3.14% of KKU-213A cell lysis at an effector to target ratio of 5:1 (Figure 5b). This might be caused by the effect of IFN-γ production from the CAR T cells (Figure 4b), which was reported to

enhance cancer cell cycle arrest, apoptosis, and necroptosis.[62] Our anti-MUC1-CAR T cells showed potent anti-cancer effect comparable to that of other studies in different cancer models using second- and third-generation anti-MUC1-CAR T cells[22, 23, 32, 34], and also inverted cytokine receptor (IL-4R/IL-7) CAR T cells. Therefore, the results of our study support the therapeutic potential of anti-MUC1-CAR T cells for further development into an alternative CCA treatment.

Cancer heterogeneity, tumor microenvironment, assessment of anti-tumor efficacy of CAR T cells, and safety are major concerns in the development of CAR T cell-based therapies. MUC1 expression was detected in CCA patient tissues from many countries, but the expression varied, and the highest MUC1 expression was 86.5%. Therefore – some, but not all, CCA patients could be treated with this therapy. MUC1 expression should be firstly examined in patient tissues before treatment selection. For patients showing low MUC1 expression, combined treatments, including dual-CAR T cells or combination of anti-MUC1-CAR T cells with CAR T cells targeting other CCA-associated antigens, should be further investigated.[66] Since the previous evidence showed that gemcitabine, a chemotherapeutic agent, induced MUC1 upregulation in primary intrahepatic cholangiocarcinoma cells from patients[67], the combination of MUC1-targeting CAR T cells with gemcitabine may be a potential treatment in patients with CCA. Elements within the tumor microenvironment, such as immune checkpoint molecules (PD-1/PD-L1) and immunosuppressive cytokines, should also be addressed. To overcome these problems, the CAR T cells should be able to disrupt or block the immune checkpoint PD-1/PD-L1[68], modify to express switch receptor (PD-1-CD28)[69], or modify by addition of inverted cytokine receptor (IL-4R/IL-7)[23] to improve the CAR T cell functions within the tumor microenvironment. To address the safety issue, the CAR T cells can be modified to express suicide genes, including herpes simplex virus thymidine kinase (HSV-tk), inducible caspase 9 (iCasp9), and truncated epithelial growth factor receptor (tEGFR), which can enable CAR T cell elimination.[70]Several previous studies have shown very impressive cancer control by using CAR T cells targeting MUC1 in various cancer models[22, 23, 32, 34] with low adverse effects.[35] At present, there are 11 active phase I/II clinical trials of anti-MUC1-CAR T cells against multiple cancer models, some of which are combined therapy with checkpoint blockade.[71, 72] Finally, our anti-MUC1-CAR T cells and their safety for use in a CCA treatment setting require further investigation in an animal model and clinical trials.

In conclusion, MUC1 is a potential target antigen for development of CAR T cell therapy for CCA since it is highly expressed on the membrane of CCA cells. We have engineered an anti-MUC1-CAR and used lentiviral vector delivery to express this CAR in human T cells, which were mainly of the $CD8^+$ subset. These anti-MUC1-CAR T cells showed anti-cancer activity against MUC1-positive CCA cells by production of anti-tumor cytokine, IFN-γ, and induction of CCA cell lysis. This is the first study showing the potential of anti-MUC1-CAR T cells in CCA. The results of this study are beneficial for further development of CAR T cell therapy against CCA.

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KS designed and performed experiments, collected and analyzed data, interpreted results, and prepared manuscript. TS performed experiments and prepared the manuscript. NPh performed IHC staining. JS, TC, NPo, SW, MJ, and PY managed the study, provided materials and reagents, designed experiments, interpreted results, and edited the manuscript.

Disclosures

The authors have no conflicts of interest to declare.

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Figure legend

Figure 1. Expression of MUC1 protein in cholangiocarcinoma tissue samples. MUC1 protein in CCA was detected by immunohistochemistry (IHC). (a) Normal bile duct tissue. (b) The adjacent area of normal (liver) and cancerous (CCA) tissues (indicated by blue and black arrow, respectively). (c-f) Four tissue samples from four individual patients with CCA. In the positive areas, MUC1 were stained in both cytoplasm and on cell surface.

Figure 2. Expression of MUC1 protein in cholangiocarcinoma cell lines. (a) Immunoblot analysis showing total protein expression of MUC1 in cholangiocytes (MMNK-1), CCA cell lines (KKU-055, KKU-100, and KKU-213A), and a breast cancer cell line (MCF-7). The protein expression levels were quantitated and plotted as bar graphs (b). Expression of MUC1 in cells was detected by immunofluorescence assay (IFA) (c), and cell surface expression of MUC1 was stained without permeabilization and examined by flow cytometry (d), which was plotted as bar graphs (e). Data represents the mean ± SD of 3 independent experiments. Staining with isotype control antibody was used as a negative control for IFA (data not shown) and flow cytometry.

Figure 3. Generation of anti-MUC1-CAR T cells and phenotype determination. (a) A schematic drawing of anti-MUC1-CAR lentiviral construct. (b) Histogram plot showed anti-MUC1-CAR expression on untransduced (UTD) and transduced (anti-MUC1-CAR) T cells (left and middle panel) detected by flow cytometry. The transduction plots of both UTD and anti-MUC1-CAR T cells were merged together (right panel). (c) A dot plot showed percentage of anti-MUC1-CAR expression summarized from five independent experiments (mean ± SD). (d) The cell phenotype detected by flow cytometry, the bars with color codes: green, yellow, and pink represent unactivated PBMCs, PHA-activated lymphocytes, and anti-MUC1-CAR T cells, respectively. The data were plotted from four independent experiments (mean ± SD).

Figure 4. IFN-γ production by anti-MUC1-CAR T cells following exposure to CCA cells. (a) The representative cell gating data of IFN-γ production in untransduced T cells, untransduced plus target cells, anti-MUC1-CAR T cells alone, and anti-MUC1-CAR T cells co-cultured with MMNK-1, KKU100, and KKU-213A. (b) The raw data were plotted as a bar graph. The light pink, pink, light green, and green bars represent untransduced cells alone, untransduced cells cocultured with target cells, anti-MUC1-CAR T cells alone, and CAR T cells with target cells, respectively. Compared to UTD T cells, the p -value of IFN-γ production from anti-MUC1-CAR T exposed to KKU-100 and KKU-213A cells was 0.0123 and 0.0157, respectively. The$p$ -value of IFN-γ in anti-MUC1-CAR T cells co-cultured with MMNK-1 compared to KKU100 and KKU-213A cells was 0.0178 and 0.0057, respectively. The p -value of IFN-γ in anti-MUC1-CAR T cells exposed to KKU-100 compared to KKU-213A was 0.0412. The data were analyzed from three independent experiments.

Figure 5. Cytotoxic function of anti-MUC1-CAR T cells on MUC1-expressing CCA cells. (a) The remaining target cells detected by fluorescence microscopic method after co-culturing with anti-MUC1-CAR T cells. (b) Specific cell lysis of anti-MUC1-CAR T cells on MMNK-1, KKU-100, and KKU-213A cells examined by luciferase assay at effector to target (E:T) ratios of 1:1, 2.5:1, and 5:1, respectively. Compared to MMNK-1 cells, the p -values of anti-MUC1-CAR T cell cytotoxic function on KKU-100 and KKU-213A cells were 0.0199 and 0.0168 (at E:T of 1:1), 0.004 and 0.0222 (at E:T of 2.5:1), and 0.0008 and 0.0014 (at E:T of 5:1), all respectively. Compared to KKU-100, the killing activity of anti-MUC1-CAR T cells against KKU-213A was significantly higher (p =0.0215).

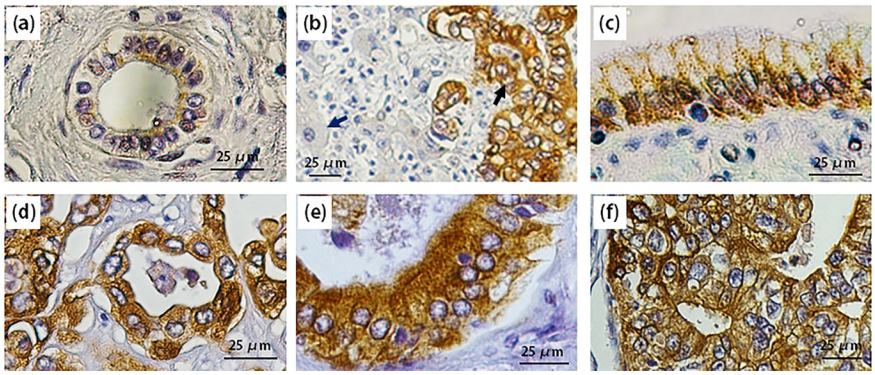

Figure 1

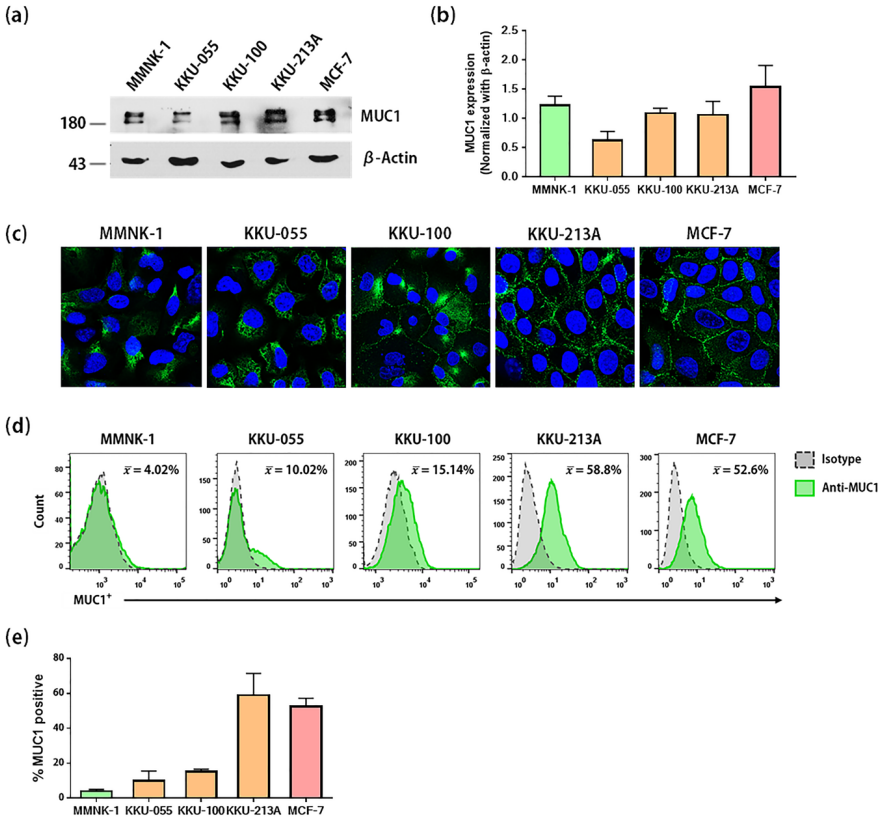

Figure 2

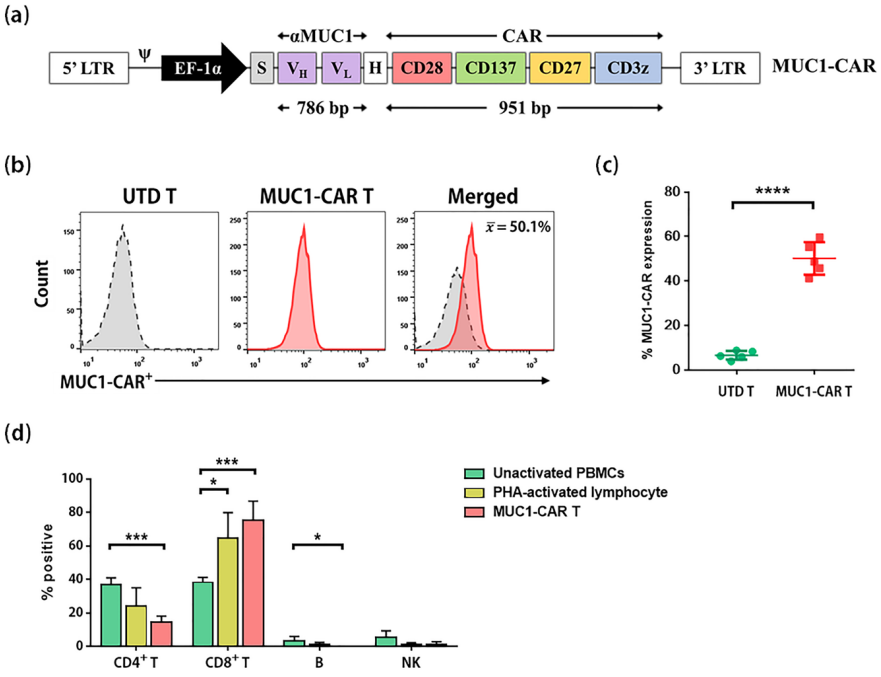

Figure 3

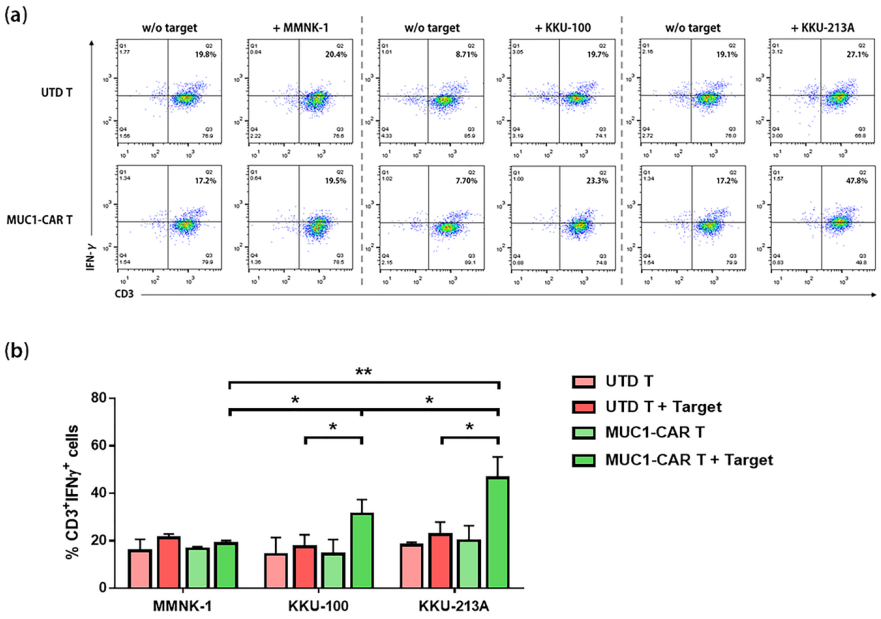

Figure 4

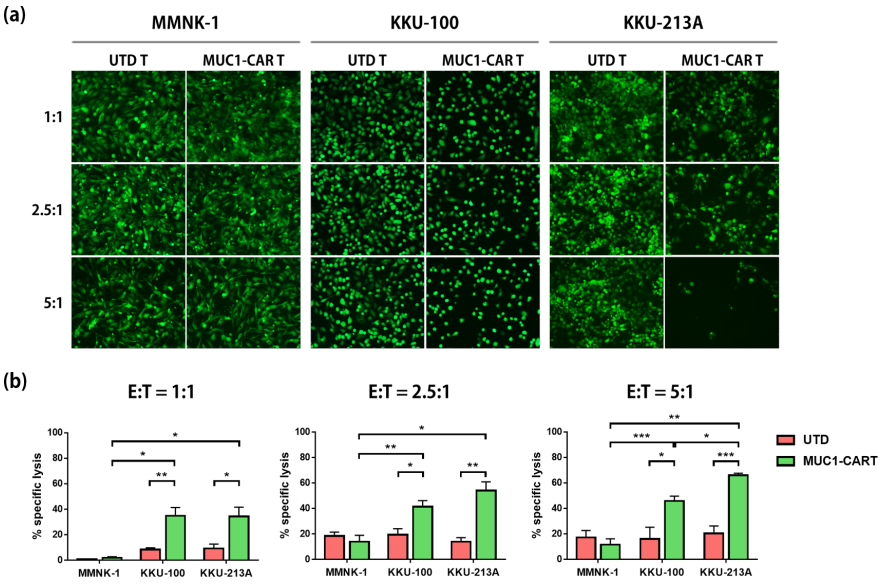

Figure 5