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Bispecific T cell engager-armed T cells targeting integrin αvβ6 exhibit enhanced T cell reeducation and antitumor activity in cholangiocarcinoma

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\textbf{A B S T R A C T}

Advanced cholangiocarcinoma (CCA) presents a clinical challenge due to limited treatment options, necessitating exploration of innovative therapeutic approaches. Bispecific T cell engager (BTE)-armed T cell therapy shows promise in hematological and solid malignancies, offering potential advantages in safety over continuous BTE infusion. In this context, we developed a novel BTE, targeting CD3 on T cells and integrin αvβ6, an antigen elevated in various epithelial malignancies, on cancer cells. The novel BTE was generated by fusing an integrin αvβ6-binding peptide (A20) to an anti-CD3 (OKT3) single-chain variable fragment (scFv) through a G4S peptide linker (A20/αCD3 BTE). T cells were then armed with A20/αCD3 BTE (A20/αCD3-armed T cells) and assessed for antitumor activity. Our results highlight the specific binding of A20/αCD3 BTE to CD3 on T cells and integrin αvβ6 on target cells, effectively redirecting T cells towards these targets. After co-culture, A20/αCD3-armed T cells exhibited significantly heightened cytotoxicity against integrin αvβ6-expressing target cells compared to unarmed T cells in both KKU-213A cells and A375.αβ6 cells. Moreover, in a five-day co-culture, A20/αCD3-armed T cells demonstrated superior cytotoxicity against KKU-213A spheroids compared to unarmed T cells. Importantly, A20/αCD3-armed T cells exhibited an increased proportion of the effector memory T cell (Tem) subset, upregulation of T cell activation markers, enhanced T cell proliferation, and increased cytolytic molecule/cytokine production, when compared to unarmed T cells in an integrin αvβ6-dependent manner. These findings support the potential of A20/αCD3-armed T cells as a novel therapeutic approach for integrin αvβ6-expressing cancers.

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\textit{Abbreviations}: 2D, Two-dimensional; 3D, Three-dimensional; A20/αCD3-Lx, A20/αCD3 BTE-secreting Lenti-X™ 293 T cells; B-ALL, B-acute lymphoblastic leukemia; BTE, Bispecific T cell engager; CAR, Chimeric antigen receptor; CCA, Cholangiocarcinoma; CD3, Cluster of differentiation 3; CD4, Cluster of differentiation 4; CD8, Cluster of differentiation 8; CD95, Cluster of differentiation 25; CD45RO, Cluster of differentiation 45RO; CD56, Cluster of differentiation 56; CD62L, Cluster of differentiation 62 L; CD69, Cluster of differentiation 69; CRS, Cytokine release syndrome; EC50, Half maximal effective concentration; FMDV, Foot-and-mouth disease virus; IFN-γ, Interferon gamma; IL-2, Interleukin-2; IL-4, Interleukin-4; IL-6, Interleukin-6; IL-7, Interleukin-7; IL-10, Interleukin-10; IL-15, Interleukin-15; IL-17A, Interleukin-17A; Lx, Lenti-X™ 293 T; MHC, Major histocompatibility complex; OV, Opisthorchis viverrine; PBMC, Peripheral blood mononuclear cell; RFP, Red fluorescence protein; scFv, Single-chain variable fragment; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TAA, tumor-associated antigen; Tem, Central memory T cells; Tem, Effector memory T cells; Temra, effector memory T cells re-expressing CD45RA; TNF-α, Tumor necrosis factor-alpha; U.S. FDA, United States Food and Drug Administration; VP1, Viral capsid protein 1.

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1. Introduction

Cholangiocarcinoma (CCA) is a malignant epithelial tumor originating from the bile duct epithelial cells lining the biliary tree [1]. Geographical variations in CCA incidence are observed, with the highest rates documented in Northeast Thailand, a region where a significant portion of the population is infected by Opisthorchis viverrini (OV), a well-known risk factor for CCA [1]. The prognosis of CCA is generally bleak, as evidenced by a 5-year survival rate ranging from 5% to 17% [2]. While resection remains the sole curative treatment; only approximately 25% of patients are eligible for surgery [3], and there is a high recurrence rate (~70%) post-surgical resection [4]. For unresectable patients, the combination of gemcitabine and cisplatin chemotherapy is recommended as the first-line therapy, but the median overall survival is only 10 months [5]. Encouragingly, various immunotherapeutic approaches for CCA, such as peptide vaccination, immune checkpoint inhibitors, and chimeric antigen receptor (CAR) T cells, are under clinical investigation [6]. Nonetheless, there is an urgent need for the exploration of novel treatments to potentially augment existing therapies and provide additional support for patients with CCA.

One promising immunotherapeutic approach involves the use of bispecific T cell engagers (BTEs) designed to redirect T cells toward cancer cells. BTEs are composed of two distinct antigen-binding domains: one targeting CD3 on T cells, and the other targets tumor-associated antigens (TAAs) on tumor cells. The interaction between T cells and tumor cells triggered by BTEs activates T cells, resulting in the elimination of tumor cells [7]. This mechanism allows T cells to directly target TAAs expressed on the tumor cell surface, independently of the major histocompatibility complex (MHC). In 2017, the United States Food and Drug Administration (U.S. FDA) approved the first BTE, which targets CD19 (αCD19/αCD3 BTE; blinatumomab), for the treatment of relapsed or refractory B-acute lymphoblastic leukemia (B-ALL) [8]. Patients treated with blinatumomab exhibited superior outcomes compared to standard-of-care chemotherapy, including improved median overall survival, event-free survival, and quality of life [8]. Currently, various other BTEs are undergoing clinical trials for both hematologic malignancies and solid tumors [9].

Nevertheless, the short half-life of BTEs mandates continuous infusion, potentially resulting in severe side effects such as cytokine release syndrome (CRS) and neurotoxicity, as observed in blinatumomab treatment [10–12]. As an alternative strategy, the concept of BTE-armed T cells has been proposed by integrating BTE therapy with adoptive T cell therapy. Clinical trials have demonstrated the efficacy of BTE-armed T cells, with the notable absence of dose-limiting toxicities in both hematological and solid malignancies [13–16]. Significantly, BTE-armed T cells require a considerably smaller amount of BTE than BTE treatment alone, potentially mitigating the severity of side effects such as CRS [17, 18]. Consequently, BTE-armed T cells hold promise as an approach to ensure potent cancer cell killing with minimal toxicity.

A crucial consideration in the application of BTEs involves the careful selection of TAAs. In the context of CCA, integrin αvβ6 emerges as a promising target antigen. Belonging to the integrin family of proteins, integrin αvβ6 forms a heterodimer on the cell membrane and is exclusively expressed on epithelial cells with strict regulation. Its upregulation is specifically associated with tissue remodeling, wound healing, and carcinogenesis [19], integrin αvβ6 represents an attractive target due to its association with cancer progression and worse overall survival in various solid tumors, including cervical squamous cell carcinoma [20], non-small cell lung cancer [21], breast cancer [22], epithelial ovarian cancer [23], pancreatic ductal adenocarcinoma [24], and CCA [25].

To target integrin αvβ6, previous studies have explored the application of the A20 peptide derived from the viral capsid protein 1 (VP1) of the foot-and-mouth disease virus (FMDV) as an integrin αvβ6-targeting domain. This peptide has found utility in many approaches, including drug conjugation [26], liposome conjugation [27], tumor imaging [28] and CAR T cells [29–31]. Our research has focused on integrin αvβ6-targeting CAR T cells using the A20 peptide as the integrin αvβ6-binding domain. These CAR T cells demonstrate highly selective killing of integrin αvβ6-expressing CCA cells compared to non-transduced T cells [30]. While CAR T cells have proven effective in cancer therapy, their production is an individualized and resource-intensive process that can be costly and time-consuming [32], potentially limiting patient accessibility to the treatment. Therefore, BTE-armed T cells present a potential alternative approach for patients who are ineligible for CAR T cell production, given the relatively less complex production process.

In this investigation, we devised a non-genetic modification strategy to undertake ex vivoarming of T cells with A20/αCD3 BTE, offering potential alternatives for patients with CCA. The BTE was designed to specifically target integrin αvβ6-expressing CCA cells. This design integrated the A20 peptide alongside the anti-CD3 (OKT3) single chain variable fragment (scFv) as the T cell binding domain. To assess the effectiveness of this approach in treating integrin αvβ6-expressing cancers, we evaluated the cytotoxic function, T cell activation, proliferation, and the secretion of cytolytic molecules/cytokines in T cells armed with A20/αCD3, comparing these outcomes with unarmed T cells. This comprehensive analysis serves as a proof of concept, demonstrating the potential utility of this strategy in the treatment of integrin αvβ6-expressing cancers, with a particular focus on CCA.

2. Materials and methods

2.1. Ethics approval

The protocol and informed consent procedures for obtaining venous blood samples from healthy volunteers received approval from the Mahidol University Multi-Faculty Cooperative Institutional Review Board in Bangkok, Thailand (COA number: MU-MOU 2023/060.1004).

2.2. Cell lines and cell culture

The human cell lines, including MMNK-1 (an immortalized cholangiocyte cell line) [33], and KU-213A (a CCA patient-derived cell line) [34], were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank in Osaka, Japan. A375.puro and A375.β6 (melanoma cell lines transduced with the pBabe puro retroviral vector or with pBabe puro encoding the integrin β6 subunit respectively) [29, 35], served as negative and positive integrin αvβ6-expressing cell lines respectively. These cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) F12 (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin at 37°C with 5% CO2. The Lenti-X™ 293 T cell line, a subclone of the transformed human embryonic kidney cell line, was acquired from Takara Bio, Inc. (Shiga, Japan). Lenti-X™ 293 T cells were cultured in DMEM (Gibco; Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin at 37°C with 5% CO2. The Raji human Burkitt lymphoma cell line was sourced from the American Type Culture Collection (ATCC) and cultured in Roswell Park Memorial Institute (RPMI)-1640 Medium (Gibco; Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin at 37°C with 5% CO2. Routine assessment for Mycoplasma contamination was performed using nested PCR.

2.3. Immunofluorescence assay

MMNK-1, KU-213A, A375.puro, and A375.β6 cells were plated on cover slips and incubated overnight. Subsequently, the cells were rinsed with phosphate-buffered saline (PBS) containing 2% FBS and fixed with 4% paraformaldehyde on ice. Afterward, a washing step with 2% FBS in...
PBS was followed by blocking with 5% bovine serum albumin (BSA) in PBS for 30 minutes on ice. The cells were then subjected to probing with the anti-integrin α6 primary antibody (Clone ITGβ6; Invitrogen, Carlsbad, CA, USA) at a concentration of 2 μg/ml at 4°C overnight. In the subsequent stage, the cells underwent additional washes and were probed with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) at a dilution of 1:500. Hoechst 33342 (Molecular probes, Eugene, OR, USA) at a concentration of 1:5000 was used for nuclear counterstaining. Following a final wash with 2% FBS in PBS, the cover slips containing cells were mounted onto glass slides using mounting reagent. Visualization was achieved using a Nikon Eclipse Ti fluorescence microscope (Nikon, Tokyo, Japan).

2.4. Flow cytometry

For integrin αvβ6 staining, MMNK-1, KU213A, A375.puro, and A375,α6 cells were fixed with 4% paraformaldehyde on ice and subsequently washed with 2% FBS in PBS. The cells were then subjected to blocking with 2% human AB serum in DMEM F12. Following another round of washing, cells were probed with mouse anti-integrin αvβ6 antibody (Clone 10D5; Merck Millipore, USA) in DMEM F12 containing 0.1% BSA on ice. Afterward, the cells underwent a washing step and were probed with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) in PBS containing 2% FBS.

For the Immunophenotype analysis of T cells, the following antibodies were used: anti-CD3 FITC (Clone UCHT-1; ImmunoTools, Friesoythe, Germany), anti-CD3 APC (Clone UCHT-1; ImmunoTools), anti-CD4 APC (Clone MEM-241; ImmunoTools), anti-CD8 APC (Clone UCHT-4; ImmunoTools), anti-CD56 PE (Clone S.1H11; BioLegend, San Diego, CA, USA), anti-human CD45RO FITC (Clone UCHL1, ImmunoTools), anti-human CD62L PE (Clone H6L2, ImmunoTools), anti-CD69 APC (Clone FN50; ImmunoTools), and anti-CD25 PerCP Cy5.5 (Clone BC96; Thermo Fisher Scientific). All samples, resuspended in 100 μl of PBS containing 2% FBS, were analyzed by a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). FlowJo software (FlowJo LLC, Ashland, OR, USA) was employed for result analysis.

2.5. A20/αCD3 BTE plasmid generation, lentiviral production, and production of A20/αCD3 BTE-secreting stable cells

The A20/αCD3 BTE DNA fragment was engineered and inserted into a self-inactivating lentivirus vector (pCDH) controlled by EF-1α promoter. This sequence encompasses the CD124 signal peptide, αCD4, and αCD3 single-chain variable fragment (scFv) (Clone OKT3), MYC tag, and 6xHis tag. Detailed components of A20/αCD3 BTE and their respective amino acid sequences are delineated in Table S1. To generate lentiviruses carrying the A20/αCD3 BTE construct, Lenti-X™ 293 T cells underwent co-transfected with A20/αCD3 BTE plasmids and two packaging plasmids (psPAX2 and pMD2, G) at a ratio of 5:3:1 using the calcium phosphate transfection method. Following co-transfection, the cells were replenished with 5% FBS DMEM. The lentiviral-containing culture supernatant was collected at 48 and 72 hours, then filtrated through a 0.45 μm filter unit to eliminate cell debris. The viral particles were concentrated at 20,000×g at 4°C for 90 minutes. Lentiviruses were titrated using the qPCR Lentivirus Titration (Titer) Kit (abm® Richmond, BC, Canada). The titrated lentiviruses were employed to transduce Lenti-X™ 293 T cells using 8 μg/ml polybrene at 37°C with 5% CO2 overnight. Since the pCDH vector contained a puromycin resistance gene, transduced Lenti-X™ 293 T cells were selected in a culture medium containing 4 μg/ml puromycin. A20/αCD3 BTE-secreting Lenti-X™ 293 T cells (A20/αCD3-Lx) were assessed using a Nikon Eclipse Ti fluorescence microscope (Nikon) and a CytoFLEX flow cytometer (Beckman Coulter) to visualize and quantify the expression of red fluorescence protein (RFP) as a transduction efficiency reporter.

2.6. A20/αCD3 BTE production and concentration

A20/αCD3-Lx cells were cultured in Opti-MEM medium (Gibco; Thermo Fisher Scientific) for 3 days to collect the secreted BTE. Subsequently, the culture supernatant containing A20/αCD3 BTE was concentrated using Amicon® Ultra-15 Centrifugal Filter Devices (Merck Millipore). A20/αCD3 BTE was subjected to analysis through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie Brilliant Blue (CB) staining of the polyacrylamide gel was then performed to assess the purity of A20/αCD3 BTE, while immunoblot analysis was conducted to detect the presence of cMyc and 6xHis tags on A20/αCD3 BTE. The concentration of A20/αCD3 BTE was quantified using the His-Tag Protein ELISA Kit (Cell Biolabs, San Diego, CA, USA).

2.7. T cell isolation and activation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood samples obtained from healthy donors using Lymphocyte Separation Medium (Corning, Inc., Corning, NY, USA) following the manufacturer’s instructions. The non-adherent cells were collected as a source of CD3+ lymphocytes (T cells) and activated for three days using 5 μg/ml phytohemagglutinin-L (PHA-L) (Sigma-Aldrich Corporation, Saint Louis, MO, USA), cultured in AIM-V medium (Gibco; Thermo Fisher Scientific) supplemented with 5% human AB serum, 10 ng/ml recombinant human interleukin (rhIL)-2, 10 ng/ml rhIL-7, and 5 ng/ml IL-15 (ImmunoTools).

2.8. Specific binding, arming, and engaging of A20/αCD3 BTE

To assess the binding capability of A20/αCD3 BTE to integrin αvβ6-expressing cells and CD3 on T cells, varying amounts of A20/αCD3 BTE (1.56–800 ng/50,000 cells) were incubated with target cells including lymphocytes, Raji, A375.puro, and A375,α6 cells. Following incubation, cells were washed with 2% FBS in PBS and subsequently incubated with anti-MYC tag FITC (Clone ab1394, Abcam, Cambridge, UK). The stained cells were then resuspended in 100 μl of 2% FBS in PBS before being analyzed by the CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). The results were analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA) to generate a dose-response curve, and the half maximal effective concentration (EC50) was determined using GraphPad software (La Jolla, CA).

For T cell arming, PHA-L-activated lymphocytes were incubated with A20/αCD3 BTE (200 ng/50,000 cells) for 30 minutes at 37°C and then washed once with RPMI. The percentage of A20/αCD3 BTE-armed T cells was analyzed by staining with anti-MYC tag FITC (Clone ab1394, Abcam, Cambridge, UK) and determined using the CytoFLEX flow cytometer (Beckman Coulter, CA).

To further validate the ability of A20/αCD3 BTE to engage T cells with A375,α6 cells, mCherry-expressing A375,α6 cells were cultured on flat-bottomed plates overnight (20,000 cells/well). Subsequently, Cell-Tracker™ Green 5-chloromethylfluorescein diacetate (CMFDA) (Thermo Fisher Scientific)-labeled lymphocytes armed with A20/αCD3 BTE (200 ng/50,000 cells) were co-cultured to A375,α6 at an effector:target (E:T) ratio of 5:1 for two hours. After washing with PBS three times, the cells were fixed with 4% paraformaldehyde. The crosslinking between A375,α6 and T cells mediated by BTEs was determined using a Nikon Eclipse Ti fluorescence microscope (Nikon). The remaining lymphocytes were then quantified.

2.9. Cytotoxicity assay

On 96-well plates, 10,000 cells/well of mCherry-expressing MMNK-1, KU213A, A375.puro, and A375,α6 cells were seeded one day prior to the addition of either unarmed or armed T cells at E:T ratios of 1.25:1, 2.5:1, and 5:1. Mean fluorescence intensity (MFI) was assessed using a
MvuiCyte live-cell imaging system (PerkinElmer, Waltham, MA, USA) at 24- and 48-hours after co-culture. Cytotoxicity was calculated using the following formula: ([MFI of target cell alone – MFI of treated condition] / (MFI of target cell alone)) × 100.

A three-dimensional (3D) spheroid model was employed to validate the killing activity of armed T cells. In brief, 2000 MMNK-1 and KUU-213A cells were labelled with CellTracker™ Blue 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF₂HC) (Thermo Fisher Scientific), mixed with 3% Corning Matrigel matrix (Corning, Inc.), and added to ultraflat attachment 96-well round-bottomed plate (Corning, Inc.). The plate was then centrifuged at 1000×g at 4°C for 10 minutes and cultured for 48 hours to form a single spheroid. Unarmed or armed T cells labeled with CellTracker™ Green CMFDA (Thermo Fisher Scientific) at a concentration of 200 ng/50,000 cells of A20/αCD3 BTE were added to the spheroids at an E:T ratio of 5:1 in the presence of 2 μg/mL propidium iodide (PI). The dead spheroids were analyzed by quantitating the increase in PI mean fluorescence intensity (MFI) using a Nikon Eclipse Ti fluorescence microscope (Nikon) and quantified with NIS-Elements software. Cytotoxicity was calculated using the following formula: ([MFI of treated condition – MFI of spheroid alone]/MFI of positive control – MFI of spheroid alone) × 100. The positive control was a spheroid treated with 0.1% Triton-X 100.

2.10. T cell proliferation assay

For the assessment of T cell proliferation, T cells were pre-labeled with CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) (eBioscience, San Diego, CA, USA) for 10 minutes at 37°C and subsequently co-cultured with MMNK-1 or KUU-213A cells at an E:T ratio of 2:1 (30,000:15,000) for 48 hours. The dilution of CFSE was analyzed using a CytoFLEX flow cytometer (Beckman Coulter).

2.11. Cytolytic molecule/cytokine production

A20/αCD3 BTE-armed T cells or unarmed T cells were co-cultured with MMNK-1 or KUU-213A cells at an E:T ratio of 5:1 for 48 hours. Subsequently, the culture supernatants were collected, centrifuged to remove cell debris, and stored at −70°C. The levels of cytolytic molecules and cytokines in the culture supernatants were assessed using the Cytokine Bead Array (CBA) from the LEGENDplex™ Human CD8/NK cell panel (#741065, BioLegend), following the manufacturer’s protocol. This panel enables the simultaneous quantification of 13 human cytokines and proteins, including IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, soluble Fas, soluble Fasl, granzyme A, granzyme B, perforin, and granulysin. The samples were analyzed using a CytoFLEX flow cytometer (Beckman Coulter).

2.12. Statistical analysis

The experimental results were derived from a minimum of three independent experiments and are presented as the mean ± standard error of the mean (SEM). Student’s t-test was employed for comparisons between two treatment groups, while one-way analysis of variance (ANOVA) was utilized for comparisons involving more than two treatment groups. Statistical significance for each comparison was assessed using GraphPad software (La Jolla, CA). Differences with a p-value less than 0.05 were considered statistically significant.

3. Results

3.1. Integrin αvβ6 expression in target cell lines

In this investigation, we assessed the presence of integrin αvβ6 in target cell lines, namely MMNK-1 (an immortalized cholangiocyte cell line) and KUU-213A cells (a cholangiocarcinoma cell line), utilizing both flow cytometry and immunofluorescence assays. A375.puro and A375.β6 melanoma cells, known for their negative and positive integrin αvβ6 expression, respectively, were employed as control cell lines [29, 35]. The analysis disclosed a minimal percentage of integrin αvβ6 expression on the cell surface of MMNK-1 (0.63 ± 0.03%) and A375. puro (3.30 ± 0.67%) cells. In contrast, KUU-213A (97.33 ± 3.38%) and A375.β6 (98.73 ± 0.18%) cells exhibited robust integrin αvβ6 expression, surpassing 90% (Fig. 1A-B). Importantly, immunofluorescence analysis confirmed these flow cytometry findings. Specifically, integrin αvβ6 was observed in KUU-213A and A375.β6 cells, whereas no detectable expression of integrin αvβ6 was evident in MMNK-1 and A375.puro cells (Fig. 1C).

3.2. A20/αCD3 BTE production

We generated the A20/αCD3 BTE construct by fusing the A20 peptide sequence, which targets integrin αvβ6, with the anti-CD3 scFv sequence (OKT3). This construct was incorporated into a lentiviral backbone plasmid, utilizing the self-cleaving peptide T2A to enable co-expression of red fluorescence protein (RFP) as a transduction efficiency reporter. Successful gene transfer of the A20/αCD3 BTE construct into Lenti-X™ 293 T (Lx) cell line was achieved through lentiviral vectors (Fig. 2A). Following puromycin selection, RFP expression was evident in Lenti-X™ 293 T cells (Fig. 2B). Flow cytometric analysis of cells transduced with the A20/αCD3 BTE-encoded lentiviral vector (A20/αCD3-Lx) revealed a remarkably high expression level (99.93 ± 0.03%) compared to non-transduced Lx cells (0.98 ± 0.08%) (p<0.0001) (Fig. 2C-D). Culture supernatant from A20/αCD3-Lx confirmed the successful secretion of A20/αCD3 BTE, verified through Coomassie Brilliant Blue (CB) staining and immunoblot analysis using anti-cMyc and anti-6xHis antibodies. The A20/αCD3 BTE protein was detected at the predicted molecular weight of approximately 35 kDa (Fig. 2E). Complete triplicate immunoblots are depicted in Fig. S1. Suggesting that we successfully generated A20/αCD3 BTE.

3.3. A20/αCD3 BTE bound specifically to T cells and integrin αvβ6-expressing cells

To assess the binding affinity of A20/αCD3 BTE for its specific antigens on both T cells and integrin αvβ6-expressing cells, Fig. 3A illustrates schematic representations of A20/αCD3 BTE binding as analyzed by flow cytometry. A20/αCD3 BTE binds to T cells through the anti-CD3 scFv domain and to integrin αvβ6 via the A20 peptide domain. Flow cytometry results revealed significantly higher A20/αCD3 BTE binding on lymphocytes expressing CD3 molecules compared to control Raji cells at the highest A20/αCD3 BTE dose (800 ng/50,000 cells) (77.5 ± 3.51% vs. 1.08 ± 0.05%, p<0.0001), with a half maximal effective concentration (EC50) of 27.72 ng/50,000 cells (Fig. 3B-C). Similarly, A20/αCD3 BTE (800 ng/50,000 cells) exhibited significantly higher binding to A375.β6 cells compared to A375.puro cells (86.37 ± 6.6% vs. 4.18 ± 0.56%, p<0.001), with an EC50 of 31.36 ng/50,000 cells (Fig. 3D-E), confirming the selective binding of A20/αCD3 BTE to its target antigens, including CD3 and integrin αvβ6. Fig. S2 illustrates the levels of CD3 expression in lymphocytes or Raji cells and integrin αvβ6 in A375.puro or A375.β6 cells, and indicates control for binding assay.

To validate the specific bridging ability of A20/αCD3 BTE between T cells and integrin αvβ6-expressing cells, we utilized PHA-L-activated lymphocytes as a source of T cells, demonstrating a higher proportion of T cells compared to non-activated lymphocytes (Fig. S3). Then, we armed PHA-L-activated T cells with A20/αCD3 BTE at a concentration of 200 ng/50,000 cells (referred to as A20/αCD3-armed T cells). This concentration, selected for its robust binding capacity in lymphocytes comparable to the highest dose (800 ng/50,000 cells), demonstrated significant binding to lymphocytes compared to Raji cells (76.2 ± 3.325% vs. 1.879 ± 0.058%, p<0.0001) (Fig. 3B-C). CMFDA-labeled T cells armed with A20/αCD3 BTE at 200 ng/50,000 cells were co-cultured with mCherry-expressing A375.β6 cells. Following washing,
A20/αCD3-armd T cells were observed surrounding A375.β6 cells, with a significantly higher number of remaining lymphocytes compared to unarmed T cells (187.3 ± 35.63 cells vs. 16.33 ± 7.54 cells, p < 0.01) (Fig. 3F–G). These results indicate that A20/αCD3 BTE specifically binds on its target molecules including CD3 and integrin αβ6 which promotes T cell redirection toward integrin αβ6-expressing target cells.

3.4. Antitumor activity of A20/αCD3-armd T cells against integrin αβ6-expressing cells

We proceeded to evaluate the antitumor efficacy of A20/αCD3-armd T cells using the Muvicycle live-cell imaging system. A20/αCD3-armd T cells and unarmed T cells were co-cultured with mCherry-expressing target cell lines, including MMNK-1, KKKU-213A, A375.puro, and A375.β6 cells. Following a 24-hour co-culture, A20/αCD3-armd T cells exhibited significantly highest cytotoxicity against KKKU-213A cells (28.37 ± 5.26% vs. 10.97 ± 4.7%, p < 0.05) and A375.β6 cells (28.82 ± 2.95% vs. 7.92 ± 5.62%, p < 0.05) at an E:T ratio of 5:1 compared to unarmed T cells. After 48 hours of co-culture, the E:T ratio of 5:1 also yielded the highest cytotoxicity in KKKU-213A cells (51.5 ± 3.78% vs. 15.2 ± 3.79%, p < 0.0001) and A375.β6 cells (46.17 ± 2.56% vs. 21.79 ± 7.91%, p < 0.05) when comparing A20/αCD3-armd T cells and unarmed T cells. Additionally, A20/αCD3-armd T cells demonstrated increased specific killing at 48 hours compared to 24 hours at an E:T ratio of 5:1 in KKKU-213A cells (51.5 ± 3.78% vs. 28.37 ± 5.26%, p < 0.05) and at E:T ratios of 1:25:1 (35.44 ± 1.42% vs. 16.7 ± 2.1%, p < 0.001), 2:5:1 (41.51 ± 1.45% vs. 26.44 ± 2.5%, p < 0.01), and 5:1 (46.17 ± 2.56% vs. 28.82 ± 2.95%, p < 0.01) in A375.β6 cells (Fig. 4C–D and G–H). However, A20/αCD3-armd T cells did not exhibit significant killing activity against integrin αβ6-negative cells, including MMNK-1 and A375.puro (Fig. 4A–B and E–F). These findings indicate that A20/αCD3-armd T cells display specific cytotoxicity against integrin αβ6-expressing cell lines, demonstrating superior function in a time-dependent manner.

To better replicate in vivo conditions characterized by complex cell-extracellular matrix interactions, we employed a three-dimensional (3D) spheroid culture model, in contrast to the traditional two-dimensional (2D) culture model where cells grow as a monolayer. Spheroids, mimicking solid tumor masses, were formed by culturing CMFDA-labelled A20/αCD3-armd T cells or unarmed T cells at an E:T ratio of 5:1 in the presence of propidium iodide (PI). The destruction of KKKU-213A spheroids was evident through increased PI intensity in A20/αCD3-armd T cells compared to unarmed T cells (27.64 ± 5.52% vs. 1.47 ± 3.67%, p < 0.05) and day 5 after co-culture (34.08 ± 4.37% vs. 2.4 ± 2.04%, p < 0.01). A20/αCD3-armd T cells also exhibited toxicity to KKKU-213A spheroids at day 3 and 5, significantly higher than day 1 after co-culture (27.64 ± 5.52% vs. 3.39 ± 5.24%, p < 0.05 and 34.08 ± 4.37% vs. 3.39 ± 5.24%, p < 0.05, respectively) (Fig. 5C–D). In MMNK-1 spheroids, A20/αCD3-armd T cells showed non-significant cytotoxicity compared with unarmed T cells during the observed time (Fig. 5A–B). Thus, A20/αCD3-armd T cells specifically demonstrate antitumor activity against integrin αβ6-expressing spheroids.

3.5. A20/αCD3 BTE-armd T cells specifically exhibited expansion of effector memory T cell subset, T cell activation, and T cell proliferation through co-culture with integrin αβ6-expressing cells

We then examined the impact of A20/αCD3 BTE on T cells following co-culture with target cells. In KKKU-213A cells, we observed a significant decrease in the proportion of naïve T cells (CD3+, CD45RO–, CD62L–) in A20/αCD3-armd T cells compared to unarmed T cells (22.97 ± 14.15% vs. 60.23 ± 8.39%, p < 0.05). Conversely, the population of effector memory T cells (Tem) (CD3+, CD45RO+, CD62L–) increased in A20/αCD3-armd T cells compared to unarmed T cells (29.25 ± 11.24%...
However, both A20/αCD3-armed T cells and unarmed T cells did not exhibit significant differences in the memory T cell subset profile when cultured alone (no target cells) or co-cultured with MMNK-1 cells (Fig. 6A–B). Moreover, the proportion of T (CD3+, CD56−), NK (CD3−, CD56+), and NKT (CD3+, CD56+) cells from unarmed T cells and A20/αCD3-armed T cells were not significantly different after co-culture with MMNK-1 or KU-213A or during culture alone (Fig. S4A–B). Additionally, unarmed T cells and A20/αCD3-armed T cells did not show significant changes in the percentage of both CD4+ and CD8+ T cells (Fig. S4C–D). T cell activation markers, including CD69 and CD25, were significantly increased in A20/αCD3-armed T cells compared with unarmed T cells when co-

**Fig. 2.** Generation of A20/αCD3 BTE protein. (A) Schematic representation of the A20/αCD3 BTE construct and the process of A20/αCD3 BTE production. (B) Fluorescence images depicting RFP expression in non-transduced Lenti-X™ 293 T cells (non-transduced-Lx) (upper panel) and A20/αCD3 BTE-secreting Lenti-X™ 293 T cells (A20/αCD3-Lx) (lower panel). Scale bars represent 100 μm. (C) Flow cytometry histogram illustrating RFP expression in A20/αCD3-Lx cells compared to non-transduced-Lx cells (light gray). (D) Quantification of RFP expression percentages. (E) A20/αCD3 BTE protein obtained from the culture medium of A20/αCD3-Lx cells were detected using Coomassie Brilliant Blue (CB) staining and immunoblot analysis. Results are presented as mean ± standard error of the mean (SEM) (n = 3). Statistical differences were determined by Student’s t-test, and asterisks denote the following p-value: ****p<0.0001.
cultured with KUK-213A, the percentage of CD69 was 54.25 ± 5.01% vs. 30.58 ± 3.67% (p<0.01), and CD25 was 73.78 ± 7.72% vs. 30.17 ± 8.02% (p<0.01). In the absence of target cells and during co-culturing with MMNK-1, unarmed T cells and A20/αCD3-armed T cells showed non-significant differences in both CD69 and CD25 (Fig. 6C–F). This suggests that A20/αCD3 BTE promotes Tem expansion and induces T cell activation in an integrin αvβ6-dependent manner.

T cell proliferation was assessed in A20/αCD3-armed T cells and unarmed T cells following 72 hours of co-culture with MMNK-1 and KUK-213A cells at an E:T ratio of 2:1. Proliferation was quantified as the percentage of T cells with diluted CFSE intensity. A20/αCD3-armed T cells exhibited a significantly heightened proliferation rate when co-cultured with KUK-213A cells compared to unarmed T cells (76.6 ± 3.54% vs. 18.85 ± 2.27%, p<0.0001), whereas no significant differences were observed when cultured alone or co-cultured with MMNK-1 cells (Fig. 6G–H). This suggests that the proliferation of A20/αCD3-armed T cells is specifically induced by integrin αvβ6 positive target cells.

### 3.6. Cytolytic molecule and cytokine production of A20/αCD3-armed T cells in response to integrin αvβ6-expressing cells

In this investigation, we explored the production of cytolytic molecules and cytokines in response to integrin αvβ6-expressing cells. Culture supernatants from A20/αCD3-armed T cells and unarmed T cells, cultured alone or co-cultured with MMNK-1 or KUK-213A cells, underwent analysis using the cytokine bead array system (Bethyl). The levels of cytolytic molecules were significantly elevated when compared to unarmed T cells. This included granulysin (10,670 ± 728 pg/ml vs. 37.81 ± 58.52 pg/ml, p<0.001), granzyme B (83,631 ± 728 pg/ml vs. 5082 ± 3359 pg/ml, p<0.001), perforin (1010 ± 72.69 pg/ml vs. 353.4 ± 68.53 pg/ml, p<0.01), granulysin (10,670 ± 582 pg/ml vs. 1608 ± 728 pg/ml, p<0.001), and soluble FAS ligand (sFASL) (283.2 ± 7.81 pg/ml vs. 58.52 ± 14.99 pg/ml, p<0.001) (Fig. 7A–E). Furthermore, T helper 1 cytokines were also increased in culture supernatants collected from A20/αCD3-armed T cells co-cultured with KUK-213A-armed T cells, including IL-2 (58.6 ± 12.1 pg/ml vs. 1.46 ± 1.46 pg/ml, p<0.01), TNF-α (439 ± 97.43 pg/ml vs. 37.17 ± 7.03 pg/ml, p<0.05), and IFN-γ (64,050 ± 6713 pg/ml vs. 2939 ± 966.4 pg/ml, p<0.001) (Fig. 7F–H). Additionally, the level of IL-17A was elevated in A20/αCD3-armed T cells compared to unarmed T cells in the presence of KUK-213A cells (60.35 ± 9.62 pg/ml vs. 3.58 ± 0.28 pg/ml, p<0.01) (Fig. 7I). Conversely, these secretory molecules did not exhibit significant differences between A20/αCD3-armed T cells and unarmed T cells in conditions of cultured alone or MMNK-1 co-culture. Moreover, the levels of IL-4, IL-6, IL-10, and IFN-γ were not significantly different under every condition when comparing A20/αCD3-armed T cells and unarmed T cells (Fig. 8A–D). These findings suggest that A20/αCD3-armed T cells respond to integrin αvβ6-expressing cancer cells by secreting cytolytic molecules and cytokines, potentially contributing to enhanced anti-tumor activity compared to unarmed T cells.

### 4. Discussion

Given the limited efficacy of standard care for advanced cholangiocarcinoma (CCA), there is an imperative to explore innovative therapeutic approaches. In this study, we devised an immunotherapy strategy utilizing the novel A20/αCD3 bispecific T cell engager (BTE) and examined the antitumor activity of T cells armed with this BTE (A20/αCD3-armed T cells). Our research investigation revealed that A20/αCD3-armed T cells demonstrated activation, proliferation, production of cytolytic molecules/cytokines, and elimination of target cells in an integrin αvβ6-dependent manner, highlighting their potential for the development of CCA treatments.

The upregulation of integrin αvβ6 has been documented in CCA tissues, ranging from 25–88%, and is associated with poor patient survival [25,30,36,37]. In our previous study, we found that 73.3% of liver fluke associated CCA specimens from Thai patients exhibited integrin αvβ6 positivity, with high-level expression correlating with reduced survival time [30]. This elevated expression of integrin αvβ6 in CCA tissues suggests a substantial proportion of CCA patients may benefit from integrin αvβ6-targeted treatment. In this study, we confirmed integrin αvβ6 expression in four cell lines, including MMNK-1 (an immortalized human cholangiocyte cell line), KUK-213A (a CCA patient-derived cell line), A375.puro, and A375.I6 cells (Fig. 1A–C). These cell lines were employed as target cells to evaluate A20/αCD3-armed T cell antitumor responses.

The primary limitation of BTEs is their short half-life in serum, necessitating continuous administration to maintain therapeutic concentrations [11,40]. Elevated BTE levels have been associated with adverse events [12,13]. Consequently, an alternative approach involving the arming of T cells with BTEs has been proposed, demonstrating notable efficacy, a favorable safety profile, and the absence of dose-limiting toxicities in clinical trials [14–17]. Therefore, in this study, we generated A20/αCD3-armed T cells to evaluate their anti-tumor activity against integrin αvβ6 expressing cells. The A20/αCD3 BTE was produced from A20/αCD3 BTE-secreting Lenti-X™ 293 T cells (A20/αCD3-Lx) (Fig. 2). This A20/αCD3 BTE exhibited the ability to bind cells expressing CD3 or integrin αvβ6. T cells armed with A20/αCD3 BTE at 200 ng/50,000 cells effectively redirected to integrin αvβ6-positive target cells (A375.I6) (Fig. 3). Previous findings demonstrate the specific binding of A20 to integrin αvβ6 [29]. These results confirm the specificity and capacity of A20/αCD3 BTE in redirecting T cells toward integrin αvβ6-expressing cancer cells.

Our 2D killing assays revealed that A20/αCD3-armed T cells exhibited specific cytotoxicity against integrin αvβ6-expressing target cells, including KUK-213A and A375.I6 cells, in a time-dependent manner. Conversely, minimal killing activity was observed in MMNK-1 and A375.puro cells, lacking integrin αvβ6 expression (Fig. 4). These results indicate that the specific killing activity of A20/αCD3-armed T cells is influenced by integrin αvβ6, in a time-dependent manner. Acknowledging the limitations of traditional 2D culture systems, which may not replicate the natural structure of tumor masses, we extended our studies to include 3D culture models that mimic tumor mass, cell–cell, and cell–extracellular matrix interactions [38]. Notably, A20/αCD3-armed T cells induced cytotoxicity in KUK-213A spheroids in a time-dependent manner, with lower killing activity observed in MMNK-1 spheroids (Fig. 5). It is noteworthy that although A20/αCD3-armed T cells exhibited comparable killing activity to 4th generation CAR T cells targeting integrin αvβ6 (A20-4G CAR T cells) in a 2D co-culture model, they demonstrated relatively inferior killing activity against CCA spheroids [30]. This discrepancy may be attributed to the absence of...
Fig. 4. Assessment of the killing activity of A20/αCD3-armed T cells against target cells. The killing assay was conducted to measure the remaining mCherry-expressing target cells following co-culture with unarmed T cells or A20/αCD3-armed T cells for 24 and 48 hours. Representative images depict the residual target cells, and the bar graphs illustrate the percentages of cytotoxicity for (A–B) MMNK-1, (C–D) KU-213A, (E–F) A375.puro, and (G–H) A375.β6 cells after co-culture at 24 and 48 hours across different E:T ratios. Results are presented as mean ± standard error of the mean (SEM). (n = 4). Statistical differences were determined using Student’s t-test, with asterisks denoting the following p-values: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
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Fig. 5. Cytotoxic effects of A20/αCD3-armed T cells on cancer spheroids. Representative images and a bar graph illustrating the killing activity of A20/αCD3 BTE-armed T cells against (A–B) MMNK-1 and (C–D) KKU-213A spheroids at an E:T ratio of 5:1 for 1, 3 and 5 days post co-culture compared to unarmed T cells. The scale bar represents 200 nm. Spheroid destruction was evaluated through propidium iodide (PI) incorporation (depicted in red). Results are presented as mean ± standard error of the mean (SEM). (n = 3). Statistical differences were determined using Student’s t-test, with asterisks denoting the following p-values: *p<0.05, **p<0.01.

Concomitantly, costimulatory signals provided to A20/αCD3-armed T cells, present in A20-CAR-4G (CD28, 4-1BB, CD27), with these costimulatory molecules enhancing T cell activation, proliferation, survival, and cytokine production to collectively augment the antitumor activity of T cells [39]. However, the manufacturing process of CAR T cells is notably intricate compared to BTE-armed T cells. Given that genetic modification is not a prerequisite, the arming process simply involves the incubation of T cells with BTEs, rendering it more versatile and less complex in terms of production. This streamlined approach has the potential to enhance accessibility to the treatment for patients who are ineligible for CAR T cell production.

The effector memory T cell (Tem) subset emerges as a particularly advantageous population for BTEs, displaying high cytotoxic potency [40]. Our findings revealed a selective increase in the Tem population within A20/αCD3-armed T cells upon encountering KKU-213A cells (Fig. 6A–B). This observation aligns with previous studies, emphasizing the predominant expansion of Tem in patients treated with BLINATMAB [41]. Present data also substantiate Tem as the main subpopulation among proliferating T cells, hypothesized to mediate cytotoxicity in response to BTE treatment [42]. Furthermore, A20/αCD3-armed T cells demonstrated exclusive upregulation of both early (CD69) and late (CD25) T cell activation markers following exposure to KKU-213A cells (Fig. 6C–F). These activation markers are directly upregulated upon TCR-CD3 complex activation [43] and play crucial roles in facilitating T cell recruitment to inflamed tissues (CD69) [44–46] or maintaining the high-affinity of IL-2 receptor expression (CD25) [47]. Additionally, A20/αCD3-armed T cells exhibited increased proliferation specifically in response to KKU-213A cell exposure (Fig. 6G–H), potentially stemming from TCR-CD3 complex activation and enhanced IL-2 receptor affinity via CD25 upregulation. These findings offer insights into the distinctive responses of A20/αCD3-armed T cells, suggesting their specificity for integrin αvβ6-expressing cancer cells, thereby promoting Tem subset expansion, T cell activation, and proliferation.

BTEs are intricately engineered to concomitantly engage a chosen tumor-associated antigen (TAA) and CD3ε, thus possessing signaling capacities. Upon interaction with both T cells and tumor cells, BTEs facilitate the formation of an artificial immune synapse, prompting T cell activation and bolstering antitumor responses [7]. The most plausible explanation underlying BTE-mediated targeted cytotoxicity involves the specific binding of the anti-CD3 scFv component to a surface molecule on target cells, likely the CD3 complex. This binding event could emulate natural T cell activation, instigating downstream signaling molecules such as PKC-θ, which activates NF-κB pathway [48]. Consequently, there is an upsurge in cytolytic molecule and cytokine production. Cytolytic molecules directly compromise the integrity of the target cell membrane, while cytokines recruit and activate cytotoxic T lymphocytes (CTLs), thereby further amplifying target cell death. Following co-culture with KKU-213A cells, A20/αCD3-armed T cells demonstrated heightened production of cytolytic molecules including granzyme A, granzyme B, perforin, and granulysin compared to unarmed T cells (Fig. 7A–D). Additionally, elevated levels of soluble FAS ligand (sFASL), the cleaved form of FASL facilitated by matrix metalloproteinase, were observed in the culture supernatant collected from A20/αCD3-armed T cells after co-culturing with KKU-213A cells (Fig. 7E). Notably, sFASL has the capacity to induce target cell apoptosis through association with extracellular matrix proteins or spontaneous aggregation [63]. Moreover, A20/αCD3-armed T cells exhibited heightened production of IL-2, TNF-α, and IFN-γ in an integrin αvβ6-dependent manner compared to unarmed T cells (Fig. 7F–H). These findings align with previous clinical studies that demonstrated increased levels of T helper 1 cytokines in metastatic breast cancer patients treated with aHER2/αCD3-armed T cells, fostering an antitumor environment [49]. Conversely, levels of T helper 2 cytokines, including IL-4 and IL-10, remained comparable between unarmed T cells and...
Figure A: Flow cytometry analysis of CD62L-PE expression on T cells from Unarmed and Armed T cells with No target, MMNK-1, and KKKU-213A cells. The results are presented as dot plots and bar graphs showing the percentage of positive cells.

Figure B: Bar graph showing the percentage of Temra, Tem, Tcm, and Naive cells in Unarmed and Armed T cells with No target, MMNK-1, and KKKU-213A cells. Significant differences are indicated by asterisks.

Figure C: Flow cytometry analysis of CD69-APC expression on T cells from Unarmed and Armed T cells with No target, MMNK-1, and KKKU-213A cells. The results are presented as dot plots and bar graphs showing the percentage of positive cells.

Figure D: Bar graph showing the percentage of CD3+CD69+ cells in Unarmed and Armed T cells with No target, MMNK-1, and KKKU-213A cells. Significant differences are indicated by asterisks.

Figure E: Flow cytometry analysis of CD25-PerCP Cy5.5 expression on T cells from Unarmed and Armed T cells with No target, MMNK-1, and KKKU-213A cells. The results are presented as dot plots and bar graphs showing the percentage of positive cells.

Figure F: Bar graph showing the percentage of CD3+CD25+ cells in Unarmed and Armed T cells with No target, MMNK-1, and KKKU-213A cells. Significant differences are indicated by asterisks.

Figure G: Flow cytometry analysis of CFSE expression on T cells from Unarmed and Armed T cells with No target, MMNK-1, and KKKU-213A cells. The results are presented as dot plots and bar graphs showing the percentage of positive cells.

Figure H: Bar graph showing the percentage of proliferation in Unarmed and Armed T cells with No target, MMNK-1, and KKKU-213A cells. Significant differences are indicated by asterisks.
A20/αCD3-armed T cells when exposed to MMNK-1 or KKU-213A cells (Fig. S6A–B). These T helper 2 cytokines are implicated in promoting tumor growth and counteracting T helper 1-mediated responses [50]. Previous reports have indicated that the presence of T helper 2 cells is correlated with a poor prognosis in various solid tumors [51–55]. Additionally, higher levels of IL-17A were observed in A20/αCD3-armed T cells compared to unarmed T cells after encountering KKU-213A cells (Fig. 7). IL-17A, a proinflammatory cytokine, plays a dual role in tumor progression and metastasis, promoting inflammatory angiogenesis that can enhance tumor growth and metastasis. However, it also exhibits antitumor effects by modulating adaptive immune responses, recruiting T cells, enhancing NK cell activity, and promoting the generation and activation of cytotoxic T cells [56,57]. Our results suggest that A20/αCD3-armed T cells facilitate the killing of integrin αvβ6-expressing cancer cells through the secretion of cytolytic molecules and preferential release of T helper 1 cytokines, thereby supporting an antitumor environment.
A20/αCD3-armed T cells is specifically dependent on integrin αvβ6 expressed on cancer cells. Remarkably, previous studies have reported that BTE-armed T cells exhibit lower cytokine production compared to BTE plus unarmed T cells in mouse model. This effect is achieved through the removal of excess BTE, which has the potential to induce toxicity. Importantly, despite this reduction in cytokine levels, BTE-armed T cells still demonstrate comparable trafficking ability and antitumor activity [60]. In addition, clinical studies of BTE-armed T cells also confirmed the absence of severe CRS, with observed improvements in clinical outcomes [13,61,62]. These results verified the efficacy of BTE-armed T cells, highlighting their safety profile by mitigating excessive cytokine production, thereby potentially reducing the risk of life-threatening CRS.

To ensure both efficacy and safety in clinical application, forthcoming studies should prioritize validating the effectiveness of A20/αCD3-armed T cells in animal models. This validation constitutes a critical step toward establishing the foundation for subsequent clinical trials. Moreover, addressing a major obstacle in the context of solid tumors, including tumor heterogeneity, could be achieved by employing multiple BTEs targeting various antigens on tumor cells. Utilizing dual BTEs-armed T cells has been reported to exhibit superior efficacy compared to the use of a single BTE [63]. For CCA, several TAAs have been proposed as targets for cancer immunotherapy, including epidermal growth factor receptor (EGFR) [64], mucin-1 (MUC-1) [65], CD133 [66], and PD-L1 [67]. These antigens represent potential targets for the development of BTE-armed T cells, in combination with A20/αCD3 BTE, to address immune escape mechanisms such as antigen downregulation or loss. Additionally, it is noteworthy that A20/αCD3 BTE provides only the initial signal from anti-CD3 scFv, potentially falling short of completing the T cell activation process. To address this, the introduction of a costimulatory signal to achieve full T cell activation is a viable strategy. Previous proposals have suggested the use of BTEs that bind to T cells on CD28 [68]. In this proposed approach, αPD-L1/αCD28 BTE could be combined with αCD19/αCD3 BTE. It is suggested that T cells activated by these two BTEs would receive a complete activation signal and are protected from inhibitory signal from PD-L1, an immune checkpoint molecule expressed on tumor cells. Importantly, aberrant PD-L1 expression has been identified in CCA tissues [69], suggesting that the application of αPD-L1/αCD28 BTE in combination with A20/αCD3 BTE could enhance antitumor efficacy by countering immune evasion mechanisms and concurrently co-activating T cells.

5. Conclusion

In summary, our findings demonstrate that the use of armed T cells with A20/αCD3 BTE induces T cell redirection, potentially leading to the elimination of cancer cells expressing integrin αvβ6 in both monolayer and spheroid culture system. Furthermore, A20/αCD3-armed T cells exhibited increased expression of activation markers, enhanced proliferation, and heightened production of cytokytic molecules/cytokines upon exposure to target cells. These results collectively provide initial proof-of-concept for the utilization of A20/αCD3 BTE-armed T cells as an alternative approach in the treatment of CCA.

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CRediT authorship contribution statement

Kwanpirom Suwanchiwasiri: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft preparation. Nattaporn Phanthaphol: Conceptualization, Methodology, Validation, Project administration, Supervision, Writing – review & editing. Chalermchai Somboonpataarun: Methodology. Pornpimon Yuti: Methodology. Jatupon Sujjitjoon: Methodology. Piirya Luangwattananun: Conceptualization, Methodology. John Maher: Resources, Writing – review & editing. Pa-thai Yenchitsomanus: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. Mutita Junking: Conceptualization, Data curation, Validation, Funding acquisition, Project Administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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Institutional review board statement

The study was conducted in compliance with the principles outlined in the Declaration of Helsinki and was approved by the Mahidol University Multi-Faculty Cooperative Institutional Review Board in Bangkok, Thailand (COA number: MU-MOU 2023/060.1004).

Informed consent statement

Written informed consent was obtained from all participants prior to their inclusion in the study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116718.

References

D. Bhutani, L.G. Lum, Activated T cells armed with bispecific antibodies kill tumor
M. Zhu, B. Wu, C. Brandl, J. Johnson, A. Wolf, A. Chow, et al., Blinatumomab, a
N. Ahmed, C. Riley, G.E. Rice, M.A. Quinn, M.S. Baker, Alpha(v)beta(6) integrin-A
K. Suwanchiwasiri et al.
M.-E. Goebeler, R.C. Bargou, T cell-engaging therapies
S. Zhou, M. Liu, F. Ren, X. Meng, J. Yu, The landscape of bispecific T cell engager in
678, https://doi.org/10.1097/s410678.6.
N. Phantholph, C. Somboonpattarakas, K. Suwanchiwasiri, T. Chieochanin, J.
S. Juijtoon, S. Wongkhamp, et al., Chimeric antigen receptor T cells targeting
gene expression in solid tumors using chimeric antigen receptor-engineered T cells, Mol.
L. Whilding, L. Halim, B. Draper, A.C. Parente-Pereira, T. Zabinski, D.M. Davies,
et al., CAR T cells targeting the integrin αvβ6 and Co-Expressing the chemokine
recognition for CXCR2 demonstrated anti-tumor immunity and efficacy against
P. Salmikangas, N. Kinsella, P. Chamberlain, Chimeric antigen receptor T-cells (CAR
T-cells) for cancer immunotherapy - moving target for industry? Pharm. Res.
M. Maruyama, K. Kobayashi, K.A. Westerman, M. Sakagushi, J.E. Allain,
targeting liposomal alendronate for combinatory
and cisplatin for advanced biliary tract cancer, Crit. Rev. Oncol./Hematol. 80 (1)
B. Sofia, R. Leoni, A. Zucchetti, L. Musi, A. Coccia, et al., The role of anti-CD138
in dextran sulphate sodium-induced diseases, Theranostics 10 (7) (2020) 2930–2942,
https://doi.org/10.7150/thno.37807.
N. Kizaki, T. Kondo, W. Przybysz, A. Teresaki, V. Filas, et al., 2D and 3D cell cultures - a comparison of different types of cancer cell cultures, Arch.
R. Weinkove, P. George, N. Danyan, A.D. McLean, Selecting cancerous
immunological synapses in T cell-redirecting strategies for cancer immunotherapy,
M. Klinger, J. Benjamín, R. Kischel, S. Stienenger, G. Zugaibier, Harnessing T cells to
fight cancer with BiTe® antibody constructs—past developments and future
R. Bargou, E. Leo, G. Zugaibier, M. Klinger, M. Goebeler, S. Knop, et al., Tumor
regression in cancer patients by very low doses of a T cell
N.D. Bhattacharyya, G.F. Feng, Regulation of T cell β2 γ3 signaling by
T. Miki-Hosokawa, A. Hasagawa, C. Iwamura, K. Shinoda, S. Tofukui, Y. Watanabe, et al., CD69 controls the pathogenesis of allergic airway
pone.0065494.


