A humanized 4-1BB-targeting agonistic antibody exerts potent antitumor activity in colorectal cancer without systemic toxicity

Lian-sheng Cheng
University of Science and Technology of China
Yong-feng Cheng
Anhui Medical University
Wen-ting Liu
Hefei HankeMab Biotechnology Limited
Aolin Shen
Anhui Medical University
Dayan Zhang
Hefei KankeMab Biotechnology Limited
Tingjuan Xu
University of Science and Technology of China
Wu Yin
University of Science and Technology of China
Min Cheng
University of Science and Technology of China
Xiaopeng Ma
University of Science and Technology of China
Fengrong Wang
Hefei HankeMab Biotechnology Limited
Qun Zhao
Hefei HankeMab Biotechnology Limited
Xiaoli Zeng
Hefei HankeMab Biotechnology Limited
Yan Zhang
Anhui Medical University
Guodong Shen (gdshen@ustc.edu.cn)
University of Science and Technology of China https://orcid.org/0000-0003-2521-8738

Research Article

Keywords: 4-1BB/CD137, Monoclonal antibody, Targeted therapy, Cancer immunotherapy, Antitumor immunity, Toxicity

Posted Date: February 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1352893/v1

Abstract

Background Colorectal cancer (CRC) is one of the most common malignancies and the patient survival rate remains unacceptably low. The anti-programmed cell death-1 (PD-1)/programmed cell death ligand 1 (PD-L1) antibody-based immune checkpoint inhibitors have been added to CRC treatment regimens, however, only a fraction of patients benefits. As an important co-stimulatory molecule, 4-1BB/CD137 is mainly expressed on the surface of immune cells including T and natural killer (NK) cells. Several agonistic molecules targeting 4-1BB have been clinically unsuccessful due to systemic toxicity or weak antitumor effects. We generated a humanized anti-4-1BB IgG4 antibody, HuB6, directed against a unique epitope and hypothesized that it would promote antitumor immunity with high safety. Methods The antigen binding specificity, affinity and activity of HuB6 were determined by enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) and flow cytometry. The antitumor effects were evaluated in humanized mice bearing syngeneic tumors, and possible toxicity was evaluated in humanized mice and cynomolgus monkeys. Results HuB6 showed high specificity and affinity for a binding epitope distinct from those of other known 4-1BB agonists, including utomilumab and urelumab, and induced CD8+ T, CD4+ T and NK cell stimulation dependent on FcγR crosslinking. HuB6 inhibited CRC tumor growth in a dose-dependent manner, and the antitumor effect is superior to utomilumab in humanized mouse models of syngeneic CRC. Furthermore, HuB6 combined with an anti-PD-L1 antibody significantly inhibited CRC growth in vivo. Additionally, HuB6 induced antitumor immune memory in tumor model mice rechallenged with 4 × 106 tumor cells. Toxicology data for humanized 4-1BB mice and cynomolgus monkeys showed that HuB6 could be tolerated up to a 180 mg/kg dose without systemic toxicity. Conclusions These results demonstrate that HuB6 is a suitable candidate for further clinical development and potential agent for CRC immunotherapy.

Background

Colorectal cancer (CRC) is one of the most common malignancies, and the patient survival rate remains unacceptably low [1]. Recently, monoclonal antibody (mAb)-based immune checkpoint inhibitors, particularly anti-programmed cell death-1 (PD-1)/programmed cell death ligand 1 (PD-L1) mAbs, have been added to CRC treatment regimens [2]. However, only a fraction of patients benefits from the therapy [3, 4].

4-1BB, also called CD137 or tumor necrosis factor receptor superfamily member 9 (TNFRSF9), is a costimulatory molecule expressed functionally on the surface of various types of leukocytes, such as T cells, natural killer (NK) cells and subsets of dendritic cells, and can be activated by its ligand 4-1BBL or activating anti-4-1BB antibodies to enhance tumor rejection; thus, it is regarded as a potential target for cancer immunotherapy [5–7].

Several anti-4-1BB agonistic antibodies have advanced to clinical stages but have never been clinically successful because of the intolerable toxicity caused by systemic immune activation [8]. Urelumab (BMS-663513), an IgG4 mAb, caused severe hepatotoxicity in more than 5% of patients enrolled in phase I and

II clinical trials [9]. In contrast, utomilumab (PF-05082566), an IgG2 mAb, showed fewer grade III–IV adverse effects and no dose-limiting toxicity up to the highest dose of 10 mg/kg, but it produced a much milder agonistic function than urelumab [10, 11]. Therefore, new antibody drugs that effectively and safely target 4-1BB are urgently needed.

Here, we demonstrate that HuB6, a novel human recombinant anti-4-1BB mAb with high specificity, has a binding epitope distinct from those of other known anti-4-1BB mAbs and shows potent antitumor activity and immune memory induction in humanized mouse models bearing CRC tumors and no systemic toxicity in either humanized mice or cynomolgus monkeys.

Methods

Cell culture

CHO-K1 and HEK-293 cells were obtained from American Type Culture Collection (ATCC CCL-61 and CRL-1573). HEK-293/NFκB-Luci/4-1BB cells were genetically engineered and expressed human 4-1BB and a luciferase reporter driven by a response element sensitive to 4-1BB agonistic stimulation and cultured in DMEM supplemented with 1 μg/mL puromycin (Gibco, C11995500BT) and 800 μg/mL hygromycin B (Sangon Biotech, A600230-0001). CHO-K1/CD32A, CHO-K1/CD32B, CHO-K1/CD16 and CHO-K1/hu4-1BB cells were designed to expression the human proteins FcγR□A, FcγR□B, Fcγ□RA and 4-1BB on the cell membrane, respectively, and grown in DMEM/F12 (HyClone, SH30023.01) containing 1 mg/mL Geneticin (Gibco, 11811023). The murine and human CRC cell lines CT26 and Colo205 were obtained from the cell bank affiliated with the Shanghai Institute of Biochemistry and Cell Biology (SIBCB), and the murine CRC cell line MC38 was purchased from Cobioer Company (Nanjing, China), authenticated, tested for mycoplasma contamination and cultured in RPMI-1640 medium (HyClone, SH30809.01). All media were supplemented with 10% fetal bovine serum (Ausbian, VS500T) and a 1% penicillin–streptomycin solution (HyClone, SV30010), and cells were cultured at 37°C in a humidified incubator with 5% CO2.

Protein expression and purification

The antibodies and antigens used in the study were generated in-house by cloning DNA-encoding sequences independently into the multiple cloning sites of the mammalian expression vector pcDNA™ 3.4 TOPO™ (Invitrogen, A14697), followed by expression in the Expi293 expression system (Gibco, A14635). The sequences for control antibodies, including urelumab (IMGT/mAb-DB, ID: 373), utomilumab (IMGT/mAb-DB ID: 657) and an anti-CD3 antibody (clone: OKT3, IMGT/mAb-DB, ID: 92), were obtained from IMGT/mAb-DB (http://www.imgt.org/mAb-DB). The sequences for mouse 4-1BB (UniProt, P20334), cynomolgus monkey 4-1BB (UniProt, A9YYE7) and human 4-1BBL (UniProt, P41273) were obtained from UniProt (https://sparql.UniProt.org/). In addition, human IgG, used as an isotype control, was purchased from GenScript Biotech Corporation (Nanjing, China). Protein purification was performed according to a previously published protocol [10]. Briefly, medium containing a secreted protein was harvested by centrifugation and filtered before purification on a Ni Sepharose Excel column (GE) equilibrated in 25 mM

Tris pH 8.0 and 500 mM NaCl. The bound protein was washed with 25 mM Tris pH 8.0, 500 mM NaCl, and 15 mM imidazole before elution with 25 mM Tris pH 8.0, 500 mM NaCl, and 250 mM imidazole. Proteins were further purified by size exclusion chromatography on a Superdex 75 column (GE) equilibrated in PBS.

Enzyme linked immunosorbent assay (ELISA)

The indirect ELISA method was used, and plates were coated with 4-1BB-ECD-mFc (6 nM 4-1BB-ECD-mFc, 1 μg/mL mouse 4-1BB-ECD-mFc or 350 ng/mL cynomolgus 4-1BB-mFc) in carbonate buffer at 4°C overnight. After blocking with 1% BSA at 37°C for 2 hours, serially diluted test antibodies were added, incubated at room temperature for 2 hours and then subjected to detection with HRP-conjugated secondary antibodies (goat anti-human Fc, Jackson ImmunoResearch Laboratories, 146460). After washing with a PBST solution three times, TMB (Invitrogen, 002023) was added as a substrate, and the absorbance was detected at 405 nm.

Antibody affinity determination

The affinity of HuB6, utomilumab or urelumab for human 4-1BB was determined by surface plasmon resonance (SPR) using a Biacore 8K (GE Healthcare) carried out in single-cycle mode with a protein A biosensor chip (GE Healthcare) and Fortebio Octet Red96 system (Pall ForteBio Analytics, Shanghai, China) according to the manufacturers' manuals.

Competitive protein binding assay

CHO-K1-Hu4-1BB cells were cultured to 80% confluence, digested with trypsin, centrifuged at 1000 rpm for 5 min and collected in EP tubes. HuB6, utomilumab and urelumab were labeled with biotin to create the corresponding Bio-antibodies and diluted to 1.5 μg/mL with PBS. The working concentration of human 4-1BBL ranged from 90 μg/mL to 0.35 μg/mL with a 4-fold gradient dilution. Each Bio-antibody was mixed with 4-1BBL and then incubated with CHO-K1-Hu4-1BB cells for 30 min. After washing twice with a PBS buffer solution, the cells were incubated with a streptavidin-FITC secondary antibody (BioLegend, 405202) and incubated for 30 min in the dark. Finally, the prepared cells were suspended in 500 μL PBS and detected by flow cytometry (Beckman Coulter, CytoFLEX).

Luciferase assay

The activity of the mAb HuB6 was detected using the luciferase reporter gene method *in vitro*. A total of 1×10^4 HEK-293/NFκB-Luci/4-1BB cells per well were added to 96-well plates and incubated for 6 hours. HuB6, utomilumab and urelumab were added at 4 μg/mL and multiple minor concentrations by antibody dilution, and the cells were incubated for 8-24 hours. Then, both firefly luciferase activity and Renilla luciferase activity were measured with a Glomax multidetection system luminometer using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized against Renilla luciferase activity to measure antibody activity.

Mutant antigen binding detection

Extracellular amino acid sites (M101, I32, and N42) in the human 4-1BB antigen were independently mutated to synthesize different target genes, which were inserted into the pcDNA3.4 vector to obtain DNA plasmids carrying the different 4-1BB antigens. Expi293 cells expressing mutant or wild-type (WT) 4-1BB antigens on the surface were obtained by transient transfection and incubated with utomilumab, urelumab or HuB6 at a concentration of 10 μg/mL and subsequent 3-times gradient dilution for 30 min. After washing twice with PBS buffer, the cells were incubated with a FITC-conjugated goat anti-human IgG (H+L) secondary antibody (Invitrogen, H10301) for 30 min, washed and resuspended in PBS, and then subjected to an antibody binding assay evaluated by flow cytometry (Beckman Coulter, CytoFLEX).

Lymphocyte isolation and T cell-activation assay

Blood leukopaks were obtained from healthy people at the Shanghai Zhaxin Hospital of Integrated Traditional Chinese & Western Medicine under institutional review board-approved protocols. Peripheral blood mononuclear cells (PBMCs) were isolated according to the manufacturer's instructions (Ficoll 400, F8636, Sigma–Aldrich). Human CD4+ and CD8+ T cells were purified using BD IMag anti-human CD4 (No. 557767) and anti-human CD8 beads (No. 557766), and CD3-CD56+ NK cells were prepared by separation with magnetic beads (NK purification kit, Miltenyi Biotec). The purities of CD4+ T, CD8+ T and NK cells were confirmed with flow cytometry (Beckman Coulter, CytoFLEX). For a cell proliferation assay, CD8+ T cells were labeled with 10 μmol/l CFSE (Invitrogen) according to the manufacturer's protocol and assayed by flow cytometry. To determine the IFNγ secretion activity of CD4+ T, CD8+ T and NK cells, 96-well cell culture plates (Corning) were pretreated with an anti-CD3 antibody (clone: OKT3, 0.4 μg/mL, Biosciences) for CD4+ T and CD8+ T cells suspended in 50 μL PBS for 1 hour at 37°C and 100 U/mL recombinant human IL-2 (rhIL-2, PeproTech) was included in the complete culture medium for NK cells. After washing twice with PBS, 1×10^5 CD4+ T, CD8+ T or NK cells in 200 μL complete RPMI-1640 medium were added and treated with HuB6, urelumab or utomilumab across a range of doses (0.04 μg/mL, 0.4 μg/mL and 4 μg/mL). After 3 days of culture in a CO2 incubator at 37℃, the IFN-γ content in the cell culture supernatant was determined with an ELISA kit (BioLegend), and the absorbance was read with an ELISA plate reader (BioTek) at the wavelengths specified in the kit.

Model mouse

To establish two types of CRC tumor-grafted mouse models, 8-week-old human 4-1BB/4-1BBL double knock-in C57BL/6 and B-NDG B2m KO plus mice, in which human PBMCs were transplanted to reconstitute human immune cells, were purchased from Biocytogen Corporation (Beijing, China) and used according to the appropriate experimental protocol. Briefly, 2×10^6 MC38 or CT26 cells mixed with Corning Matrigel in a 1:1 volume ratio were inoculated subcutaneously into human 4-1BB/4-1BBL double knock-in C57BL/6 mice. Similarly, 2×10^6 Colo205 cells mixed with 1:1 Matrigel were inoculated subcutaneously into B-NDG B2m KO plus mice. After palpable tumors were established, the mice were randomized on the basis of tumor volume and body weight. Subsequently, treatment with a mAb or an isotype control was

performed twice a week for up to 3 weeks by intraperitoneal injection. Tumor growth was monitored twice a week by measuring tumor length and width. Tumor volume was calculated according to the following equation: 0.5 × length × width × width.

Toxicology study

HuB6 toxicity studies were conducted with humanized model mice and purpose-bred cynomolgus monkeys. Humanized 4-1BB mice were intraperitoneally injected with a low dose (3 mg/kg) or a high dose (30 mg/kg) of HuB6, utomilumab, urelumab or an isotype antibody once every 3 days for 6 total injections. Cynomolgus monkeys were administered repeated intravenous doses of 3, 10, and 30 mg/kg/week for 5 weeks or single doses of 60 and 180 mg/kg for toxicity studies. Two male and 2 female cynomolgus monkeys were randomly assigned to each group, and the antibodies were administered via intravenous infusion at a dose of 5 mL/kg administered at a rate of 1 mL/min. Mouse necropsies were performed according to a standard protocol, and the major organs were collected for histological evaluation. All the tissues were fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE) and analyzed by a professional pathologist. In addition, blood and serum were collected for clinical hematological and chemical analyses using a Sysmex BX4000.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software), and one-way or two-way ANOVA was used to compare intergroup differences. A p value of <0.05 was considered significant.

Results

Characterization of the mAb HuB6

Previous reports have demonstrated that the IgG4 form of recombinant human IgG mAbs is useful in various therapeutic applications to reduce FcγR activation and Fc-mediated toxicity, including the complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) pathways [12, 13]. Therefore, we first screened twelve humanized 4-1BB-targeted single-chain variable fragments (scFvs) belonging to the IgG4 subtype derived from a hybridoma mAb (No. 37G10F4) with high affinity for the human 4-1BB extracellular domain (ECD) and physiological activity in activating T cell functions using artificial intelligence computer aided design technology, and then the superior candidate mAb HuB6 was generated from the scFvB60103 colony using unbiased functional screening.

To determine the possible binding epitope of the mAb HuB6, important extracellular amino acid sites in the human 4-1BB antigen were mutated, and the affinity changes were analyzed. It is known that amino acid I64 of the 4-1BB protein is the key binding site for 4-1BBL, M101 and I132 are the binding amino acids for utomilumab, and N42 is fundamental for urelumab binding [10]. Therefore, the mutations

in the 4-1BB antigen were targeted to the key amino acids M101, I132 and N42 by making point mutations, and the mAb-bound epitopes were analyzed using flow cytometry. Our results showed that utomilumab hardly bound the M101-mutated or I132-mutated 4-1BB antigen (Fig. 1a) and urelumab did not bind the N42-mutated antigen (Fig. 1b), but HuB6 could bind all the mutated antigens (Fig. 1c). Therefore, the epitope recognized by HuB6 exists in both cysteine-rich domain 1 (CRD1) and CRD2 of human 4-1BB, which is unique and distinct from those recognized by utomilumab (CRD3 and CRD4) and urelumab (CRD1) (Fig. 1d, structural data for the HuB6/4-1BB complex not shown).

To define the specificity and affinity of HuB6, both the monomeric form (mono-Hu4-1BB) and dimeric form (Hu4-1BB-mFc WT) of the human 4-1BB ECD were prepared, and HuB6 showed different binding affinities for them [half-maximal effective dose (EC50): 0.086 nM for mono-Hu4-1BB, EC50: 0.046 nM for Hu4-1BB-mFc WT; Fig. 2a]. Furthermore, HuB6 could bind to the 4-1BB protein of cynomolgus monkeys with an affinity similar to that of utomilumab (HuB6 EC50: 0.035 nM, utomilumab EC50: 0.038 nM), but urelumab did not show any affinity (Fig. 2b). Moreover, none of the three 4-1BB-specific antibodies crossreacted with mouse 4-1BB, while the control anti-mouse 4-1BB mAb could bind to mouse 4-1BB (Fig. 2c). By a flow cytometry assay, HuB6 was found to bind to activated CD8+ T cells with higher affinity than utomilumab at concentrations ranging from 0.016 μg/mL to 10 μg/mL (Fig. 2d). The Kd value of HuB6 binding to human 4-1BB was similar to that of utomilumab but higher than that of urelumab, as determined by the Biacore and ForteBio methods. Furthermore, through competitive binding experiments, 4-1BBL was shown to compete with HuB6 and utomilumab for binding to 4-1BB in a concentration-dependent manner, but no competition was observed for urelumab (Fig. 2e). These results indicate that HuB6 has high antigen specificity and affinity, similar to utomilumab.

HuB6 increases T cell activation dependent on FcR crosslinking

It was reported that antibodies targeting 4-1BB can enhance the proliferation of antigen-stimulated T cells *in vitro* and promote CD8+ T cell-dependent antitumor immunity in preclinical cancer models [14]. Here, the effects of the mAb HuB6 on CD8+ T cells were tested. By the carboxyfluorescein succinimidyl ester (CFSE) labeling method, the percentages of proliferating cells were shown to be 64.84%, 78.16% and 90.32% after treatment with HuB6 at 2.5 μg/mL, 10 μg/mL and 40 μg/mL, respectively; in comparison, the percentages in the blank control and only anti-CD3 antibody treatment groups were 0.54% and 22.36%, respectively (Fig. 3a). Moreover, HuB6 increased the secretion of IFN-γ by CD8+ T, CD4+ T and NK cells in a dose-dependent manner (Fig. 3b).

A previous study indicated that the FcγR interaction is critical for the bioactivity of 4-1BB-specific mAbs [15]. To evaluate the effect of Fc-mediated crosslinking on the mAb HuB6, different FcγRs were used in a CD8+ T cell costimulation assay. HuB6 activated CD8+ T cells in the presence of FcγR□A, FcγR□B or FcγR□A expressed on CHO-K1 cells, similar to the IgG4 isotype mAb urelumab, although the activation extent was lower than that achieved with urelumab. In comparison, the IgG2 isotype mAb utomilumab exhibited an activating function in the presence of FcγR□B or FcγR□A but little activity in the presence of only FcγR□A (Fig. 3c and d). In the absence of FcγR-expressing CHO-K1 cells, no activity was observed for

HuB6 or utomilumab, whereas urelumab could still activate CD8+ T cells (Fig. S1). Furthermore, HuB6 did not activate human PBMCs without FcγR crosslinking, as determined by measuring routine cytokines including TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10 and IL-17A (Fig. S2). These data indicate that HuB6 induces T cell activation in a manner dependent on FcγR crosslinking.

HuB6 exerts potent antitumor effects

Humanized mouse models bearing CRC tumors were established to evaluate the antitumor effect of HuB6 *in vivo,* and a schematic diagram of HuB6 or control mAb treatment is shown in Fig. 4a. First, a dose escalation study was performed with HuB6 in humanized 4-1BB mouse models bearing MC38 or CT26 tumors, and the HuB6-treated groups showed dose-dependent antitumor effects on both tumor volume ($p < 0.05$, Fig. 4b) and tumor weight ($p < 0.05$, Fig. 4c). Moreover, the average tumor volumes in the high-dose HuB6 group (3 mg/kg) were significantly smaller than those in the middle-dose HuB6 or utomilumab group (1 mg/kg, $p < 0.05$), and especially for the CT26 model, HuB6 had a better antitumor effect than utomilumab at the same dose (10 mg/kg, $p < 0.05$). Notably, some tumors in the MC38 model completely regressed in all three HuB6 groups, showing a dose-dependent relationship (1, 2 and 3 tumors receded in the low-, middle- and high-dose groups, respectively), whereas no tumor regression was observed in the utomilumab group (Fig. S3).

Next, the combined antitumor effect of HuB6 and an anti-PD-L1 mAb was investigated. It was reported that the MC38 cell line has a positive response to the anti-PD-L1 mAb atezolizumab [16]. Therefore, the humanized 4-1BB mouse model bearing MC38 tumors was chosen and treated with 1 mg/kg atezolizumab alone, 0.3 mg/kg HuB6 or utomilumab alone and their combinations. As shown in Fig. 4d, although atezolizumab alone inhibited tumor growth ($p < 0.05$), combination with HuB6 or utomilumab significantly improved the antitumor effect ($p < 0.05$), and one tumor completely regressed in the HuB6 plus atezolizumab group. Similarly, HuB6 plus atezolizumab produced a synergistic antitumor effect in human PBMC-engrafted mice bearing human CRC cell line Colo205 transplants compared with either mAb monotherapy ($p < 0.05$). Furthermore, by measuring tumor weight, a remarkable difference was found between atezolizumab combined with HuB6 or utomilumab and any mAb alone in the MC38 model ($p < 0.05$), but a significant difference was found between only atezolizumab plus HuB6 and atezolizumab alone in the Colo205 model ($p < 0.05$, Fig. 4e).

HuB6 induces antitumor immune memory

According to the reported experimental procedure for evaluating antitumor immune memory [17], we tested the ability of HuB6 to induce immune memory against cancer (Fig. 5a). First, humanized 4-1BB mice were inoculated with 2×10^6 MC38 cells in one flank and then intraperitoneally injected with 10 mg/kg HuB6, utomilumab or an isotype control only once when the tumor volume reached approximately 150 mm^3 (eight days later). Remarkably, all the treated mice exhibited complete tumor regression on the 18^{th} day in the HuB6 group or on the 21^{st} day in the utomilumab group. Next, on the 45^{th} day, the surviving tumor-free mice were rechallenged with 4×10^6 MC38 cells injected into the other flank and

showed complete tumor rejection (Fig. 5b). Similarly, 10 mg/kg HuB6 treatment administered twice per week (five times total) induced a robust immune memory response against cancer in the humanized 4-1BB mouse model bearing B16F10 melanoma transplants, and no tumor relapse was observed at the end of the experiment on day 65 (Fig. 5c).

HuB6 exhibits high safety in animal models

To assay the possible toxicity of HuB6 *in vivo*, humanized 4-1BB mice and cynomolgus monkeys were employed. Humanized 4-1BB mice were intraperitoneally injected with a low dose (3 mg/kg) or a high dose (30 mg/kg) of HuB6, utomilumab, urelumab or an isotype control once every 3 days (6 times total), and no significant differences in hematological markers, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were found between HuB6 or utomilumab and the isotype control in either the low-dose or high-dose group ($p > 0.05$. Fig. 6a). However, the ALT level in the high-dose urelumab group was significantly higher than that in the isotype group on the 18^{th} day at the end of the experiment ($p < 0.001$, Fig. 6a). In addition, histopathological evaluation of major organs, including the heart, liver, lungs, kidneys and spleen, confirmed the high safety of HuB6 (Fig. S4).

For the cynomolgus monkey study, 5-week repeat-dose toxicity and single-dose toxicity tests were performed. First, 8 males and 8 females were randomly divided into HuB6 groups treated with a low dose (3 mg/kg), middle dose (10 mg/kg) or high dose (30 mg/kg) and an isotype control group, and the antibodies were administered via repeated intravenous infusions (once a week for 5 weeks) at a dose of 5 mL/kg administered at a rate of 1 mL/min. Hematological indexes, including red blood cells (RBCs), white blood cells (WBCs), platelets (PLTs) and lymphocytes (LYMPHs), and serum biochemical markers, including ALT, AST, creatinine (CREA) and urea, were determined, and no abnormal changes were observed in any of the groups on day 35 (Fig. 6b and c). In our study, HuB6 was well tolerated up to a dosage of 30 mg/kg/week without any abnormalities in general condition (e.g., decreased food consumption or body weight) or hematological changes (e.g., decrease in neutrophils or PLTs). In contrast, utomilumab, despite having much weaker agonistic activity, was reported to cause dose-limiting toxicity at dosages greater than 5 mg/kg/week [11] and elicited systemic toxicity at a dosage of 30 mg/kg/week in a 4-week toxicity study [8]. Moreover, we conducted a single-dose toxicity study with cynomolgus monkeys. HuB6 was well tolerated at 60 mg/kg without any abnormalities, and when the dose reached 180 mg/kg, mild and transient liver toxicity (less than 2-fold increases in ALT and AST) was found on day 2. The indexes were restored to normal on day 9, and no other abnormalities occurred (Fig. 6d and e). These results demonstrate that HuB6 has a good safety profile.

Discussion

In this study, we demonstrated that the humanized anti-4-1BB mAb Hub6 induced T cell proliferation and activity and had potent efficacy in tumor inhibition and immune memory induction without systemic toxicity; thus, it was regarded as a potential candidate for cancer immunotherapy. As 4-1BB is one of the costimulatory receptors of immune cells, 4-1BB-targeting mAbs have shown promising antitumor effects

in preclinical models [18]. However, the clinical development of two leading molecules, utomilumab and urelumab, is facing serious challenges due to low efficacy or severe systemic toxicity [8, 11].

Recently, several bispecific tumor antigen-targeted 4-1BB agonists have been developed [19–22]. However, their therapeutic efficacy relies fully on the expression of tumor antigens, limiting their clinical application to patients with antigen overexpression. In addition, tumor antigen, such as EGFR, is widely expressed in normal, non-neoplastic tissues, and its use as a target antigen can lead to severe on-target, off-tumor immunotoxicity [19, 22]. It follows that the selected tumor antigens should be highly tumor-specific and their high expression is often limited to only specific types of cancer [8]. In contrast, the success of immune checkpoint inhibitors, such as anti–PD-1/PD-L1 antibodies, is partially attributed to their broad applicability in a variety of cancers regardless of the antigen expression status. Therefore, a novel humanized anti-4-1BB agonistic antibody that has strong agonistic activity, a high safety profile and broad applicability without depending on tumor antigen expression is urgently needed.

Our previous studies showed that HER2-targeted antibodies with different binding epitopes exhibited different antitumor properties [23, 24]. Here, we screened twelve humanized 4-1BB-targeted IgG4 subtype scFvs and then generated a novel anti-4-1BB mAb, HuB6, with an antigen epitope distinct from those of other known antibodies, such as utomilumab and urelumab; thus, HuB6 has unique antitumor efficacy and a high safety profile. As shown in Fig. 1d, the binding epitope of HuB6 is between CRD1 and CRD2 of the 4-1BB protein, while urelumab binds to CRD1, and utomilumab binds between CRD3 and CRD4. The binding site of 4-1BBL overlaps with those of HuB6 and utomilumab but not with that of urelumab; thus, both HuB6 and utomilumab are ligand-blocking antibodies, while urelumab is a non-ligand-blocking antibody, which was confirmed by the 4-1BBL competitive binding assay result. As a 4-1BB agonist, HuB6 increased the proliferation of CD8 + T cells and production of the antitumor cytokine IFN-γ, inhibited tumor growth in all the mouse models tested, induced potent antitumor immune memory and exerted an enhanced tumor-inhibiting effect in combination with an anti-PDL1 mAb, similar to utomilumab, urelumab and several other potential therapeutics [8, 25–28].

However, obvious differences in in agonistic activity and toxicity are also easily found among these antibodies. First, the affinity of HuB6 is similar to that of utomilumab, but the tumor inhibitory efficacy in several mouse models seemed to be greater than that of utomilumab. Second, Hub6 was constructed as a recombinant human IgG4 mAb, which is the same subtype as urelumab, but urelumab causes more severe liver toxicity, consistent with a previous study [9]. The above differences could be interpreted with the epitope accessibility theory [10]. Namely, the binding site on the very N-terminus of CRD1 targeted by urelumab orients the antibody Fc domain to be optimally exposed for interaction with FcγR, which potentially enhances ADCC and CDC. The utomilumab epitope, which is closer to the cell surface, orients the antibody parallel to the membrane, where engagement of FcγR may be more restricted, and the HuB6 binding epitope makes the interaction with FcγR mild, potentially between the interactions of urelumab and utomilumab. Furthermore, IgG4 is known to engage FcγRI and FcγRIIB more than does IgG2, although both isotypes are generally characterized by relatively low FcγR interactions [29]. Our results showed that the CD8 + T cell activation induced by HuB6 was dependent on FcγR crosslinking, as was

that induced by utomilumab, but urelumab activated T cells without FcγR crosslinking, which might be associated with the agonistic activity and toxicity of the antibodies. From here we see that the difference among HuB6, Urelumab and Utomilumab in agonist activity and toxicity may be deciphered by the epitope accessibility and FcγR interaction, which provides new evidence in understanding how therapeutic mAb works.

Several important limitations should be considered in our study. First, the antitumor efficacy of HuB6 is mainly studied on CRC, which is also one of routinely selected types for cancer immunotherapy [30, 31], but more cancer types should be tested to explore the indication of HuB6 therapy. In addition, the capacity of HuB6, such as lymphocyte recruitment and activation in tumor microenvironment, needs to be determined in the future. Currently, HuB6 has been approved for evaluation in clinical trials based on the preclinical evidence, and multiple types of solid cancers including CRC will be investigated.

when interpreting the results of our study. our study included a limited number of cases, which is difficult to overcome given the low incidence of these disorders. Our relatively small number of patients with preeclampsia hindered us from making inferences from some of our analyses. Further studies involving multiple centers and a larger number of patients are necessary in order to validate our fndings. In addition, although we compared many potential confounders, we cannot exclude the possibility of the other confounding factors from unmeasured covariates.

Conclusions

We generated a novel humanized anti-4-1BB mAb, HuB6, that was shown to exert agonistic activity in tumors without systemic toxicity. HuB6 monotherapy demonstrated potent antitumor efficacy against CRC without systemic immune activation and had an enhanced tumor inhibitory effect when administered in combination with an anti-PDL1 antibody. These results strongly support the translation of HuB6 into clinical testing for the treatment of CRC and other solid tumors.

Abbreviations

ADCC
antibody-dependent cell-mediated cytotoxicity
CDC
complement-dependent cytotoxicity
CFSE
carboxyfluorescein succinimidyl ester
CRC
colorectal cancer
CRD
cysteine-rich domain
ECD

extracellular domain
EC50
half-maximal effective dose
ELISA
enzyme-linked immunosorbent assay
HE
hematoxylin and eosin
mAb
monoclonal antibody
NK
natural killer cell
PBMC
peripheral blood mononuclear cell
PD-1
programmed cell death-1
PD-L1
programmed cell death ligand 1
scFv
single-chain variable fragment
SPR, surface plasmon resonance
TNFRSF9
tumor necrosis factor receptor superfamily member 9.

Declarations

Supplementary Information

The online version contains supplementary material.

Author Contributions LC and GS are responsible for the study design and supervision. LC, YC, AS and WL are responsible for the study implementation and writing of the manuscript. YZ and XM are responsible for data analysis and interpretation. TX, WY and MC are responsible for the model animal experiments. DZ, XZ, FW and QZ are responsible for the expression, purification and quality control of the antibodies. All authors participated in drafting the manuscript and approved the final version of the manuscript.

Acknowledgments

We thank Prof. Xiaohu Zheng and Prof. Haiyan Weng at USTC for their technical assistance.

Competing interests

The authors declare no competing interests.

Availability of data and materials

This article includes the datasets that support our findings.

Consent for publication

Not applicable.

Ethics approval and consent to participate The study was conducted with the approval of the Ethics Committee of the Shanghai Zhaxin Hospital of Integrated Traditional Chinese & Western Medicine (approval no. 2021-03) and in accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. All animal experiments were approved by the Ethics Committee for Animal Experiments of The First Affiliated Hospital of the University of Science and Technology of China [2021-N(A)-083] and performed in accordance with the guidelines for animal experiments in laboratories. Endpoints such as euthanasia were considered at the appropriate time if the animals had intolerable pain (such as gait disturbance, water/feeding impairment, tumor diameter of 20 mm or more, tumor volume of 4000 mm^3 or more, and 25% or more weight loss in 7 days).

Funding

This work was supported by the Key Project of Science and Technology of Anhui Province (202004b11020019, 202004j07020020), the Hefei Municipal Natural Science Foundation (2021005) and the Natural Science Foundation for the Higher Education Institutions of Anhui Province (KJ2019A0943).

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209–49.
2. Morse MA, Hochster H, Benson A. Perspectives on Treatment of Metastatic Colorectal Cancer with Immune Checkpoint Inhibitor Therapy. Oncologist. 2020;25(1):33–45.
3. Fan X, Wang D, Zhang W, Liu J, Liu C, Li Q, Ma Z, Li H, Guan X, Bai Y, et al. Inflammatory Markers Predict Survival in Patients With Advanced Gastric and Colorectal Cancers Receiving Anti-PD-1 Therapy. Front Cell Dev Biol. 2021;9:638312.
4. Liao W, Overman MJ, Boutin AT, Shang X, Zhao D, Dey P, Li J, Wang G, Lan Z, Li J, et al. KRAS-IRF2 Axis Drives Immune Suppression and Immune Therapy Resistance in Colorectal Cancer. Cancer Cell. 2019;35(4):559–72 e557.
5. Houot R, Goldstein MJ, Kohrt HE, Myklebust JH, Alizadeh AA, Lin JT, Irish JM, Torchia JA, Kolstad A, Chen L, et al. Therapeutic effect of CD137 immunomodulation in lymphoma and its enhancement by Treg depletion. 2009;114(16):3431–3438.

6. Croft M. The role of TNF superfamily members in T-cell function and diseases. Nat Rev Immunol. 2009;9(4):271–85.
7. Hong JP, Reynoso GV, Andhey PS, Swain A, Turner JS, Boon ACM, Krammer F, Ellebedy AH, Zanini F, Artyomov M, et al. An Agonistic Anti-CD137 Antibody Disrupts Lymphoid Follicle Structure and T-Cell-Dependent Antibody Responses. Cell Rep Med. 2020. Doi:10.1016/j.xcrm.2020.100035.
8. Kamata-Sakurai M, Narita Y, Hori Y, Nemoto T, Uchikawa R, Honda M, Hironiwa N, Taniguchi K, Shida-Kawazoe M, Metsugi S, et al. Antibody to CD137 Activated by Extracellular Adenosine Triphosphate Is Tumor Selective and Broadly Effective In Vivo without Systemic Immune Activation. Cancer Discov. 2021;11(1):158–75.
9. Segal NH, Logan TF, Hodi FS, McDermott D, Melero I, Hamid O, Schmidt H, Robert C, Chiarion-Sileni V, Ascierto PA, et al. Results from an Integrated Safety Analysis of Urelumab, an Agonist Anti-CD137 Monoclonal Antibody. Clin Cancer Res. 2017;23(8):1929–36.
10. Chin SM, Kimberlin CR, Roe-Zurz Z, Zhang P, Xu A, Liao-Chan S, Sen D, Nager AR, Oakdale NS, Brown C, et al. Structure of the 4-1BB/4-1BBL complex and distinct binding and functional properties of utomilumab and urelumab. Nat Commun. 2018;9(1):4679.
11. Fisher TS, Kamperschroer C, Oliphant T, Love VA, Lira PD, Doyonnas R, Bergqvist S, Baxi SM, Rohner A, Shen AC, et al. Targeting of 4-1BB by monoclonal antibody PF-05082566 enhances T-cell function and promotes anti-tumor activity. Cancer Immunol Immunother. 2012;61(10):1721–33.
12. Gurjar SA, Derrick JP, Dearman RJ, Thorpe R, Hufton S, Kimber I, Wadhwa M. Surrogate CD16-expressing effector cell lines for determining the bioactivity of therapeutic monoclonal antibodies. J Pharm Biomed Anal. 2017;143:188–98.
13. Stein R, Qu Z, Chen S, Solis D, Hansen HJ, Goldenberg DM. Characterization of a humanized IgG4 anti-HLA-DR monoclonal antibody that lacks effector cell functions but retains direct antilymphoma activity and increases the potency of rituximab. Blood. 2006;108(8):2736–44.
14. Buchan SL, Dou L, Remer M, Booth SG, Dunn SN, Lai C, Semmrich M, Teige I, Martensson L, Penfold CA, et al. Antibodies to Costimulatory Receptor 4-1BB Enhance Anti-tumor Immunity via T Regulatory Cell Depletion and Promotion of CD8 T Cell Effector Function. Immunity. 2018;49(5):958–70 e957.
15. Ho SK, Xu Z, Thakur A, Fox M, Tan SS, DiGiammarino E, Zhou L, Sho M, Cairns B, Zhao V, et al. Epitope and Fc-Mediated Cross-linking, but Not High Affinity, Are Critical for Antitumor Activity of CD137 Agonist Antibody with Reduced Liver Toxicity. Mol Cancer Ther. 2020;19(4):1040–51.
16. Homet Moreno B, Zaretsky JM, Garcia-Diaz A, Tsoi J, Parisi G, Robert L, Meeth K, Ndoye A, Bosenberg M, Weeraratna AT, et al. Response to Programmed Cell Death-1 Blockade in a Murine Melanoma Syngeneic Model Requires Costimulation, CD4, and CD8 T Cells. Cancer Immunol Res. 2016;4(10):845–57.
17. Poggio M, Hu T, Pai CC, Chu B, Belair CD, Chang A, Montabana E, Lang UE, Fu Q, Fong L, et al. Suppression of Exosomal PD-L1 Induces Systemic Anti-tumor Immunity and Memory. Cell. 2019;177(2):414–27. e413.

18. Williams JB, Horton BL, Zheng Y, Duan Y, Powell JD, Gajewski TF. The EGR2 targets LAG-3 and 4-1BB describe and regulate dysfunctional antigen-specific CD8 + T cells in the tumor microenvironment. J Exp Med. 2017;214(2):381–400.
19. Compte M, Harwood SL, Munoz IG, Navarro R, Zonca M, Perez-Chacon G, Erce-Llamazares A, Merino N, Tapia-Galisteo A, Cuesta AM, et al. A tumor-targeted trimeric 4-1BB-agonistic antibody induces potent anti-tumor immunity without systemic toxicity. Nat Commun. 2018;9(1):4809.
20. Hinner MJ, Aiba RSB, Jaquin TJ, Berger S, Durr MC, Schlosser C, Allersdorfer A, Wiedenmann A, Matschiner G, Schuler J, et al. Tumor-Localized Costimulatory T-Cell Engagement by the 4-1BB/HER2 Bispecific Antibody-Anticalin Fusion PRS-343. Clin Cancer Res. 2019;25(19):5878–89.
21. Claus C, Ferrara C, Xu W, Sam J, Lang S, Uhlenbrock F, Albrecht R, Herter S, Schlenker R, Husser T, et al. Tumor-targeted 4-1BB agonists for combination with T cell bispecific antibodies as off-the-shelf therapy. Sci Transl Med. 2019. doi:10.1126/scitranslmed.aav5989.
22. Mikkelsen K, Harwood SL, Compte M, Merino N, Molgaard K, Lykkemark S, Alvarez-Mendez A, Blanco FJ, Alvarez-Vallina L. Carcinoembryonic Antigen (CEA)-Specific 4-1BB-Costimulation Induced by CEA-Targeted 4-1BB-Agonistic Trimerbodies. Front Immunol. 2019;10:1791.
23. Wang Z, Cheng L, Guo G, Cheng B, Hu S, Zhang H, Zhu Z, Niu L. Structural insight into a matured humanized monoclonal antibody HuA21 against HER2-overexpressing cancer cells. Acta Crystallogr D Struct Biol. 2019;75(Pt 6):554–63.
24. Zhou H, Zha Z, Liu Y, Zhang H, Zhu J, Hu S, Shen G, Cheng L, Niu L, Greene MI, et al. Structural insights into the down-regulation of overexpressed p185(her2/neu) protein of transformed cells by the antibody chA21. J Biol Chem. 2011;286(36):31676–83.
25. Qi X, Li F, Wu Y, Cheng C, Han P, Wang J, Yang X. Optimization of 4-1BB antibody for cancer immunotherapy by balancing agonistic strength with FcgammaR affinity. Nat Commun. 2019;10(1):2141.
26. Chen S, Lee LF, Fisher TS, Jessen B, Elliott M, Evering W, Logronio K, Tu GH, Tsaparikos K, Li X, et al. Combination of 4-1BB agonist and PD-1 antagonist promotes antitumor effector/memory CD8 T cells in a poorly immunogenic tumor model. Cancer Immunol Res. 2015;3(2):149–60.
27. Tolcher AW, Sznol M, Hu-Lieskovan S, Papadopoulos KP, Patnaik A, Rasco DW, Di Gravio D, Huang B, Gambhire D, Chen Y, et al. Phase Ib Study of Utomilumab (PF-05082566), a 4-1BB/CD137 Agonist, in Combination with Pembrolizumab (MK-3475) in Patients with Advanced Solid Tumors. Clin Cancer Res. 2017;23(18):5349–57.
28. Sanmamed MF, Rodriguez I, Schalper KA, Onate C, Azpilikueta A, Rodriguez-Ruiz ME, Morales-Kastresana A, Labiano S, Perez-Gracia JL, Martin-Algarra S, et al. Nivolumab and Urelumab Enhance Antitumor Activity of Human T Lymphocytes Engrafted in Rag2-/-IL2Rgammanull Immunodeficient Mice. Cancer Res. 2015;75(17):3466–78.
29. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, Daeron M. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood. 2009;113(16):3716–25.

30. Johdi NA, Sukor NF. Colorectal Cancer Immunotherapy: Options and Strategies. Front Immunol. 2020;11:1624.
31. Kuang C, Park Y, Augustin RC, Lin Y, Hartman DJ, Seigh L, Pai RK, Sun W, Bahary N, Ohr J, Rhee JC, Marks SM, Beasley HS, Shuai Y, Herman JG, Zarour HM, Chu E, Lee JJ, Krishnamurthy A. Pembrolizumab plus azacitidine in patients with chemotherapy refractory metastatic colorectal cancer: a single-arm phase 2 trial and correlative biomarker analysis. Clin Epigenetics. 2022;14(1):3.

Figures

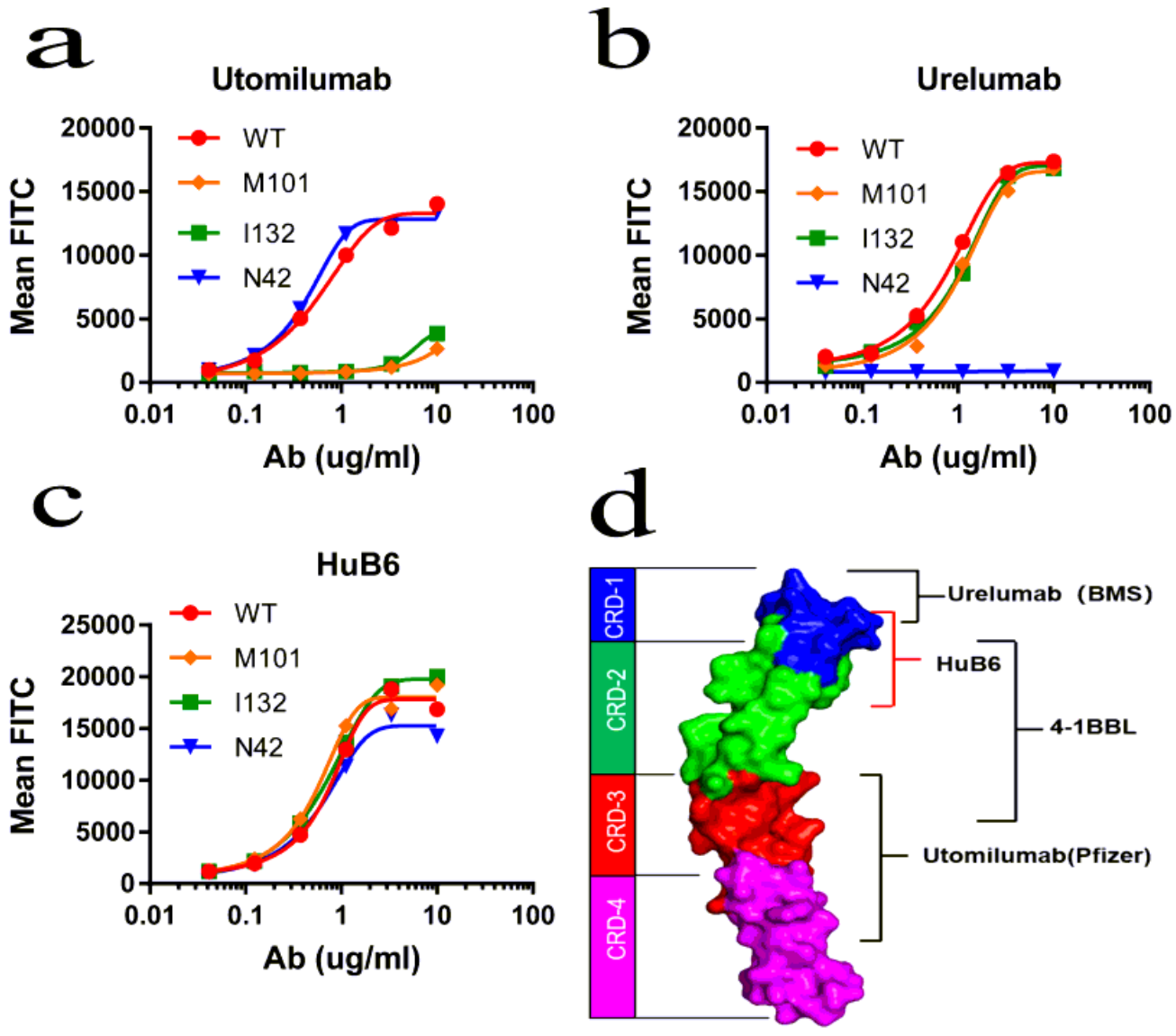

Figure 1

HuB6 binds a unique epitope within 4-1BB. The key binding sites of (a) utomilumab, (b) urelumab and (c) HuB6 were analyzed with an amino acid point mutation binding assay using flow cytometry. The results

are representative of three different experiments and expressed as the mean values. Hu4-1BB antigens with a mutation in the key amino acid M101, I132 or N42 were transfected into Expi293 cells. (d) The binding epitopes of utomilumab, urelumab and HuB6 are shown on the structural model of 4-1BB.

Figure 2

HuB6 has high binding specificity and affinity. (a) HuB6 bound to both the monomeric form (mono-Hu4-1BB) and dimeric form (Hu4-1BB-mFc WT) of human 4-1BB, as determined by ELISA. (b) The dynamic curves for HuB6, utomilumab and urelumab binding to cynomolgus monkey 4-1BB (Cy4-1BB) determined by ELISA. (c) The dynamic curves for HuB6, utomilumab and urelumab binding to murine 4-1BB (Mu4-1BB) determined by ELISA. (d) The fluorescence values of activated CD8+ T cells binding with FITC-labeled HuB6, utomilumab or an isotype antibody determined by FACS. The results are representative of three different experiments and expressed as the mean value ± SD. **$p < 0.001$ compared to the utomilumab group. *$p < 0.001$ compared to the IgG group. (e) The Kd values of HuB6, utomilumab and urelumab binding to human 4-1BB measured by the Biacore and ForteBio methods and competitive binding capacity of 4-1BBL detected by FACS. The Kd data are representative of three different experiments and expressed as the mean value ± SD. Yes/No indicates whether 4-1BBL can compete for binding to 4-1BB.

Figure 3

HuB6 promotes the proliferation and activation of T cells in manner dependent on FcγR crosslinking. (a) The proliferation of CD8+ T cells was induced by HuB6, and the cell percentage was determined by CSFE labeling. HuB6 was used at three concentrations: 2.5, 10 and 40 μg/ml. (b) CD4+ T, CD8+ T and natural killer (NK) cell activation by HuB6 at the indicated concentrations was monitored through detection of INF-γ in the supernatant by ELISA. *$p < 0.01$ compared to the utomilumab group. (c) The activation of CD8+ T cells by HuB6, urelumab or utomilumab dependent on FcγR crosslinking (in the presence of FcγR□A, FcγR□A or FcγR□B expressed on CHO-K1 cells) was monitored through detection of INF-γ in the supernatant by ELISA. (d) The activation of CD8+ T cells by HuB6, urelumab or utomilumab dependent on FcγR crosslinking (in the presence of FcγR□A, FcγR□A or FcγR□B expressed on CHO-K1 cells) was monitored by a luciferase reporter gene assay. The results are representative of three different experiments and expressed as the mean values.

Figure 4

HuB6 exerts potent antitumor activities in humanized mouse models. (a) Schematic diagram for *in vivo* antibody treatment of human 4-1BB knock-in mice bearing murine colorectal cancer transplants or human PBMC-engrafted mice bearing human colorectal cancer transplants. After tumors were established, the mice were randomized into groups of 8 animals per group on day 8. Treatment with HuB6 or utomilumab was administered six times (indicated by vertical arrows) to MC38 model mice or five times to CT26 and Colo205 model mice. (b) The changes in tumor volume in the HuB6 (MC38 model: l, 0.3 mg/kg; m, 1 mg/kg; and h, 3 mg/kg. CT26 model: l, 1 mg/kg; m, 3 mg/kg; and h, 10 mg/kg) and utomilumab (MC38 model: 1 mg/kg; CT26 model: 10 mg/kg) groups. (c) Relative tumor weight determined by comparison with the isotype control. Tumor tissues were removed and weighed at the end of the experiment in (b). (d) The changes in tumor volume in the HuB6 or utomilumab monotherapy (antibody doses: 0.3 mg/kg for the MC38 model, 10 mg/kg for the Colo205 model) and anti-PD-L1 combination therapy (atezolizumab doses: 0.5 mg/kg for the MC38 model, 10 mg/kg for the Colo205 model) groups. (e) Relative tumor weight determined by comparison with the isotype control. Tumor tissues were removed and weighed at the end of the experiment in (d). In all panels, n = 8 biologically independent animals. Statistical analysis was performed using two-way ANOVA; *$p < 0.05$ compared to the isotype group, **$p < 0.05$ compared to the single antibody group. Error bars within the figure represent the mean ± SD.

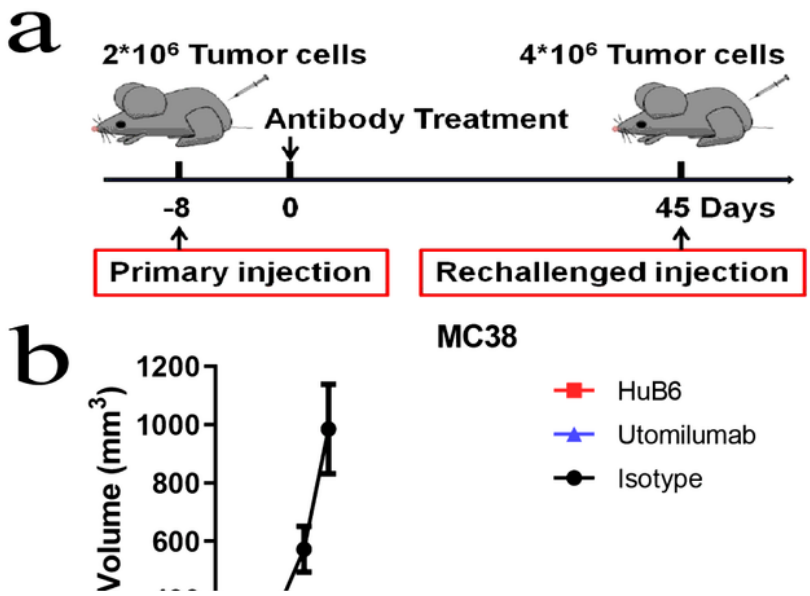

Figure 5

HuB6 induces antitumor immune memory. (a) Schematic of the experimental design for testing antitumor immune memory. (b) Tumor volume changes in humanized 4-1BB mice subcutaneously inoculated with 2 × 10^6 MC38 cells and then intraperitoneally injected with 10 mg/kg HuB6 or utomilumab only once eight days later; the mice were given a secondary subcutaneous injection of 4×10^6 MC38 cells on day 45 (n=6). (c) Tumor volume changes in humanized 4-1BB mice subcutaneously inoculated with 2 × 10^6 B16F10

cells and then intraperitoneally injected with 10 mg/kg HuB6 twice per week beginning 8 days later (five times total); the mice were given a secondary subcutaneous injection of 4×10^6 B16F10 cells on day 45 (n=6).

Figure 6

HuB6 has a good safety profile. (a) Humanized 4-1BB model mice were intraperitoneally injected with HuB6, utomilumab, urelumab or an isotype control once every 3 days (6 times total). The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the low-dose (3 mg/kg) group and high-dose (30 mg/kg) group were measured on the 16th day. *$p < 0.001$. (b, c) Eight male and 8 female cynomolgus monkeys were randomly divided into the low-dose (3 mg/kg), middle-dose (10 mg/kg) and high-dose (30 mg/kg) HuB6 groups and an isotype group, and the antibodies were administered via repeated intravenous infusions (once a week for 5 weeks). The evaluated serum biochemical markers included ALT, AST, creatinine (CREA) and UREA (b), and the hematological indexes included red blood cells (RBCs), white blood cells (WBCs), platelets (PLTs) and lymphocytes (LYMPHs) (c). (d, e) Six male and 6 female cynomolgus monkeys were randomly divided into the low-dose (60 mg/kg) and high-dose (180 mg/kg) HuB6 groups and an isotype control group, and the antibodies were administered only once via intravenous infusion. The evaluated serum biochemical markers included ALT, AST, CREA and UREA (d), and the hematological indexes included RBCs, WBCs, PLTs and LYMPHs (e).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfigure.docx