Am J Cancer Res 2023;13(7):3246-3256
www.ajcr.us /ISSN:2156-6976/ajcr0151328

Original Article

A novel 4-1BB/HER2 bispecific antibody shows potent antitumor activities by increasing and activating tumor-infiltrating T cells

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Received May 20, 2023; Accepted June 12, 2023; Epub July 15, 2023; Published July 30, 2023

Abstract: Resistance to HER2-targeted therapy narrows the efficacy of cancer immunotherapy. Although 4-1BB/CD137 is a promising drug target as a costimulatory molecule of immune cells, no therapeutic drug has been approved in the clinic because of systemic toxicity or limited efficacy. Previously, we developed a humanized anti-HER2 monoclonal antibody (mAb) HuA21 and anti-4-1BB mAb HuB6 with distinct antigen epitopes for cancer therapy. Here, we generated an Fc-muted IgG4 HER2/4-1BB bispecific antibody (BsAb) HK006 by the fusion of HuB6 scFv and HuA21 Fab. HK006 exhibited synergistic antitumor activity by blocking HER2 signal transduction and stimulating the 4-1BB signaling pathway simultaneously and strictly dependent on HER2 expression *in vitro* and *in vivo*. Strikingly, HK006 treatment enhanced antitumor immunity by increasing and activating tumor-infiltrating T cells. Moreover, HK006 did not induce nonspecific production of proinflammatory cytokines and had no obvious toxicity in mice. Overall, these data demonstrated that HK006 should be a promising candidate for HER2-positive cancer immunotherapy.

Keywords: Cancer immunotherapy, monoclonal antibody, 4-1BB/CD137, HER2, antitumor immunity

Introduction

Human epidermal growth factor receptor 2 (HER2) is a key member of the epidermal growth factor receptor (EGFR) family and has a dimerization priority with the family receptors, which initiates a variety of signaling pathways leading to carcinogenesis, cancer metastasis and chemo/radio-therapy resistance [1]. Amplification or overexpression of HER2 occurs in breast cancer, gastric/gastroesophageal cancer and other cancers, such as ovarian, endometrial, bladder, lung and colon cancers, and serves as a prognostic and predictive biomarker [2]. HER2-targeted therapy represented by trastuzumab has fundamentally revolutionized the treatment of various cancers. However, a large proportion of cancer patients do not respond to trastuzumab, highlighting the need to find new therapeutic strategies to enhance antitumor efficacy [3, 4].

4-1BB (CD137) belongs to the tumor necrosis factor receptor (TNFR) superfamily and is an important costimulatory molecule expressed functionally on the surface of T and other types of leukocytes, and its clustering on the cell membrane initiates downstream signaling for cellular proliferation and activation [5-7]. Over the past decade, the potential of 4-1BB costimulation as an effective strategy for cancer immunotherapy has been extensively demonstrated in multiple studies [8-10]. Nevertheless, several 4-1BB agonistic molecules have suffered setbacks due to notable toxicity or suboptimal efficacy, such as urelumab (BMS) with

dose-limiting liver toxicity and utomilumab with insufficient activity even in combination with anti-PD-1 treatment [11-14]. Recently, the 4-1BB receptor, as a compelling target to avert the exhaustion of tumor-infiltrating lymphocytes (TILs) and overcome resistance to immune checkpoint inhibitor (ICI) therapy, has been supported by new immunological theories and findings [15, 16]. As expected, the addition of anti-4-1BB monoclonal antibody (mAb) to anti-HER2 mAb has been demonstrated to strengthen the cytotoxic antitumor response [17]. Moreover, one bispecific antibody (BsAb), PRS-343, targeting HER2 and 4-1BB has been reported to exhibit high antitumor effects with low toxicity in preclinical models, which demonstrates that it should be a promising treatment option using an effective BsAb targeting HER2 and 4-1BB [18].

Previously, we developed the humanized anti-HER2 mAb HuA21 and anti-4-1BB mAb HuB6 with distinct antigen epitopes for cancer therapy [10, 19]. By the fusion of HuB6 scFv and HuA21 Fab with a mutated Fc fragment, we generated a fully human IgG4 HER2/4-1BB BsAb HK006 to activate 4-1BB signaling strictly dependent on HER2 expression and simultaneously block HER2 signaling within the tumor microenvironment (TME). Functional studies *in vitro* and in humanized mouse models indicated that HK006 had potent antitumor efficacy by increasing and activating tumor-infiltrated T cells.

Material and methods

Cell culture

FcγRIIIa (158 V) Jurkat-NFAT-Luc effector cells were purchased from Genomeditech (Shanghai, China). CHO-K1-hu4-1BB cells expressing full-length human 4-1BB were generated by lentiviral transduction. All of the engineered cells were cultured in DMEM/F12 (HyClone) supplemented with 10% FBS, 1% P/S and 1 mg/ml geneticin (Gibco). Human peripheral blood mononuclear cells (PBMCs) were purchased from Sailybio Corporation (Shanghai) with informed consent from all donors and cultured in RPMI 1640 medium supplemented with 10% FBS. The human cancer cell lines NCI-N87 (N87), SKOV3 and MCF7 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The MCF7 cell line overexpressing human HER2 (MCF7/HER2) was generated and cultured in RPMI 1640 medium supplemented with 10% FBS, 1% P/S and 1 mg/ml Geneticin. All the cell lines were cultured at 37°C in a humidified incubator with 5% CO_2.

Protein expression and purification

The antibodies and antigens used in the study were generated by cloning DNA-encoding sequences independently into the multiple cloning sites of the mammalian expression vector pcDNA3.4 TOPO (Invitrogen), followed by expression in the Expi293F expression system (Gibco). The sequences of the anti-CD3 antibody (clone: OKT3) and PRS-343 were obtained from IMGT/mAb-DB (http://www.imgt.org/mAb-DB) and the patent US-10865250-B2, respectively. The sequences for 4-1BB and HER2 proteins were obtained from UniProt (human 4-1BB: Q07011, human HER2: P04626). Proteins were purified by protein A chromatography or Ni^{2+} chromatography (Cytiva).

Antibody specificity assays

HER2-expressing cancer cells or CHO-K1-hu4-1BB cells were used to determine the specificity of HK006. The cells were incubated for 1 hour with a series of dilutions of the antibodies and then incubated with FITC-conjugated goat anti-human Fc antibody (H10301, Invitrogen) for 30 min at 4°C. The stained cells were analyzed on the CytoFlex system (Beckman, USA), and median fluorescent intensity (MFI) values were plotted against the concentration of primary antibody. Human IgG used as an isotype control was purchased from GenScript Biotech (Nanjing, China).

Next, the binding of HK006 to human HER2 or 4-1BB proteins was analyzed by ELISA. The Nunc Maxisorp plate was coated with 1 μg/mL of proteins in carbonate buffer at 4°C overnight. After blocking with 1% BSA (Gibco) at 37°C for 2 hours, serially diluted test antibodies were added to each well and incubated at room temperature for 2 hours. The wells were washed and incubated for 1 hour at 37°C with horseradish peroxidase (HRP)-conjugated goat anti-human IgG Fc (146460, Jackson). After washing with a PBST solution three times, TMB (Invitrogen) was added as a substrate, and the absorbance was detected at 450 nm by VersaMax (Molecular Devices). For the dual-

antigen capture ELISA, human HER2 antigen was coated, and HK006 binding was detected using human 4-1BB and HRP-conjugated secondary antibody (115-035-062, Jackson).

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

To test the Fc function of the antibody, ADCC effector cells (Jurkat-NFAT-Luc, 3 × 10^4 cells/well) and target cells (SKOV3, 1 × 10^4 cells/well) were mixed with HK006 and incubated at 37°C and 5% CO_2 for 6 hours. Trastuzumab acted as a positive control. Luminescence was detected by SpectraMax (Molecular Devices) following the addition of an equal volume of firefly luciferase reagent.

Cytokine release assay

Human PBMCs from healthy donors in RPMI-1640 medium with 10% FBS in 96-well flat-bottom plates (2 × 10^5 cells/well) were treated with 10 μg/ml of the tested antibodies for 48 hours. HK006 was compared with the isotype controls IgG4, HuB6 and Urelumab. The levels of the cytokines IFN-γ, TNF-α, IL-10, IL-2, IL-6, IL-4 and IL-17a in the culture medium were measured by cytometric bead array assay (C60021, QuantoBio) according to the manufacturer's instructions. Fluorescence signals were measured by the CytoFlex system.

Cytotoxicity assay for cancer cells

To test the cytotoxicity mediated by HK006, HER2-positive cancer cell lines N87, SKOV3, MCF7/HER2 or HER2-negative cell line MCF7 were cultured at 1.0 × 10^5 cells/mL in a 96-well microplate coated with 0.5 μg/mL OKT3 overnight and then cocultured with 5 × 10^5 human PBMCs for 48 hours in a CO_2 incubator at 37°C. After incubation, the cytotoxicity for cancer cells was observed using a microscope (IX71, Olympus) and determined according to the release quantity of lactate dehydrogenase (LDH) by the kit (CK12, Dojindo). Similarly, 1.0 × 10^5 cells/mL cancer cells were cultured in a 96-well microplate coated with 0.5 μg/mL OKT3 overnight, treated with 20 μg/mL mitomycin C (Sigma Aldrich) for 2 hours, and then incubated with 5 × 10^5 cells/mL primary CD8 T cells from PBMCs using an isolation kit (557766, BD) and HK006 or other control antibodies at the indicated concentration for 3 days. The IFN-γ level in the cell supernatant was measured by ELISA.

Mouse models

Eight-week-old NOD. CB17-Prkdcscidll2rgtm1/Bcgen (B-NDG) mice, in which human PBMCs were transplanted to reconstitute human immune cells, were purchased from Biocytogen Corporation (Beijing, China) and used according to the approved experimental protocol. Briefly, 2 × 10^6 N87 or SKOV3 cells mixed with the same volume of Corning Matrigel were inoculated subcutaneously into the right flanks of huPBMC-B-NDG mice. After the tumor size reached ~50 mm^3, the tumor-bearing animals were randomized on the basis of tumor volume and body weight. Subsequently, antibodies were injected intraperitoneally into mice at a dose of 2.5 mg/kg twice a week for up to 3 weeks (n=6). 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. Mice were monitored for body weight and blood parameters.

Histopathology analysis

Immunohistochemistry (IHC) and hematoxylin and eosin (HE) assays were performed by staining 4-μm-thick formalin-fixed, paraffin-embedded whole tissue sections. For the IHC method, briefly, deparaffinized slides received one night of incubation using primary antibody (CD4 or CD8, Abcam) at 4°C. Next, the biotinylated secondary antibody and subsequent streptavidin-horseradish peroxidase complex were employed for treating tissue sections. Slides were photographed by an Aperio Versa 8 tissue imaging system (Leica). Images were analyzed using Indica Halo software.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and Dunnett's T3 test using GraphPad Prism 8.0 (GraphPad Software). A p value of < 0.05 was considered statistically significant.

Results

Construction and characterization of HK006

HK006 was generated as a novel Fc-mutated IgG4 HER2x4-1BB BsAb by fusing a single-

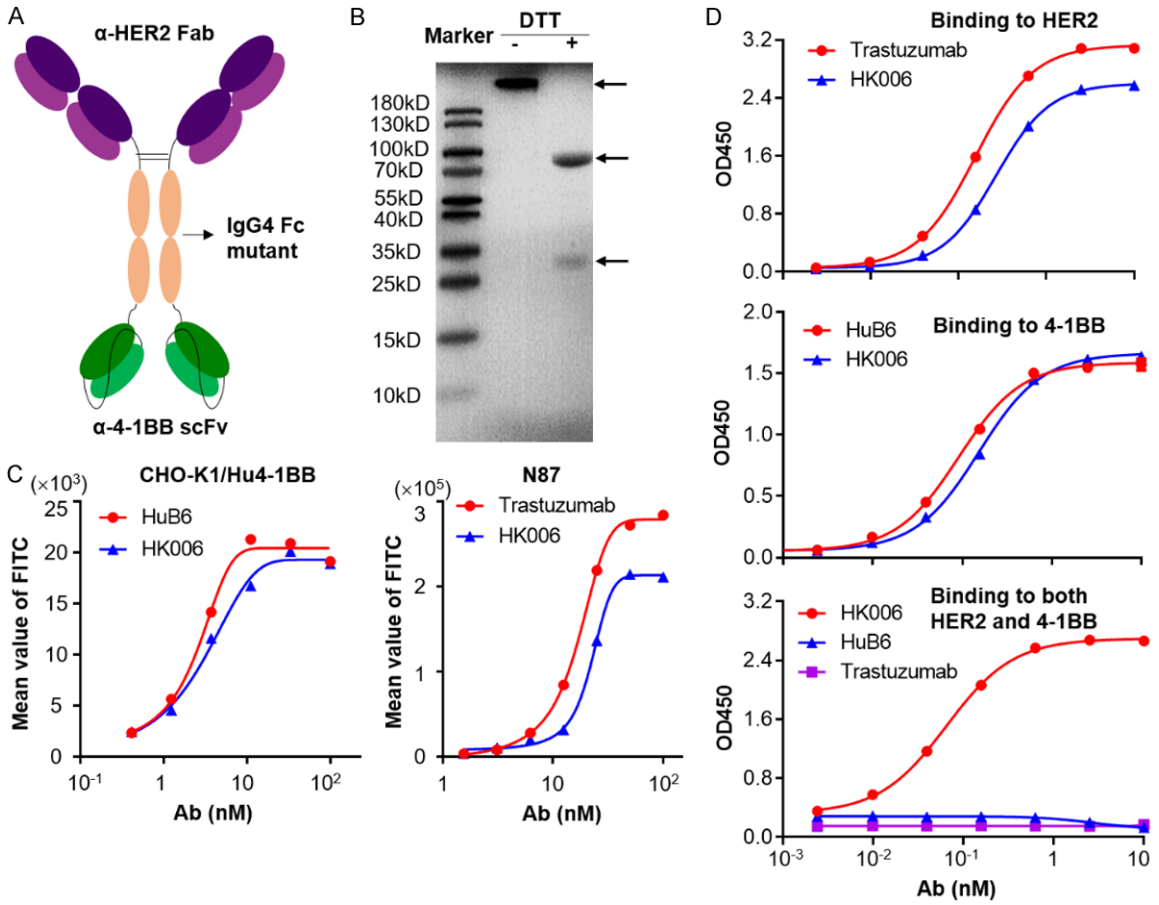

Figure 1. HK006 binds 4-1BB and HER2 simultaneously. A. Schematic diagram of the bispecific single chain antibody HK006. B. Electrophoresis pattern of HK006 with or without DTT. The bands indicated by black arrows represent HK006 or its fragments. C. HK006 binds both human 4-1BB-expressing CHO-K1 cells and HER2-positive N87 cells, as measured by flow cytometry. D. HK006 binds to both 4-1BB and HER2 proteins, as determined by ELISA. The curves are presented as one representative of three independent experiments.

chain variable fragment (scFv) targeting 4-1BB at the C-terminus of the heavy chain of a proprietary anti-HER2 IgG4 monoclonal antibody (mAb, patent CN104225594A) with three amino acid mutations (S228P, F234A, L235A) in the Fc region to mute its function (**Figure 1A**). The anti-4-1BB scFv was modified from the previously generated anti-4-1BB antibody HuB6 [10] (patents CN112794906B, CN1127949-05A, and WO2021093753A1). The structure of HK006 was confirmed by its electrophoretic pattern with or without the reducing agent dithiothreitol (DTT, **Figure 1B**). The cell binding assays demonstrated that HK006 bound human 4-1BB to an equivalent level as the parental mAb HuB6, and the affinity with HER2 was reduced compared to trastuzumab using the FACS method (**Figure 1C**), which was also confirmed by ELISA (**Figure 1D**). Moreover, HK006 could simultaneously bind to both 4-1BB and HER2, as shown by a dual-antigen capture ELISA (**Figure 1D**).

HK006 selectively kills cancer cells in a HER2-dependent manner

HER2-positive/negative cancer cell lines were cocultured with human PBMCs and treated with HK006 or trastuzumab to evaluate their cytotoxicity. As expected, both trastuzumab and HK006 induced no significant change in HER2-negative MCF7 cells and remarkable cytotoxicity in HER2-positive cells, including N87, SKOV3 and the constructed MCF7/HER2, at a concentration of 5 μg/mL. Obviously, HK006 induced more cytotoxicity in HER2-positive tumor cells than trastuzumab (**Figure 2A**, **2B**). To further confirm that HK006 acti-

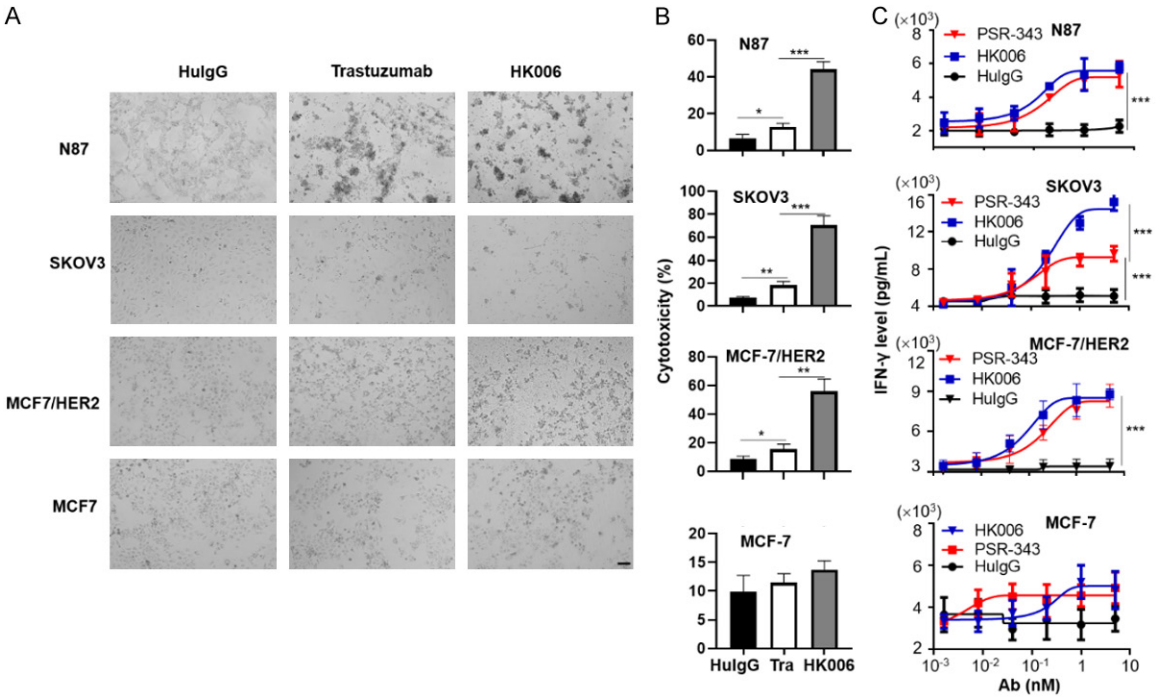

Figure 2. HK006 selectively kills cancer cells in a HER2-dependent manner. A. The cytotoxicity of HK006 in HER2-positive cancer cells was observed under a microscope. Scale, 100 μm. B. The cytotoxicity of HK006 in HER2-positive cancer cells was determined using the LDH release assay. C. HK006 induces IFN-γ secretion by human primary CD8+ T cells cocultured with HER2-positive cancer cells, as determined by ELISA. A human IgG4 protein was used as an isotype control. The values are presented as the mean ± SD from one representative of three independent experiments. **P < 0.01, ***P < 0.001.

vates 4-1BB-expressing immune cells in a strictly HER2-dependent manner, HER2-positive/negative cancer cells were cocultured with human primary CD8 T cells and treated with HK006 or PRS-343 at 5-fold dilutions from 5 nM. Notably, HK006 or PRS-343 increased IFN-γ secretion of CD8 T cells cocultured with HER2-positive cancer cells compared with HuIgG when the concentration was more than 0.2 nM, but there was no significant effect for HER2-negative cancer cells (**Figure 2C**). These results demonstrated that HK006 should have selective cytotoxicity for HER2-positive cancer cells. In particular, HK006 induced more IFN-γ secretion than PRS-343 in the SKOV3 cell line ($P < 0.001$, **Figure 2C**).

HK006 shows potent antitumor efficacy in vivo

To investigate the antitumor effect of HK006 *in vivo*, a human PBMC-reconstituted mouse model engrafted with HER2-positive SKOV3 or N87 tumor cells was given mAb treatment according to the schematic diagram (**Figure 3A**). HK006 treatment resulted in a significant reduction of tumor volume and weight in the mouse model bearing SKOV3 transplant, as well as PRS-343 and Trastuzumab plus HuB6, compared with the HuIgG control. Moreover, HK006 showed more tumor growth inhibition of SKOV3 cells than either PRS-343 or trastuzumab plus HuB6 (**Figure 3B**, **3C**, $P < 0.05$). Similarly, HK006 also induced an obvious decrease in tumor volume and weight in the mouse model bearing N87 cell transplants (**Figure 3D**, **3E**, $P < 0.001$). Taken together, these data indicated that HK006 had notable antitumor activity *in vivo* and showed some superiority to the combination of anti-HER2 mAb and 4-1BB agonist.

HK006 increases the abundance of tumor-infiltrated T cells

The *in vivo* capacity of HK006 to target T cells to HER2-positive tumor cells was assessed by histopathology of SKOV3 tumors. HE staining results indicated that HK006 could induce lymphocytes to infiltrate into tumor tissue and resulted in tumor apoptosis and necrosis (**Figure 4A**). Moreover, IHC results confirmed that HK006, PRS-343 and trastuzumab plus

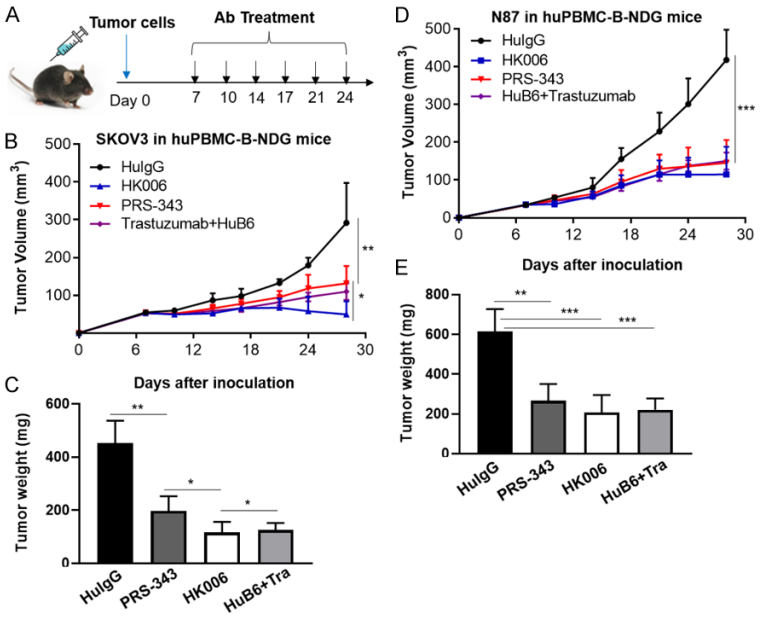

Figure 3. HK006 shows strong antitumor activity *in vivo*. A. Schematic diagram of antibody treatment of humanized mice bearing HER2-positive cancer transplants. When the mean tumor size reached approximately 50 mm^3, mice were randomized into groups of 6 animals per group. Treatment with HK006 or control antibody six times (indicated by vertical arrows). B, C. HK006 induced a significant reduction in SKOV3 tumor volume and weight in a mouse model. D, E. HK006 induced a significant reduction in N87 tumor volume and weight in a mouse model. The values are presented as the mean ± SD from one representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

HuB6 all increased the number and percentage of tumor-infiltrated lymphocytes (TILs), including CD4 and CD8 T cells, in the tumor tissue compared with the HuIgG control ($P < 0.001$), thereby rescuing the exhausted TILs and exerting potent antitumor immunity *in vivo* (**Figure 4A**, **4B**). According to immunohistochemistry results, compared with PRS-343 or trastuzumab plus HuB6, HK006 exhibited obviously upregulated CD4 and CD8 expression ($P < 0.05$, **Figure 4B**).

Taken together, HK006 could simultaneously bind to the HER2 receptor of tumor cells and 4-1BB on the surface of T cells, resulting in the blockade of HER2 signal transduction and activation of 4-1BB signaling, which induced the activation and cytotoxic cytokine release of T effector cells within the TME and thus effectively and specifically killed HER2-positive tumor cells (**Figure 4C**).

HK006 has no obvious toxicity

HK006 was confirmed not to mediate ADCC, whereas trastuzumab induced strong ADCC activity as a positive control (**Figure 5A**). Moreover, HK006 did not induce more proinflammatory cytokine (IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α) release than HuB6 and control BsAb PRS-343, which had been shown to have no systemic toxicity in preclinical studies and even maintained similar cytokine levels to the PBS control in an *in vitro* evaluation assay for antigen-independent cytokine release (**Figure 5B**). Moreover, no drug-related abnormalities in body weight or serum ALT/AST were observed in the treated mice (**Figure 5C-E**). These results suggest that HK006 should have a safe profile as a potential immunotherapy drug.

Discussion

Low objective response limits the clinical use of trastuzumab even in combination with chemotherapy and anti-PD-1/PD-L1 mAb, although it has obtained great success [20-22]. A 4-1BB agonist has been shown to activate downstream signaling cascades that enhance T-cell function and is undoubtedly a promising candidate for cancer immunotherapy [23-25]. However, therapeutic attempts to target 4-1BB have been hampered due to systemic toxicity or suboptimal agonistic potency [12, 26, 27]. In our study, an Fc-muted HER2/4-1BB BsAb, HK006, was generated and shown to inhibit HER2 signal transduction in tumor cells and stimulate 4-1BB signaling in T cells simultaneously in the TME without systemic toxicity.

HK006 should have unique antigen epitopes for HER2 and 4-1BB according to its parental mAbs HuB6 and HuA21 with distinct epitopes [10, 19, 28] and was further confirmed to bind to both antigens simultaneously using *in vitro* assays. Functionally, HK006 showed increased cytotoxicity for HER2-positive cancer cells compared with trastuzumab by activating immune cells from PBMCs. Moreover, HK006-triggered cytokine release of CD8 T cells was strictly

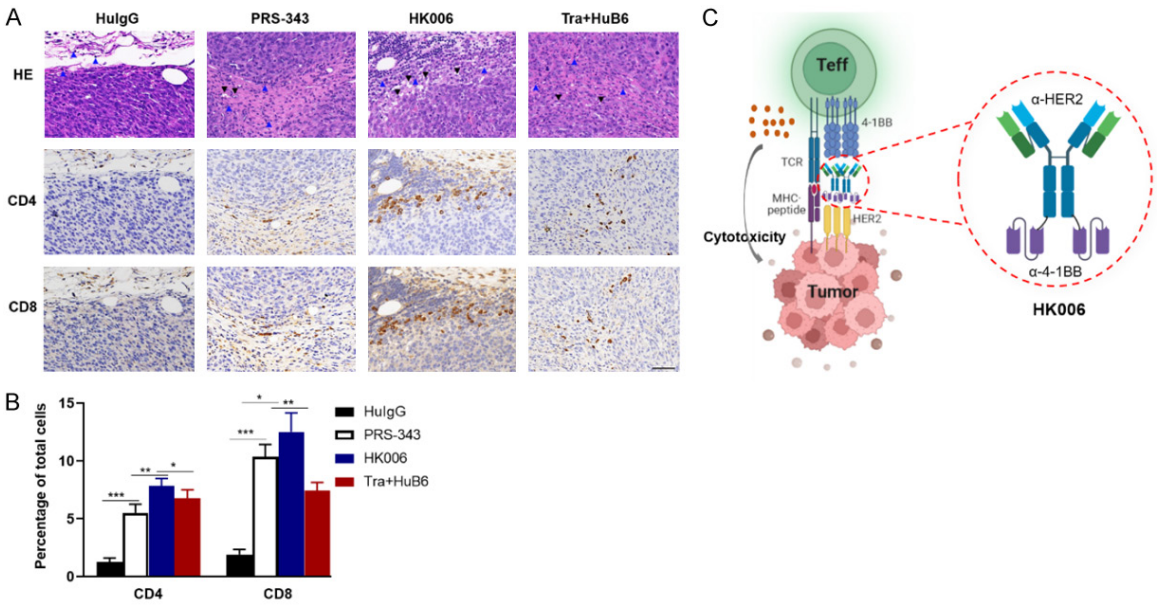

Figure 4. HK006 increases the abundance of tumor-infiltrating T cells. A. HE and IHC staining for CD4 and CD8 T cells in SKOV3 tumor tissue. Black and blue arrows mark tumor cell apoptosis and immune cells, respectively. B. HK006 increases the percentages of tumor-infiltrating CD8 T and CD4 T cells among total cells. The values are presented as the mean ± SD from the analysis results using Indica Halo software. *P < 0.05, **P < 0.01, ***P < 0.001. C. Schematic illustration of HK006 action. HK006 simultaneously binds to HER2 expressed on tumor cells and 4-1BB expressed on T cells and results in the blockage of HER2 signal transduction and clustering of 4-1BB receptors on the surface of T cells, which induces the activation and cytokine release of T effector cells and thus effectively and specifically kills tumor cells.

dependent on HER2 expression in cancer cells, thereby ensuring a preferential distribution of HK006 to HER2-expressing cells in the TME to locally stimulate antigen-specific T cells.

In humanized mouse models bearing human HER2-expressing tumors, HK006 displayed a similar antitumor efficacy against N87 cell transplantation and even a more potent inhibitory effect on tumor growth against SKOV3 cell transplantation than the positive control BsAb PRS-343 or trastuzumab combined with HuB6. The data were in accordance with the *in vitro* results for CD8 T cells cocultured with SKOV3 cells treated with HK006 and PRS-343, which implied that HK006 might be superior for ovarian cancer immunotherapy.

Mechanistically, PRS-343 has been proven to increase the number of TILs by targeting 4-1BB and HER2 simultaneously. Similarly, our data demonstrated that HK006 could increase tumor-infiltrated CD8 T and CD4 T cells, even better than PRS-343 and trastuzumab combined with HuB6, in SKOV3 tumors and resulted in the apoptosis and necrosis of tumor cells.

Previously, we generated a PD-L1x4-1BB bispecific antibody (BsAb) HK010 to simultaneously block PD-1/PD-L1 signaling and stimulate 4-1BB signaling strictly dependent on the PD-L1 receptor and found that HK010 treatment recruited more CD8 T cells into tumor tissue, reduced TAMs and resulted in more tumor apoptosis and necrosis than the anti-PD-L1 mAb [29]. Moreover, it has been reported that anti-4-1BB mAb treatment results in the expansion of CD8 T cells in lymphoid organs and tumors [30]. Therefore, we speculated that the function of HK006 in increasing and activating TILs in HER2-positive tumor tissue should be through stimulating the 4-1BB signaling pathway. It has been reported that T-cell dysfunction resulting from the immune-suppressive milieu and T-cell exhaustion in the TME leads to resistance to anti-PD-1/PD-L1 immunotherapy in malignancies [31, 32]. Therefore, it is possible that HK006 may be used in the therapeutic regimen of resistant cancer for current immunotherapy.

Previously, an anti-4-1BB mAb was reported to mediate 4-1BB clustering by binding the Fc

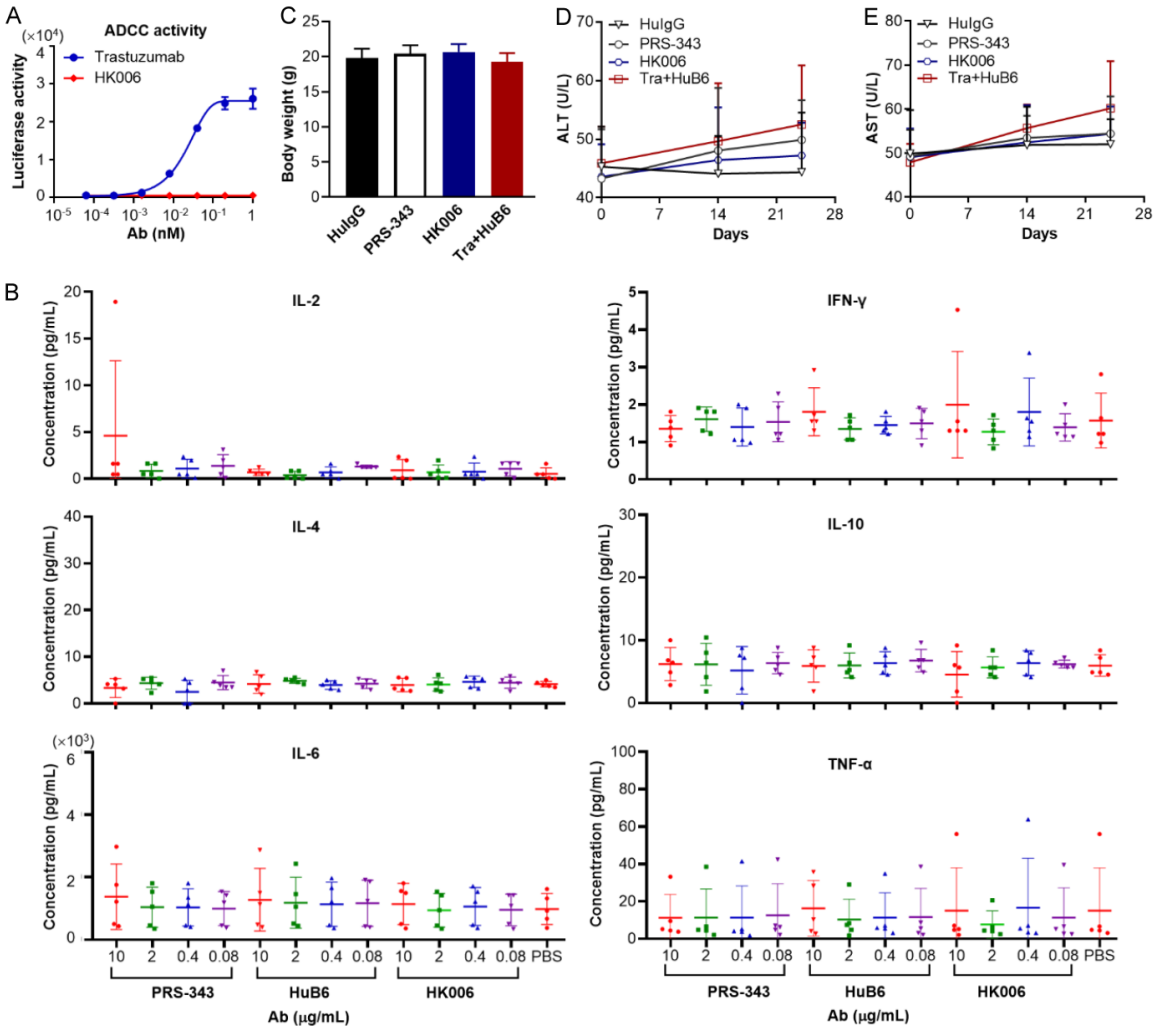

Figure 5. HK006 has no systemic toxicity. A. HK006 does not induce ADCC in Jurkat/NFAT-luc cells, as determined by the luciferase reporter assay. Trastuzumab was used as the positive control. B. HK006 does not induce antigen-independent cytokine release, as determined by a cytokine bead array (CBA) across a gradient of mAb concentrations (n=5). PBS was used as the negative control. The values are presented as the mean ± SD from one representative of three independent experiments. C. Body weight comparison of mice. D, E. Comparison of serum ALT/AST levels in mice.

domain to FcγR-expressing cells, especially to the inhibitory FcγRIIb [33, 34]. However, this approach was limited due to undesirable toxicity associated with FcγR interactions in the liver and suboptimal efficacy by differential FcγR expression [35]. In addition, FcγRIIb has been described to mediate internalization [36, 37], which may reduce the efficacy of molecules. Meanwhile, the full Fc fragment has ADCC activity, which can induce severe adverse effects such as thrombocytopenia, and Fc engineering has proven to be a good strategy to ameliorate safety liabilities [10, 38]. Therefore, HK006 was generated using an Fc-muted human IgG4 backbone to further minimize the interactions with FcγRs and functional activity, which was confirmed by our data. It has been reported that PRS-343 can stimulate 4-1BB-expressing T cells only in the presence of HER2-expressing cancer cells, which provides a more localized activation of the immune system with higher efficacy and reduced peripheral toxicity compared with current monospecific approaches [18]. Similarly, HK006 was also found not to induce nonspecific production of proinflammatory cytokines and overt toxicity in the treated mice. These results suggested that HK006 has a safety profile to support further development in clinical studies.

Taken together, we generated a novel anti-HER2 × 4-1BB BsAb, HK006, by fusing an anti-4-1BB scFv and anti-HER2 Fab with a muted IgG4 Fc fragment. HK006 exhibited synergistic antitumor activity by blocking HER2 signal transduction and enhancing antitumor immunity simultaneously and strictly dependent on HER2 expression without systemic toxicity.

Acknowledgements

This work was supported by the Key Project of Science and Technology of Anhui Province (202004b11020019), the Hefei Municipal Natural Science Foundation (2021005) and the Scientific Research Projects for Graduate Students in Anhui Universities (YJS20210265).

Disclosure of conflict of interest

None.

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References

[1] Miglietta F, Griguolo G, Bottosso M, Giarratano T, Lo Mele M, Fassan M, Cacciatore M, Genovesi E, De Bartolo D, Vernaci G, Amato O, Conte P, Guarneri V and Dieci MV. Evolution of HER2-low expression from primary to recurrent breast cancer. NPJ Breast Cancer 2021; 7: 137.

[2] Iqbal N and Iqbal N. Human epidermal growth factor receptor 2 (HER2) in cancers: overexpression and therapeutic implications. Mol Biol Int 2014; 2014: 852748.

[3] Ling Y, Liang G, Lin Q, Fang X, Luo Q, Cen Y, Mehrpour M, Hamai A, Liu Z, Shi Y, Li J, Lin W, Jia S, Yang W, Liu Q, Song E, Li J and Gong C. circCDYL2 promotes trastuzumab resistance via sustaining HER2 downstream signaling in breast cancer. Mol Cancer 2022; 21: 8.

[4] Ong LT, Lee WC, Ma S, Oguz G, Niu Z, Bao Y, Yusuf M, Lee PL, Goh JY, Wang P, Yong KSM, Chen Q, Wang W, Ramasamy A, Hoon DSB, Ditzel HJ, Tan EY, Lee SC and Yu Q. IFI16-dependent STING signaling is a crucial regulator of anti-HER2 immune response in HER2+ breast cancer. Proc Natl Acad Sci U S A 2022; 119: e2201376119.

[5] Li Y, Tan S, Zhang C, Chai Y, He M, Zhang CW, Wang Q, Tong Z, Liu K, Lei Y, Liu WJ, Liu Y, Tian Z, Cao X, Yan J, Qi J, Tien P, Gao S and Gao GF. Limited cross-linking of 4-1BB by 4-1BB ligand and the agonist monoclonal antibody utomilumab. Cell Rep 2018; 25: 909-920, e4.

[6] Bitra A, Doukov T, Croft M and Zajonc DM. Crystal structures of the human 4-1BB receptor bound to its ligand 4-1BBL reveal covalent receptor dimerization as a potential signaling amplifier. J Biol Chem 2018; 293: 9958-9969.

[7] Yu X, James S, Felce JH, Kellermayer B, Johnston DA, Chan HTC, Penfold CA, Kim J, Inzhelevskaya T, Mockridge CI, Watanabe Y, Crispin M, French RR, Duriez PJ, Douglas LR, Glennie MJ and Cragg MS. TNF receptor agonists induce distinct receptor clusters to mediate differential agonistic activity. Commun Biol 2021; 4: 772.

[8] Menk AV, Scharping NE, Rivadeneira DB, Calderon MJ, Watson MJ, Dunstane D, Watkins SC and Delgoffe GM. 4-1BB costimulation induces T cell mitochondrial function and biogenesis enabling cancer immunotherapeutic responses. J Exp Med 2018; 215: 1091-1100.

[9] Harao M, Forget MA, Roszik J, Gao H, Babiera GV, Krishnamurthy S, Chacon JA, Li S, Mittendorf EA, DeSnyder SM, Rockwood KF, Bernatchez C, Ueno NT, Radvanyi LG, Vence L, Haymaker C and Reuben JM. 4-1BB-enhanced expansion of CD8(+) TIL from triple-negative breast cancer unveils mutation-specific CD8(+) T cells. Cancer Immunol Res 2017; 5: 439-445.

[10] Cheng LS, Cheng YF, Liu WT, Shen A, Zhang D, Xu T, Yin W, Cheng M, Ma X, Wang F, Zhao Q, Zeng X, Zhang Y and Shen G. A humanized 4-1BB-targeting agonistic antibody exerts potent antitumor activity in colorectal cancer without systemic toxicity. J Transl Med 2022; 20: 415.

[11] Segal NH, He AR, Doi T, Levy R, Bhatia S, Pishvaian MJ, Cesari R, Chen Y, Davis CB, Huang B, Thall AD and Gopal AK. Phase I study of single-agent utomilumab (PF-05082566), a 4-1BB/CD137 agonist, in patients with advanced cancer. Clin Cancer Res 2018; 24: 1816-1823.

[12] Segal NH, Logan TF, Hodi FS, McDermott D, Melero I, Hamid O, Schmidt H, Robert C, Chiarion-Sileni V, Ascierto PA, Maio M, Urba WJ, Gangadhar TC, Suryawanshi S, Neely J, Jure-Kunkel M, Krishnan S, Kohrt H, Sznol M and Levy R. Results from an integrated safety analysis of urelumab, an agonist anti-CD137 monoclonal antibody. Clin Cancer Res 2017; 23: 1929-1936.

[13] Bartkowiak T, Jaiswal AR, Ager CR, Chin R, Chen CH, Budhani P, Ai M, Reilley MJ, Sebastian

MM, Hong DS and Curran MA. Activation of 4-1BB on liver myeloid cells triggers hepatitis via an interleukin-27-dependent pathway. Clin Cancer Res 2018; 24: 1138-1151.
[14] Melero I, Hirschhorn-Cymerman D, Morales-Kastresana A, Sanmamed MF and Wolchok JD. Agonist antibodies to TNFR molecules that costimulate T and NK cells. Clin Cancer Res 2013; 19: 1044-1053.
[15] Kim HD, Park S, Jeong S, Lee YJ, Lee H, Kim CG, Kim KH, Hong SM, Lee JY, Kim S, Kim HK, Min BS, Chang JH, Ju YS, Shin EC, Song GW, Hwang S and Park SH. 4-1BB delineates distinct activation status of exhausted tumor-infiltrating CD8(+) T cells in hepatocellular carcinoma. Hepatology 2020; 71: 955-971.
[16] Hall MS, Mullinax JE, Cox CA, Hall AM, Beatty MS, Blauvelt J, Innamarato P, Nagle L, Branthoover H, Wiener D, Schachner B, Martinez AJ, Richards AD, Rich CJ, Colon Colon M, Schell MJ, Teer JK, Khushalani NI, Weber JS, Mule JJ, Sondak VK, Pilon-Thomas S and Sarnaik AA. Combination nivolumab, CD137 agonism, and adoptive cell therapy with tumor-infiltrating lymphocytes for patients with metastatic melanoma. Clin Cancer Res 2022; 28: 5317-5329.
[17] Kim HY, Choi JH, Haque MM, Park JH, Kim IH, Choi BK, Lee A and Park S. Combined treatment with anti-HER2/neu and anti-4-1BB monoclonal antibodies induces a synergistic antitumor effect but requires dose optimization to maintain immune memory for protection from lethal rechallenge. Cancer Immunol Immunother 2022; 71: 967-978.
[18] Hinner MJ, Aiba RSB, Jaquin TJ, Berger S, Durr MC, Schlosser C, Allersdorfer A, Wiedenmann A, Matschiner G, Schuler J, Moebius U, Rothe C, Matis L and Olwill SA. Tumor-localized costimulatory T-cell engagement by the 4-1BB/HER2 bispecific antibody-anticalin fusion PRS-343. Clin Cancer Res 2019; 25: 5878-5889.
[19] Wang Z, Yip LY, Lee JHJ, Wu Z, Chew HY, Chong PKW, Teo CC, Ang HY, Peh KLE, Yuan J, Ma S, Choo LSK, Basri N, Jiang X, Yu Q, Hillmer AM, Lim WT, Lim TKH, Takano A, Tan EH, Tan DSW, Ho YS, Lim B and Tam WL. Methionine is a metabolic dependency of tumor-initiating cells. Nat Med 2019; 25: 825-837.
[20] Janjigian YY, Kawazoe A, Yanez P, Li N, Lonardi S, Kolesnik O, Barajas O, Bai Y, Shen L, Tang Y, Wyrwicz LS, Xu J, Shitara K, Qin S, Van Cutsem E, Tabernero J, Li L, Shah S, Bhagia P and Chung HC. The KEYNOTE-811 trial of dual PD-1 and HER2 blockade in HER2-positive gastric cancer. Nature 2021; 600: 727-730.
[21] Wang J, Huang Q, Hu X, Zhang S, Jiang Y, Yao G, Hu K, Xu X, Liang B, Wu Q, Ma Z, Wang Y, Wang C, Wu Z, Rong X, Liao W and Shi M. Disrupting circadian rhythm via the PER1-HK2 axis reverses trastuzumab resistance in gastric cancer. Cancer Res 2022; 82: 1503-1517.
[22] Valkema MJ, Mostert B, Lagarde SM, Wijnhoven BPL and van Lanschot JJB. The effectivity of targeted therapy and immunotherapy in patients with advanced metastatic and non-metastatic cancer of the esophagus and esophago-gastric junction. Updates Surg 2023; 75: 313-323.
[23] Vanamee ES and Faustman DL. Structural principles of tumor necrosis factor superfamily signaling. Sci Signal 2018; 11: eaao4910.
[24] Chin SM, Kimberlin CR, Roe-Zurz Z, Zhang P, Xu A, Liao-Chan S, Sen D, Nager AR, Oakdale NS, Brown C, Wang F, Yang Y, Lindquist K, Yeung YA, Salek-Ardakani S and Chaparro-Riggers J. Structure of the 4-1BB/4-1BBL complex and distinct binding and functional properties of utomilumab and urelumab. Nat Commun 2018; 9: 4679.
[25] Gopal AK, Levy R, Houot R, Patel SP, Popplewell L, Jacobson C, Mu XJ, Deng S, Ching KA, Chen Y, Davis CB, Huang B, Fly KD, Thall A, Woolfson A and Bartlett NL. First-in-human study of utomilumab, a 4-1BB/CD137 agonist, in combination with rituximab in patients with follicular and other CD20(+) non-hodgkin lymphomas. Clin Cancer Res 2020; 26: 2524-2534.
[26] Segal NH, Gopal AK, Bhatia S, Kohrt HE, Levy R, Pishvaian MJ, Houot R, Bartlett N, Nghiem P, Kronenberg SA, Thall AD, Mugundu G, Huang B and Davis C. A phase 1 study of PF-05082566 (anti-4-1BB) in patients with advanced cancer. J Clin Oncol 2014; 32: 3007-3007.
[27] Tolcher AW, Sznol M, Hu-Lieskovan S, Papadopoulos KP, Patnaik A, Rasco DW, Di Gravio D, Huang B, Gambhire D, Chen Y, Thall AD, Pathan N, Schmidt EV and Chow LQM. 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: 5349-5357.
[28] Li R, Hu S, Chang Y, Zhang Z, Zha Z, Huang H, Shen G, Liu J, Song L and Wei W. Development and characterization of a humanized anti-HER2 antibody HuA21 with potent anti-tumor properties in breast cancer cells. Int J Mol Sci 2016; 17: 563.
[29] Cheng LS, Zhu M, Gao Y, Liu WT, Yin W, Zhou P, Zhu Z, Niu L, Zeng X, Zhang D, Fang Q, Wang F, Zhao Q, Zhang Y and Shen G. An Fc-muted bispecific antibody targeting PD-L1 and 4-1BB induces antitumor immune activity in colorectal cancer without systemic toxicity. Cell Mol Biol Lett 2023; 28: 47.
[30] Weulersse M, Asrir A, Pichler AC, Lemaitre L, Braun M, Carrie N, Joubert MV, Le Moine M, Do

Souto L, Gaud G, Das I, Brauns E, Scarlata CM, Morandi E, Sundarrajan A, Cuisinier M, Buisson L, Maheo S, Kassem S, Agesta A, Peres M, Verhoeyen E, Martinez A, Mazieres J, Dupre L, Gossye T, Pancaldi V, Guillerey C, Ayyoub M, Dejean AS, Saoudi A, Goriely S, Avet-Loiseau H, Bald T, Smyth MJ and Martinet L. Eomes-dependent loss of the co-activating receptor CD226 restrains CD8(+) T cell anti-tumor functions and limits the efficacy of cancer immunotherapy. Immunity 2020; 53: 824-839, e10.

[31] Liu Y, Zhang Q, Xing B, Luo N, Gao R, Yu K, Hu X, Bu Z, Peng J, Ren X and Zhang Z. Immune phenotypic linkage between colorectal cancer and liver metastasis. Cancer Cell 2022; 40: 424-437, e5.

[32] Sorrentino C, D'Antonio L, Fieni C, Ciummo SL and Di Carlo E. Colorectal cancer-associated immune exhaustion involves T and B lymphocytes and conventional NK cells and correlates with a shorter overall survival. Front Immunol 2021; 12: 778329.

[33] Eskiocak U, Guzman W, Wolf B, Cummings C, Milling L, Wu HJ, Ophir M, Lambden C, Bakhru P, Gilmore DC, Ottinger S, Liu L, McConaughy WK, He SQ, Wang C, Leung CL, Lajoie J, Carson WF 4th, Zizlsperger N, Schmidt MM, Anderson AC, Bobrowicz P, Schuetz TJ and Tighe R. Differentiated agonistic antibody targeting CD137 eradicates large tumors without hepatotoxicity. JCI Insight 2020; 5: e133647.

[34] Ho SK, Xu Z, Thakur A, Fox M, Tan SS, DiGiammarino E, Zhou L, Sho M, Cairns B, Zhao V, Xiong M, Samayoa J, Forsyth CM, Powers DB, Chao DT, Hollenbaugh D, Alvarez HM and Akamatsu Y. 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: 1040-1051.

[35] Claus C, Ferrara C, Xu W, Sam J, Lang S, Uhlenbrock F, Albrecht R, Herter S, Schlenker R, Husser T, Diggelmann S, Challier J, Mossner E, Hosse RJ, Hofer T, Brunker P, Joseph C, Benz J, Ringler P, Stahlberg H, Lauer M, Perro M, Chen S, Kuttel C, Bhavani Mohan PL, Nicolini V, Birk MC, Ongaro A, Prince C, Gianotti R, Dugan G, Whitlow CT, Solingapuram Sai KK, Caudell DL, Burgos-Rodriguez AG, Cline JM, Hettich M, Ceppi M, Giusti AM, Crameri F, Driessen W, Morcos PN, Freimoser-Grundschober A, Levitsky V, Amann M, Grau-Richards S, von Hirschheydt T, Tournaviti S, Molhoj M, Fauti T, Heinzelmann-Schwarz V, Teichgraber V, Colombetti S, Bacac M, Zippelius A, Klein C and Umana P. Tumor-targeted 4-1BB agonists for combination with T cell bispecific antibodies as off-the-shelf therapy. Sci Transl Med 2019; 11: eaav5989.

[36] Dahan R, Sega E, Engelhardt J, Selby M, Korman AJ and Ravetch JV. FcgammaRs modulate the anti-tumor activity of antibodies targeting the PD-1/PD-L1 axis. Cancer Cell 2015; 28: 285-295.

[37] Teige I, Martensson L and Frendeus BL. Targeting the antibody checkpoints to enhance cancer immunotherapy-focus on FcgammaRIIB. Front Immunol 2019; 10: 481.

[38] Oshima T, Miyashita H, Ishimura Y, Ito Y, Tanaka Y, Hori A, Kokubo T and Kurokawa T. Fc engineering of anti-Nectin-2 antibody improved thrombocytopenic adverse event in monkey. PLoS One 2018; 13: e0196422.