Bispecific anti-CD3 x anti-HER2 antibody mediates T cell cytolytic activity to HER2-positive colorectal cancer *in vitro* and *in vivo*

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Abstract. Targeting HER2 overexpressed breast cancer cells with anti-HER2 monoclonal antibodies inhibits tumor growth. Here we investigated whether HER2 can serve as a target for T cell-mediated immunotherapy of human colorectal carcinoma. Specific cytolytic activity of activated T cells (ATCs) armed with anti-CD3 x anti-HER2 bispecific antibody (HER2Bi-Ab) against $HER2^+$ tumor cells was evaluated by bioluminescent signal generated by luciferase reporter on tumor cells *in vitro* and *in vivo*. In contrast to unarmed ATCs, increased cytotoxic activity of HER2Bi-armed ATCs against $HER2^+$ tumor cells was observed. Moreover, HER2Bi-armed ATCs expressed higher level of activation marker CD69 and secreted significantly higher levels of IFN-γ than the unarmed ATC counterpart. In addition, compared with anti-HER2 mAb (Herceptin®) or unarmed ATC, HER2Bi-armed ATCs showed significant suppression against colorectal carcinoma cells. In colorectal tumor cell xenograft mice, infusion of HER2Bi-armed ATCs successfully inhibited the growth of Colo205-luc cells. The HER2Bi-armed ATCs with anti-tumor effects may provide a promising immunotherapy for colorectal carcinoma in the future.

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Abbreviations: CRC, colorectal cancer; ATCs, activated T cells; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; HER2Bi-Ab, anti-CD3 x anti-HER2 bispecific antibody; PBMCs, peripheral mononuclear blood cells; ANOVA, one-way analysis of variance; TAA, tumor-associated antigen

Key words: HER2, colorectal carcinoma, bispecific antibody, immunotherapy

Introduction

Colorectal cancer (CRC) is the third most frequent cancer worldwide and a common cause of cancer death primarily due to metastases that are resistant to conventional therapy (1). Over the last couple of years, a series of monoclonal antibodies targeting different tumor cell surface antigens have been tested in both experimental and clinical studies (2-5). Therapeutical antibodies have been successfully used for the treatment of metastatic CRC or after the failure of conventional chemo-therapy, including cetuximab and panitumumab, antibodies to endothelial cell growth factor receptor (anti-EGFR), and bevacizumab, an antibody to vascular endothelial growth factor (anti-VEGF). Several drugs are under development with proven efficacy in phase II and III trials. However, the benefit of anti-EGFR therapy was limited to patients who had *KRAS* wild-type tumors with normal PTEN expression (6-8).

HER2 is a 185-kDa transmembrane tyrosine kinase receptor, and belongs to the family of epidermal growth factor receptors (EGFRs), playing critical roles in epithelial cell growth and differentiation. HER2 overexpression has been found to be involved in pathogenesis of several human cancers (9-15). Although a dozen different ligands are known to bind to EGFRs, there is no ligand to be shown to bind directly to HER2. Instead, HER2 is thought to function as a co-receptor for other members of the ErbB/HER family, and frequently activated upon ligand binding to any of the other

ErbB/HER receptors. Therefore, it is not surprising that HER2 has also been implicated in the development of carcinomas. In CRC the prevalence of HER-2/*neu* overexpression ranges from 0 to 83% (16-18), a big variation largely due to the lack of standardization of the detection methods. The success of HER-2/*neu* directed therapy in breast cancer suggests its potential role in the treatment of other tumors expressing HER2, including CRC. Herceptin® has been shown to inhibit colony formation of the HCA-7 colon cancer cell line and HCA-7 tumor xenografts (19).

In addition to the development of therapeutics for new targets, another approach to improve current antibody therapy was the development of bispecific antibodies. Cancer immunotherapies involving bispecific antibodies mediated-killing have been widely explored. Bispecific antibodies were first developed two decades ago and several molecules targeting different tumor cell surface antigens have been tested in clinical studies (20,21). In this study, clinically approved anti-CD3 antibody was chemically conjugated with Herceptin antibody. The anti-CD3 x anti-HER2 bispecific antibody (HER2Bi-Ab) was then used to direct the activated T cells (ATCs) to kill colorectal carcinoma targets. Armed with HER2Bi-Ab, ATCs exhibited high levels of specific cytotoxicity and proliferation ability. The efficacy of HER2Bi-Ab for the inhibition of HER2-positive CRCs in SCID-Beige mouse model was also demonstrated.

Materials and methods

Cell lines and mice. Colo205-luc, HT-29-luc, BXPC-3-luc, PC-3M-luc, Hela-luc, and K562-luc cell lines were all from Caliper Life Sciences. The primary culture and the metastatic culture were derived from freshly isolated colon carcinoma cells from patients of primary colon carcinoma or hepatic metastasis of colonic carcinoma, respectively, in 302 Military Hospital of China. The consent was obtained from the patients before sample collection. The study complied with the Declaration of Helsinki and was approved by the Biomedical Research Ethics Committee of CAS Key Laboratory of Pathogenic Microbiology and Immunology. The agents for cell culture were all from Gibco Co. Beige-SCID mice (8-10 weeks) were purchased from Vital River Laboratories.

Isolation of peripheral blood lymphocytes and preparation and cryopreservation of activated T lymphocytes. Peripheral mononuclear blood cells (PBMCs) were isolated using Ficoll density gradient centrifugation from healthy donors supplied by the Beijing Blood Bank. PBMCs were cultured at $1x10^6$/ml in RPMI-1640 medium supplemented with 10% FBS and 5 μg/ml anti-CD3 mAb and 100 IU/ml recombinant human IL-2 at $1x10^6$/ml. Half-volume medium exchange was performed every 3 days with medium containing fresh 100 IU/ml recombinant human IL-2 as the method previously described (22). On day 14, ATC expansion products of donors were on average 98.85±1.06% $CD3^+$ cells (38.4±18.10% $CD3^+CD4^+$ cells, and 66.35±9.83% $CD3^+CD8^+$ T cells), the cells were used immediately or cryopreserved for further use. Based on an informed consent, this project was approved by the Biomedical Research Ethics Committee of CAS Key Laboratory of Pathogenic Microbiology and Immunology.

Synthesis of HER2Bi-Ab and arming of ATCs. Anti-HER2 (Herceptin®; Roche) was reacted with sulfo-SMCC and anti-CD3 (OKT3; eBioscience; 85-16-0037-85) was reacted with Traut's reagents as previously described (23). Cryopreserved ATCs were thawed, and armed with HER2Bi at a concentration of 50 ng/10^6 cells at room temperature for 30 min followed by washing the cells to eliminate unbound antibodies. The combination of OKT3 (50 ng/10^6 cells) and Herceptin® (50 ng/10^6 cells) pre-incubated ATCs were used as control unarmed ATCs.

Cytotoxicity assay. Cytotoxicity was measured with a luciferase quantitative assay (23-25). Target cells were seeded in duplicates in 96-well U-bottom microplates at $1x10^4$/well before the addition of HER2Bi-armed, or unarmed ATCs at various effector-to-target (E/T) ratios. Effector cells and tumor cells were allowed to interact at 37˚C for 18 h. A final concentration of 0.15 mg/ml D-luciferin (Synchem Chemie; Bc219-05) was added to each well.

IFN-γ ELISA. Target cells were plated in 96-well U-bottom microplates at a concentration of $1x10^4$/well at 37˚C overnight. HER2Bi-armed, or unarmed ATCs were then added at an E/T ratio of 5:1 to target cells and incubated for 18 h. The cell free supernatants were collected, and the IFN-γ production was measured by using a human IFN-γ ELISA kit (Thermo Scientific) according to the manufacturer's instructions.

Flow cytometric analysis. The anti-CD69-PE, anti-CD3-FITC, anti-mouse IgG-FITC secondary antibodies were from eBioscience, and anti-human IgG-FITC secondary antibody was from Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd. The cells were assayed with a Guava EasyCyte flow cytometer (Guava Technologies, Inc.) and the data analysis was carried out with FlowJo software version 7.6.1 (Tree Star, Inc.).

In vitro cell proliferation assay. For evaluating Herceptin® on colorectal tumor cell proliferation *in vitro*, colon carcinoma cells were seeded into 96-well plates in triplicates and incubated with the fresh medium or Herceptin® at the indicated concentration for 72 h. For evaluating HER2Bi-armed ATCs on colorectal tumor cell proliferation *in vitro*, HT29-luc were seeded ($2x10^4$/well) into 96-well plates in triplicates and allowed to adhere overnight. The following day, the medium was removed, and fresh medium alone or containing the unconjugated mAbs (50 ng/ml), ATCs ($2x10^5$/well), HER2Bi-armed ATCs ($2x10^5$/well, armed with 50 ng/HER2Bi/10^6 ATCs) or unarmed ATCs was added to wells. Cultures were incubated for 18 h, then medium was removed and 100 μl of fresh serum-free medium containing 1/10 (v/v) Cell Counting kit-8 (CCK8; Dojindo Laboratories) reagent was added to each well and incubated for an additional 3 h. After incubation, the absorbancy of colorectal tumor cells was measured using a 96-well plate reader at 450 nm. Cell proliferation was assessed by the absorbance values according to the manufacturer's protocol.

In vivo tumor inhibition studies. In tumor prevention studies, Colo205-luc cells ($1x10^6$/mouse) were mixed with

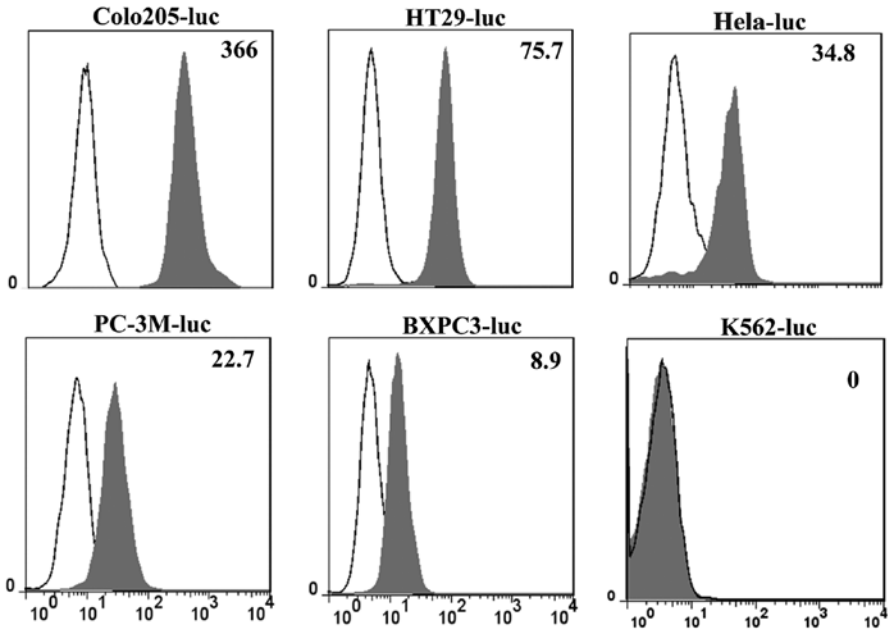

Figure 1. Expression of HER2 on different human tumor cells. HER2/neu-expressing profiles of tumor cell lines by flow cytometry. Shaded histogram represents cells stained with Herceptin® and un-shaded histogram represents cells stained with control human IgG. Mean fluorescent intensity (MFI) values obtained with Herceptin® staining subtract the control staining are indicated in the upper right of the histogram.

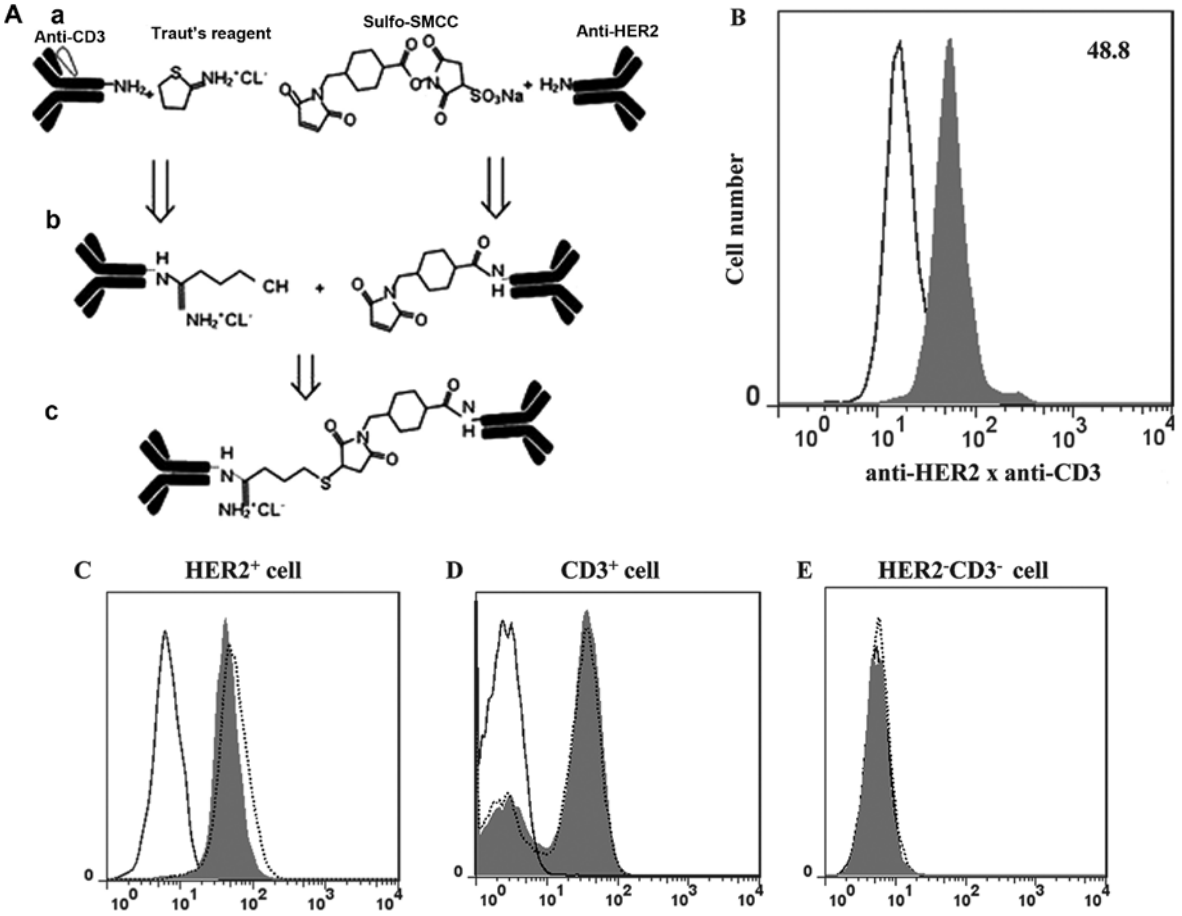

Figure 2. General scheme for the generation of anti-CD3 x anti-HER2 bispecific antibody (HER2Bi-Ab). (A) General scheme for the generation of HER2Bi-Ab. (B) Flow cytometry based binding assay for HER2Bi-Ab. Colo205-luc cells were incubated with HER2Bi-Ab (shaded histogram) or combination of OKT3 and Herceptin® (the black line), HER2Bi-Ab binding was evaluated by FITC goat anti-mouse IgG to detect the anti-CD3 moiety of the HER2Bi-Ab. (C) HT29-luc cells were incubated with HER2Bi-Ab (shaded histogram), Herceptin® (dot histogram), or control IgG (open histogram), HER2Bi-Ab binding was analyzed by FITC goat anti-human IgG to detect the anti-HER2 moiety of the HER2Bi-Ab. (D) Peripheral mononuclear blood cells (PBMCs) were incubated with HER2Bi-Ab (shaded histogram), OKT3 (dot histogram), or control IgG (open histogram), the HER2Bi-Ab binding was analyzed by FITC goat anti-mouse IgG to detect the anti-CD3 moiety of the HER2Bi-Ab. (E) K562cells were incubated with HER2Bi-Ab (shaded histogram), OKT3 (dot histogram), or control IgG (open histogram), the HER2Bi-Ab binding was analyzed by FITC-goat-anti-mouse IgG to detect the anti-CD3 moiety of the HER2Bi-Ab.

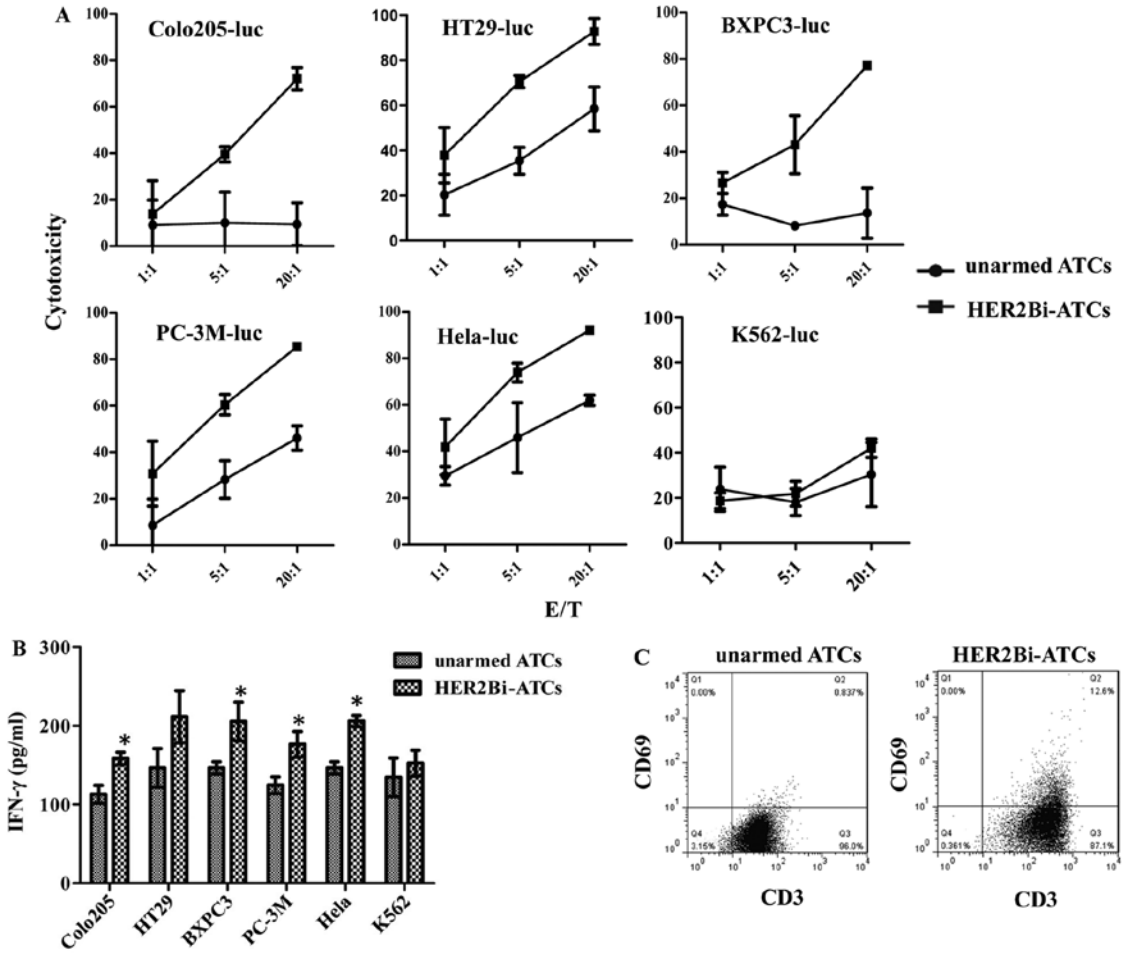

Figure 3. The antitumor effect of HER2Bi-armed activated T cells (ATCs) against different tumor cell lines. (A) Target cells were incubated either with HER2Bi-armed ATCs or a combination of OKT3 and Herceptin with ATCs (unarmed ATCs) for 18 h, luciferase quantitative assay was performed to determine cytotoxicity against different target cells at different effector-to-target (E/T) ratios. (B) IFN-γ secretion by HER2Bi-armed ATCs against different target tumor cells compared with unarmed ATCs. Supernatants of co-cultures at E/T ratio of 5:1 were harvested at 18 h and analyzed for IFN-γ level. The data are mean ± SD of triplicate determination. Shown is a representative experiment of three. (C) Expression of CD69 on HER2Bi-armed ATCs or unarmed ATCs was detected by flow cytometry after 18 h co-culture with HT-29-luc cell at E/T ratio of 5:1. *P<0.05, HER2Bi-armed ATCs compared with unarmed ATCs under similar conditions.

HER2Bi-armed ATCs ($1x10^7$/mouse) or unarmed-ATCs. The cell mixtures were immediately inoculated subcutaneously on the rear flank of five SCID-Beige mice per group. In tumor growth delay studies, SCID-Beige mice (n=5 mice per group) were injected i.p. with $3x10^6$ Colo205-luc cells. Subsequently, HER2Bi-armed ATCs ($3x10^7$/mouse) or control ATCs were administered i.p. on day 3, 10, and 17. In order to follow up the tumor growth, *in vivo* bioluminescence imaging was operated on the indicated days for 4 weeks. Bioluminescent imaging was taken using Xenogen IVIS-100 imaging system with Living Image software (Caliper Life Sciences). The signal intensity of tumor burdens was expressed as total photons/sec/cm^2 (*p/sec/cm^2/sr*).

Statistical analysis. All experiments were repeated at least twice and mostly three times. Data were analyzed using Graphpad Prism 5 software, the data are presented as the means ± SD. Unpaired Student's t-test (two-tailed) or the Mann-Whitney test was used for comparison of two groups where appropriate. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc for multiple comparison. P<0.05 was considered as statistically significant. The number with a significant difference from a control is denoted by an asterisk in the figures.

Results

HER2 overexpression in human CRC cells. The surface expression of HER2 on human tumors from different tissue origins was assessed by FACS analysis including CRC (Colo205-luc and HT-29-luc), pancreatic cancer (BXPC3-luc), prostate cancer (PC-3M-luc), cervix cancer (Hela-luc), and leukemia (K652-luc). As shown in Fig. 1, HER2 expression measured as mean fluorescent intensity (MFI) in CRC cells (Colo205-luc: 366; HT29-luc: 75.7) was much higher than that in other tumor cells (BXPC3-luc: 8.9; PC-3M-luc: 22.7; Hela-luc: 34.8). HER2 was not detected on K562 cells used as a negative control.

Preparation and characterization of HER2Bi and ATCs. Herceptin® antibody was hetero-conjugated with OKT3 chemically and named as HER2Bi (Fig. 2A). The binding

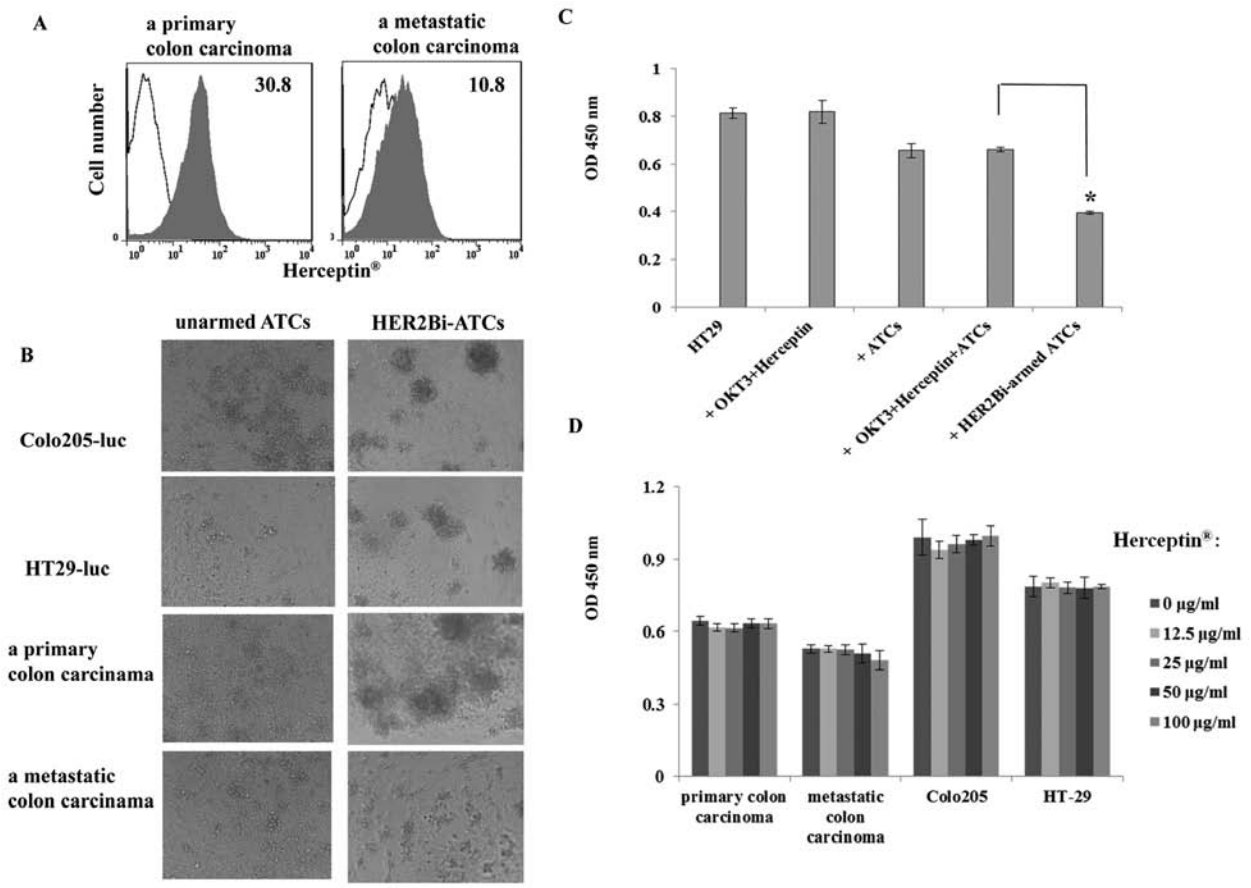

Figure 4. Anti-proliferative effects of Herceptin® or HER2Bi-armed activated T cells (ATCs) against colorectal carcinoma cells. (A) Expression of HER2 on a human primary (left) and a metastatic (right) colon carcinoma cell culture. Shaded histogram represents cells stained with Herceptin® and un-shaded histogram represents cells stained with control human IgG. Mean fluorescent intensity (MFI) values obtained with Herceptin® staining subtracting the isotype control staining are indicated in the upper right of the histogram. (B) Target colorectal carcinoma cells were incubated either with HER2Bi-armed ATCs or unarmed ATCs for 18 h at effector-to-target (E/T) ratio of 10:1, and real-time photographs were taken at x200 magnification. (C) Growth inhibition of colorectal carcinoma cells by HER2Bi-armed ATCs. Adherent HT29-luc cells were incubated either with unconjugated OKT3 and Herceptin, or ATCs, or HER2Bi-armed ATCs, or unarmed ATCs for 18 h. The proliferation of HT29-luc cells was assessed by Cell Counting kit-8 (CCK8) assay with the OD value measured at 450 nm. (D) Effects of Herceptin® on the proliferation of colorectal carcinoma cells. Colorectal carcinoma cells were treated with or without Herceptin® for 72 h and the proliferation of cells was assessed by CCK8 assay with the OD value was measured at 450 nm. The data are mean ± SD of triplicate experiments, and a representative experiment of three is shown. *P<0.05, HER2Bi-armed ATCs compared with unarmed ATCs under similar conditions.

specificity of HER2Bi against HER2 was tested. Colo205-luc cells were first stained by HER2Bi, then an anti-mouse IgG-FITC was added to detect the CD3 moiety of HER2Bi-Ab. Only functionally bispecific HER2Bi antibody was able to bind to Colo205-luc cells by HER2 recognized Herceptin® and be detected through mouse origin OKT3 by anti-mouse secondary antibody. As shown in Fig. 2B, positively stained cells were detected in 91.3% of the Colo205-luc population with an MFI of 48.8. Moreover, binding of HER2Bi-Ab on $HER2^+$ cells was confirmed by FITC goat anti-human IgG to detect the anti-HER2 moiety of the HER2Bi-Ab (Fig. 2C). To evaluate the binding of HER2Bi-Ab to $CD3^+$ cells, PBMC were incubated with HER2Bi-Ab, and the binding of HER2Bi-Ab to $CD3^+$ cells was evaluated by FITC goat anti-mouse IgG to detect the anti-CD3 moiety of the HER2Bi-Ab (Fig. 2D). In contrast, HER2Bi-Ab did not bind to $CD3^-HER2^-$-K652 cells (Fig. 2E).

Cytotoxicity of HER2Bi-armed ATC with IFN-γ production on different tumor cell lines. The amount of HER2Bi required to arm ATCs ranged from 5 to 500 ng/10^6 cells. Since 50 and 500 ng/10^6 cells showed similar cytotoxicity, we chose 50 ng/10^6 ATCs as the concentration of HER2Bi for all subsequent experiments, and ATCs mixed with both individual OKT3 and Herceptin® were used as unarmed ATC control. Cytotoxic effects of HER2Bi-armed ATCs on different $HER2^+$ tumor cells were tested *in vitro*. The assays were performed at E/T ratios of 1:1, 5:1 and 20:1. After 18 h incubation with HER2Bi-armed ATCs or unarmed ATCs, bioluminescence imaging signal in tumor cells expressed in photons per second was converted into living cell number and the cytotoxicity assays was calculated at the indicated E/T ratios. As shown in Fig. 3A, the percentage of cytotoxicity with armed ATCs was significantly greater than that with unarmed effectors at E/T ratio of 5:1 and 20:1 in Colo205-luc, HT29-luc, BXPC-3, PC-3M-luc, and Hela-luc cells.

To analyze the cytokines along with the cytotoxicity, supernatants of cell cultures were analyzed for IFN-γ production at E/T ratio of 5:1. As shown in Fig. 3B, significant increase was observed for IFN-γ secretion by HER2Bi-armed ATCs over their unarmed ATC counterparts when ATCs were co-cultured with Colo205-luc, BXPC3-luc, PC-3M-luc

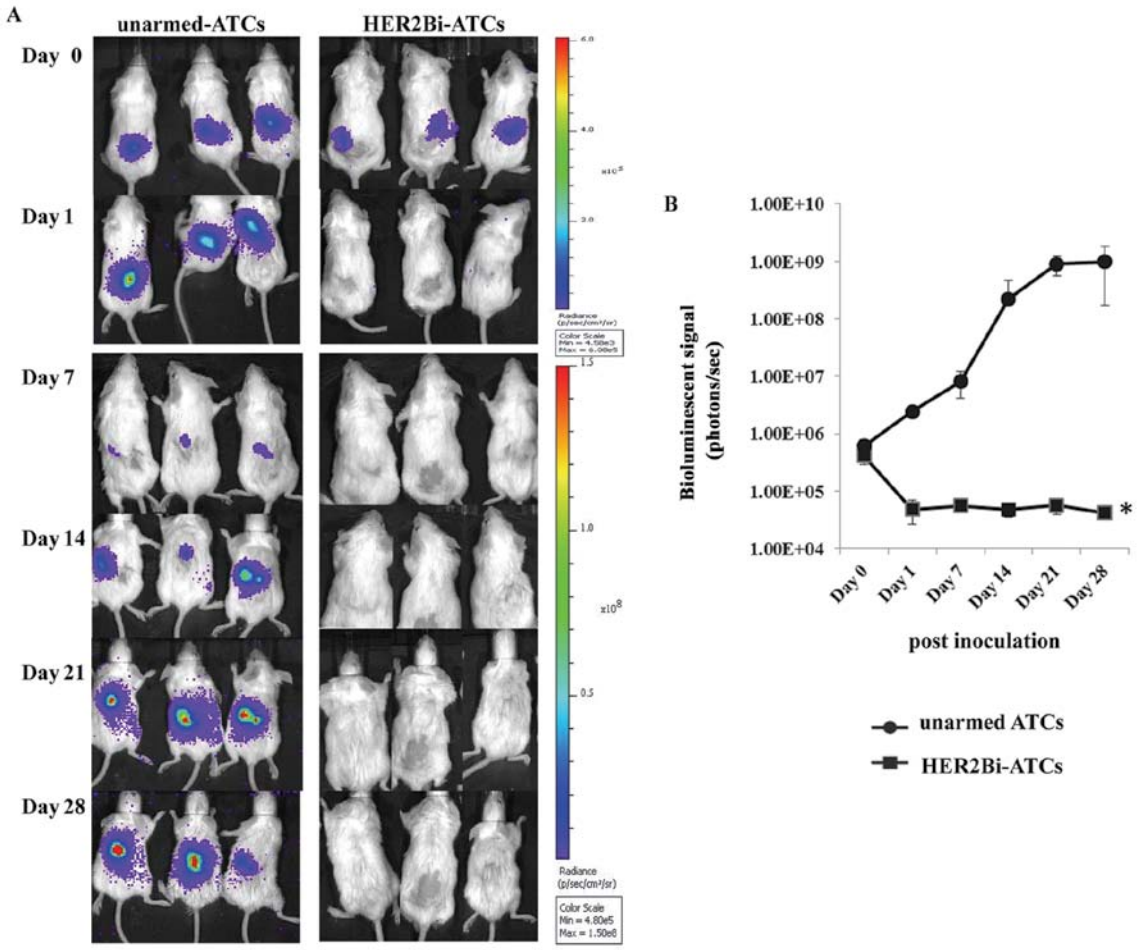

Figure 5. Prevention effects of HER2Bi-armed activated T cells (ATCs) to colorectal tumor cells *in vivo*. SCID-Beige mice were inoculated with Colo205-luc cells mixed with HER2Bi-ATCs or unarmed-ATCs, respectively, on day 0, each group contains n=5 mice. (A) Bioluminescence images of three representative mice of each group are shown on the indicated day. (B) Images were analyzed using Living Image software and the tumor values represented as total flux measurements in photons/sec, mean values of tumor growth curves are shown. *P<0.05.

or Hela-luc cells, respectively (P<0.05). Moreover, FACS analysis of HER2Bi-armed ATCs showed an increased CD69 expression over their unarmed ATCs counterparts (Fig. 3C).

Cells derived from both primary and metastatic human colorectal carcinoma were tested to evaluate whether they also expressed high levels of HER2 proteins. As shown in Fig. 4A, HER2-positive stained cells were detected by FACS analysis in 90% of the primary colorectal carcinoma cell population with an MFI of 30.8 and in 50% of the metastatic colorectal carcinoma cell population with an MFI of 10.8. Then, HER2Bi-armed ATCs were tested for cytotoxicity on HER2 positive primary cells derived from colorectal carcinoma. The assays were performed at E/T ratio of 10:1. After 18 h incubation with HER2Bi-armed ATCs or unarmed ATCs, as shown in Fig. 4B, real-time photographs of each colorectal carcinoma group were taken at x200 magnification. It was demonstrated that HER2Bi-ATCs, but not equivalent number of unarmed-ATCs, aggregated with all the four colorectal carcinoma cell types, clustering around the edge of targeting cell bulk, which showed the specific lysis of HER2Bi-ATCs.

The growth inhibition of colorectal carcinoma cells by HER2Bi-armed ATCs. Furthermore, HER2Bi-armed ATCs were tested for the inhibitory efficacy on HER2 positive colorectal carcinoma. In cell proliferation assay, unconjugated mAbs (OKT3 and Herceptin®), ATC alone, a combination of OKT3 and Herceptin® with ATC (unarmed ATC), or HER2Bi-armed ATC (E/T ratio of 10:1) were co-cultured with HT29-luc cells for 18 h, respectively. As expected, HER2Bi-armed ATCs showed a superior growth inhibition on colorectal carcinoma cells, compared to the other groups (Fig. 4C). Unexpectedly, even at the concentration of 100 μg/ml, Herceptin® did not inhibit the proliferation of colorectal tumor cells after 72 h incubation *in vitro* (Fig. 4D).

HER2Bi-armed ATCs inhibited Colo205 tumor growth in SCID-Beige mice. To determine whether HER2Bi-armed ATCs could prevent tumor growth *in vivo*, SCID-Beige mice were engrafted subcutaneously with Colo205-luc cells. The growth of tumor was monitored with bioluminescent imaging. In Fig. 5A, results of three representative mice of each group are shown. When mice were co-injected with unarmed-ATCs, the light signal increased over time from day 1 to 28. In contrast, the signal disappeared on day 1 and vanished completely from day 7 to 28 when mice were co-injected with HER2Bi-armed ATCs. Once injected, mice were given no further treatment but were monitored weekly for tumor development up through day 28 following initial injection. The mean bioluminescence

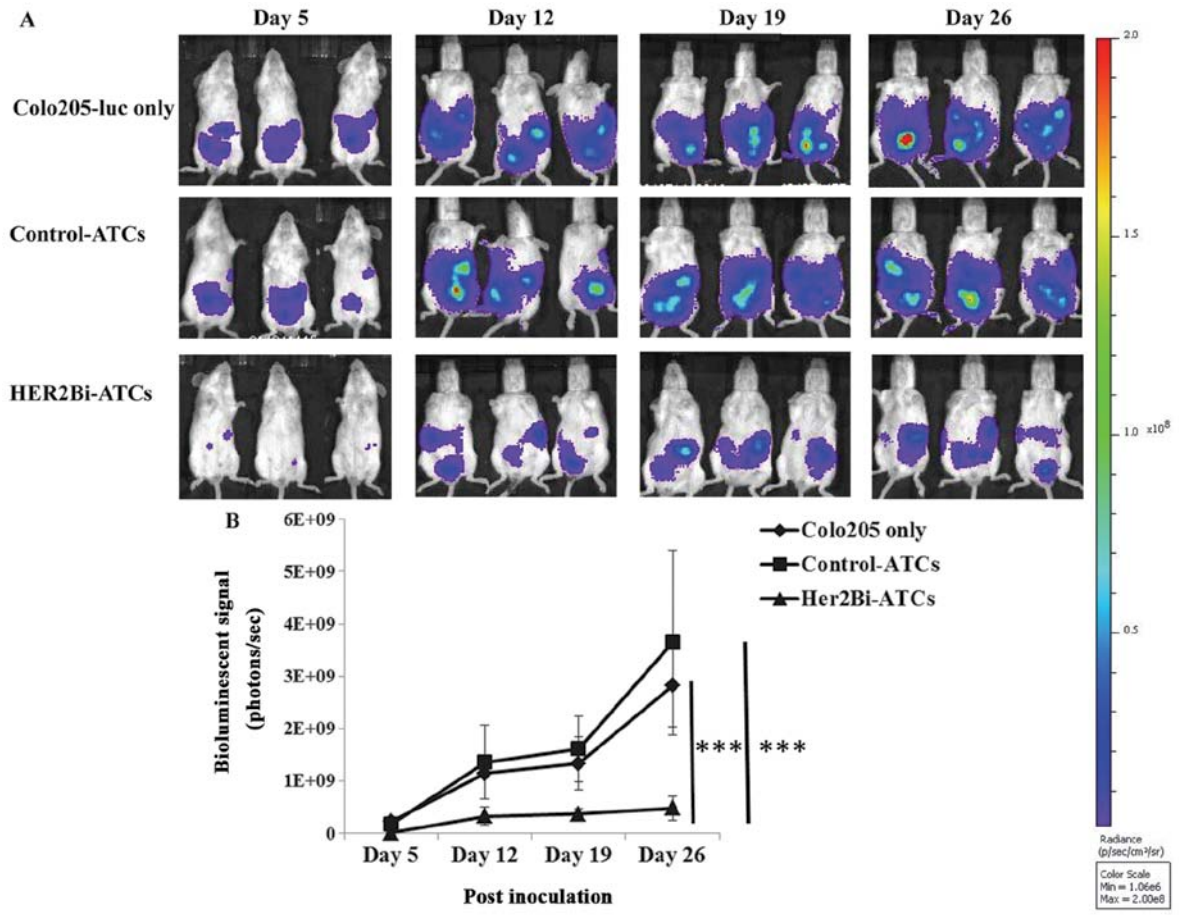

Figure 6. Suppression effects of HER2Bi-armed activated T cells (ATCs) to colorectal tumor cells *in vivo*. SCID-Beige mice were inoculated with Colo205-luc cells i.p. on day 0, and 3 days later HER2Bi-ATCs or control ATCs were administered i.p. respectively, each group contains n=5 mice. (A) Bioluminescence images of three representative mice of each group are shown on the indicated day. (B) Images were analyzed using Living Image software and tumor values represent as total flux measurement in photons/sec, mean values of tumor growth curves are shown. ***P<0.0001.

signal of each test group correlated with the number of living Colo205-luc cells as shown in Fig. 5B.

To further determine whether HER2Bi-armed ATCs could suppress tumor growth *in vivo*, SCID-Beige mice were engrafted intraperitoneally with Colo205-luc cells. Three days later, mice were treated with HER2Bi-armed ATCs or control ATCs weekly three times. The growth of tumor was monitored with bioluminescent imaging. In Fig. 6A, three representative mice of each group are shown. When Colo205-luc cells were inoculated alone, light signal increased over time. A similar kinetics of tumor growth was shown in mice that were injected with control ATCs. As for mice treated with HER2Bi-armed ATCs, the signal diminished from day 5 to 26 compared with other two groups. The mean bioluminescence signal of each test group correlated with the number of living Colo205-luc cells was shown in Fig. 6B. HER2Bi-armed ATCs inhibited the tumor growth significantly, whereas control ATCs did not, at every check point.

Discussion

Therapeutic antibodies such as bevacizumab, cetuximab, and panitumumab significantly improve survival rates of patients with advanced CRC. However, due to the limitations of conventional antibodies, including the redundancy in antibody targeted molecular pathways, inadequate exposure of the tumor due to the antibody limited tissue distribution, and immunogenicity caused by repeatable treatment, alternative approaches to improve current antibody strategy are urgently needed. One improvement is the use of T cell directed bispecific antibodies, comprising of an anti-CD3 mAb hetero-conjugated to a different mAb specific to a selected tumor-associated antigen (TAA). Such a bispecific antibody will make every T cell TAA-specific to redirect T cells to target tumor cells. HER2 is an ideal candidate used as a target by various tumor imaging and antibody-based therapeutic approaches. Phase I/II immunotherapy with HER2Bi-Ab and/or armed ATCs are currently ongoing in women with metastatic breast cancer (26,27).

In this study, we tested whether HER2 is a useful target for the development of novel bispecific Ab therapeutics in CRC, and examined *in vitro* and *in vivo* antitumor effects of HER2Bi-armed ATCs. Our study findings are relevant to the therapeutic application of target HER2 against CRC. The high expression of HER2 in colorectal carcinoma was confirmed by FACS analysis. In addition, primary or metastatic colon carcinoma cell cultures were also shown to express high level of HER2 antigen. In our present study HER2Bi-armed ATCs provided significant anti-proliferative and cytotoxic activity against HER2-positive colorectal carcinoma cells although

anti-HER2 antibody alone had no inhibitory effect to colorectal carcinoma cells tested *in vitro*. Additionally, HER2Bi-armed ATCs expressed higher level of activation marker CD69 and secreted a higher level of IFN-γ than unarmed ATC counterpart against colorectal carcinoma target cells. Furthermore, infusion of HER2Bi-armed ATCs remarkably inhibited the growth of colorectal carcinoma cells in the xenograft mouse tumor model.

Our results have shown that ATCs armed with HER2Bi released cytokine IFN-γ upon incubation with the tumor cells. The increased secretion of cytokine demonstrated that ATCs were being reactivated upon binding to tumor cells. IFN-γ secreted by HER2Bi-armed ATCs in the presence of tumor may not only cause direct tumor killing but also serve to modulate immune networks to induce local and/or systemic immune responses to tumors, capable of counteracting tumor-induced suppression by TGF-β, IL-4, and IL-10 (28,29). Flow cytometry results provided evidence that HER2Bi-armed ATCs expressed higher level of CD69 than the unarmed-ATC counterparts. CD69 represents a marker of early T-cell activation and acts as a costimulatory molecule that increased T-cell responses following TCR-ligand interaction (30). Therefore, IFN-γ produced by HER2Bi-armed ATC upon its binding to tumor antigen may be clinically beneficial. HER2Bi-Ab did not bind to $CD3^-HER2^-$-K562 cells, confirming the specificity of the HER2Bi-Ab.

In tumor growth delay studies, HER2Bi-armed ATCs prevented and furthermore significantly inhibited tumor growth in mice bearing established HER2-positive Colo205 xenografts, whereas control unarmed ATCs did not, confirming the specificity of the targets. Similarly, in a pilot study, HER2Bi-armed ATCs injected intratumorally induced remission of human hormone-refractory prostate tumor in severe combined immunodeficient mice (31). Conceivably, the binding of the effector cells at the tumor site by armed ATCs may not only augment tumoricidal activity but also increase local cytokine secretion leading to the recruitment of other immune effectors (32,33).

Although clinical studies have shown that Herceptin® significantly improves the overall survival of breast cancer patients, an unforeseen significant side-effect of cardiotoxicity manifested as left ventricular dysfunction and heart failure (34). In our study, HER2Bi-armed ATCs were highly effective in eliminating tumor cells both *in vitro* and *in vivo* at very low concentration of HER2-Bi-Ab. Also, our studies showed Herceptin® failed to inhibit proliferation of colon carcinoma cells *in vitro*, partly because of the HER2 expression on colon carcinoma cell lines were barely middle or low compared with the HER2 overexpressing breast cancer cell line SKBR3 (35). Therefore, more importantly, our study provided a new strategy for treatment of colon cancer in the event when the expression of the target tumor antigen e.g., HER2, is not high.

In conclusion, taken together with the *in vitro* cytotoxicity and cytokine secretion studies, the ability of HER2Bi-armed ATCs to prevent the development and suppress the growth of tumors in xenograft mice suggests that HER2Bi-armed ATCs could be used as a good strategy for the treatment of HER^+ CRC patient and produce clinically significant antitumor effects.

Acknowledgements

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