

A HER2-Target Antibody Drug Conjugate Combined with anti-PD-(L)1 Treatment Eliminates hHER2 + Tumors in hPD-1 Transgenic Mouse Model and Contributes Immune Memory Formation

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Research Article

Keywords: Antibody-drug conjugates, HER2-positive breast cancer, checkpoint inhibitor combination therapy

Posted Date: July 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-534358/v1>

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Version of Record: A version of this preprint was published at Breast Cancer Research and Treatment on October 16th, 2021. See the published version at <https://doi.org/10.1007/s10549-021-06384-4>.

A HER2-target antibody drug conjugate combined with anti-PD-(L)1 treatment eliminates hHER2 + tumors in hPD-1 transgenic mouse model and contributes immune memory formation

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Abstract

Purpose: Disitamab vedotin (RC48) is an HER2-directed antibody-drug conjugate, emerging as an effective strategy for cancer therapy, not only enhance antitumor immunity in previous animal models but also improve clinical outcomes for patients such as with gastric cancer, urothelium carcinoma and HER2 low-expressing breast cancer. Here, we explore the combination therapeutic efficacy of this novel HER2-targeting ADC with immune checkpoint inhibitors in a human HER2-expressing syngeneic breast cancer model.

Methods: The human HER2+ cancer cell line is constructed by stably transfection and individual clones were isolated by single-cell sorting. Flow cytometry was performed to determine its binding activity. Cytotoxic effect was determined using an MTT assay with the supplement of RC48. Human PD-1 transgenic mice were used to analyze the *in vivo* anti-tumor effects of the ADC and its combination therapy with PD-1/PD-L1 antibody.

Results: The combination of RC48 and PD-1/PD-L1 immune checkpoint inhibition significantly enhanced tumor suppression and antitumor immunity. Tumor rejection in the synergistic groups was accompanied by massive T-cell infiltration and immune marker activation. Furthermore, the combination therapy promoted immunological memory formation in the tumor-eradication animals, protecting them from tumor rechallenge.

Conclusion: A novel HER2-targeting ADC combined with immune checkpoint inhibitors can achieve remarkable effects in mice and elicit long-lasting immune protection in a hHER2+ murine breast cancer model. This study provides insights into the efficacy of RC48 therapeutic activity and a rationale for potential therapeutic combination strategies with immunotherapy.

Keywords: Antibody-drug conjugates, HER2-positive breast cancer, checkpoint inhibitor combination therapy

Introduction

Amplification and overexpression of human epidermal growth factor receptor 2 (HER2) are commonly observed in human breast cancers and often associated with poor patient survival¹⁻⁵. This genetic alteration promotes cancer cell proliferation and survival, suggesting that HER2 is a promising therapeutic target for cancers. Trastuzumab is one of successful monoclonal antibodies against HER2, which has been clinically proven to be significantly efficient in multiple indications^{6,7}. Nonetheless, de novo and acquired resistance to HER2 blockade eventually occurs in most patients with advanced disease. Therefore, new strategies are clearly needed for HER2-positive breast cancer, which to date remains poor clinical outcomes.

Antibody-drug conjugate (ADC) drugs are loaded with a small toxin molecule and exploit the function of monoclonal antibodies, which can enrich a large number of toxin molecules around the targeted tumor cells, thereby exerting a more powerful killing effect⁸⁻¹¹; for example, ado-trastuzumab emtansine (T-DM1)¹² and trastuzumab deruxtecan (DS-8201a)¹³, which were approved by the FDA in 2013 and 2019, both use trastuzumab as the antibody backbone. Another promising ADC is disitamab vedotin (RC48)^{14,15}, which combines disitamab—novel HER2 target specificity—with a potent microtubulin polymerization inhibitor monomethyl auristatin E (MMAE) as a payload, allowing for targeted drug delivery. Given that resistance often occurs in patients receiving ADC therapy post treatment. Likewise, despite the favorable efficacy results, most patients treated with T-DM1 eventually progress in HER2-positive breast cancers¹⁶. Moreover, ADCs reduce the number of targeted tumor cells, thereby minimizing the exposure of tumor-derived antigens, which may render them nonfunctional¹⁷.

In previous studies, immune-mediated mechanisms for microtubule-depolymerizing agents have been reported, such as dolastatin-10 and its synthetic analog MMAE¹⁸. Brentuximab vedotin is a representative of using MMAE as a cytotoxic payload, which results in sustained clinical responses in patients with CD30+ lymphomas¹⁹. Indeed, brentuximab vedotin induces activation of patient DCs, T cells, and B cells, reflecting augmentation of tumor-specific immunity. Given the observation of immune engagement for the microtubule-depolymerizing cytotoxic moieties used in ADCs, and chemotherapeutic agents induced tumor cell death²⁰, we hypothesized that the clinical activity observed with RC48 partly relies on an efficient antitumor immune response. The latter would allow the combination of RC48 treatment with immunotherapeutic strategies such as immune checkpoint inhibitors, including programmed cell death protein 1 (PD-1) and its ligand (PD-L1), which may help overcome resistance and potentially result in long-term benefit.

To explore the therapeutic response of the combination of RC48 with immune checkpoint inhibition, we employed a human (h) HER2+ syngeneic breast cancer model and humanized immune checkpoint inhibitors in our transgenic hPD-1 mouse model.

Material and Methods

Antibodies and compounds

Disitamab vedotin (RC48) and its parental anti-human HER2 (anti-hHER2) antibody were prepared as previously described^{14,15}. The drug-to-antibody ratio was 4, as determined by hydrophobic interaction chromatography.

For flow cytometry, fixable viability stain 450 (562247; BD Biosciences, Franklin Lakes, NJ), PercP/Cy5.5-labeled anti-mouse CD45 (30-F11, 550994; BD Biosciences), APC-labeled anti-mouse CD3e (100236; BioLegend, San

Diego, CA), FITC-labeled anti-mouse CD4 (100405; BioLegend), FITC-labeled anti-mouse CD8a (100705; BioLegend), and APC-labeled anti-mouse CD274 (B7-H1, PD-L1) (124312; BioLegend) antibodies were used.

Cell lines and cell culture

The human breast cancer cell line BT474, mouse triple-negative breast cancer cell line E0771, and 293T cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle medium (Hyclone, Waltham, MA) supplemented with 10% fetal bovine serum (Cegrogen Biotech, Stadtallendorf, Germany), 100 U/mL penicillin (Gibco, Waltham MA), and 100 µg/mL streptomycin (Gibco) at 37 °C and 5% CO₂. All cell lines were tested for no contamination.

Construction of the hHER2+ mouse cancer cell line

E0771-hHER2 is a murine breast cancer cell line expressing hHER2. Human full-length HER2 cDNA from BT474 was cloned into the transposon-based vector pLVX-IRES-ZsGreen1 and co-transfected into murine wild-type (WT) E0771 cells together with pMD2.G and psPAX2 for four rounds. All the vectors were maintained in our lab and proved for sequences. Then, cell pools stably expressing hHER2 were selected in the presence of puromycin, and individual clones were isolated by single-cell sorting using flow cytometry (FACS Aria II; BD Biosciences).

Cell counting kit (CCK)-8

E0771-hHER2 cells (5×10^3) were plated in 96-well plates, after which RC48 (0, 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , 10^0 , and 10 µg/mL) was added to the wells (4 replicate wells per concentration). After 96 h, metabolic activity was determined using CCK-8 assay (HY-K0301; MCE, New Jersey). IC₅₀ values were calculated using GraphPad Prism v6 (GraphPad Software, Inc., La Jolla, CA) using a four-parameter logistic (4PL) regression model.

Flow cytometry

E0771 WT and E0771-hHER2 (1×10^6 cells/mL, 100 µL) were separately treated on ice for 1 h with the indicated concentration (0.0001–100 µg/mL) of disitamab, followed by incubation for 30 min with IgG Fc-APC (409306; BioLegend) and fluorescence-activated cell sorting (FACSCalibur; BD Biosciences). Data were analyzed using FlowJo 7.6.2 software (TreeStar, Inc., Ashland, OR).

Immunofluorescence staining

For tumor immunofluorescence staining, samples were fixed immediately after excision in 4% paraformaldehyde overnight at 4 °C. Excised tumor tissues were frozen using OCT compound (4583; Solarbio, Beijing, China) and sectioned for immunohistochemical staining of CD3, and Ki67 (6B111141, Servicebio, Wuhan, China).

Mouse models and treatments

All procedures relating to animal care, handling, and treatment were performed according to the European Union Directive 2010/63/EU and approved by the Animal Research Ethics Committee at Tongji University (no. TJLAC-018-032). Human PD-1 transgenic mice were obtained from Shanghai Model Organisms Co., Ltd. (Shanghai, China) and kept in a pathogen-free environment. Mice (aged 5 to 6 weeks) were inoculated with 2×10^6 E0771-hHER2 cells suspended in PBS into the right flank by subcutaneous (s.c.) injection. Tumor volume was defined as $1/2 \text{ length} \times \text{width}^2$. When the average volume of tumors reached approximately 100–200 mm³, the mice were randomized divided into control and treatment groups based on tumor volumes, and treatment was initiated (day 0). RC48 (10 mg/kg), anti-hHER2 antibody (10 mg/kg), and vehicle (PBS) were

administered intravenously (i.v.) at a volume of 100 μ L/mouse. Tumor volume was analyzed by two-way ANOVA using GraphPad Prism v6. Mouse body weight was determined using an electronic scale.

In drug combination experiments, mice were randomly divided into 6 groups with similar mean tumor sizes, and treated with PBS, ADC (5 mg/kg), PD-1 antibody (10 mg/kg; Pembrolizumab; Merck & Co., Kenilworth, NJ), PD-L1 antibody (10 mg/kg; Atezolizumab, Roche, Mannheim, Germany), or their combination (ADC + PD-1 antibody or ADC + PD-L1 antibody). RC48 was administered on days 0 and 7. Anti-PD-1 and anti-PD-L1 antibodies were administered on days 0, 3, 7, and 10.

For the rechallenge study, mice E0771-hHER2 tumors cured by RC48 and PD-1/PD-L1 treatment were divided into two groups. The mice were then s.c. inoculated with 2×10^6 E0771-WT or E0771-hHER2 cells into the left flank. Naive mice (previously not inoculated with tumor cells) were also inoculated with each cell line for comparison.

Tumor digestion and analysis of intratumoral cells

On day 7 after treatment initiation, mice were euthanized, and tumors were mechanically dissociated and digested with 0.1% collagenase type I (17100-017; Invitrogen, Carlsbad, CA) supplemented with Hanks' balanced salt solution medium (HBSS; 2005368; Gibco) for 20 min at 37 °C. Primary cells were harvested, washed, and resuspended in HBSS containing 1% bovine serum albumin. Single-cell suspensions were prepared and stained with the indicated markers for flow cytometry. The resultant single cells were stained with live/dead agent, and followed with antibodies against mouse CD3, CD4, CD8, CD45, NK1.1, and PD-L1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v6. Results are shown as the mean \pm SEM of at least triplicate technical or biological replicates. Statistical significance was analyzed by an unpaired two-tailed *t*-test unless otherwise indicated. Differences were considered significant at $p < 0.05$.

Results

RC48 decreases hHER2+ cancer cell viability

To study on RC48 and the parental anti-hHER2 antibody which does not cross-react with mouse HER2, we constructed a mouse cell line that stably expresses the human target gene. The human *her2* gene was introduced into the murine triple-negative breast cancer cell line E0771 using the pLVX-IRES-ZsGreen1 plasmid. After four rounds of fluorescent-activated cell sorting (FACS), cells with high hHER2 expression levels were obtained, which bound to anti-hHER2 antibody. Monoclonal culture was conducted, after which clone #33 with the highest expression level was selected and named E0771-hHER2 in subsequent animal experiments (Fig. 1a). A series of concentration gradients of anti-hHER2 antibody staining were used to determine the binding ability of E0771-hHER2 (Fig. 1b).

In *in vitro* cell viability assays of E0771-hHER2 after RC48 treatment, the IC₅₀ value was determined to be 90 ng/mL (Fig. 1c). Subsequently, E0771-hHER2 cells treated with RC48 (0, 100, and 500 ng/mL) for 48 h and 72 h were imaged. Off-target effects of RC48 were assessed on breast cancer lines lacking expression of hHER2 (E0771 WT). Compared with E0771 WT, E0771-hHER2 cell viability was significantly reduced by RC48. (Fig. 1d). These results indicate that the E0771-hHER2 cell line can be used for further studies.

RC48 treatment increases intratumoral T cell infiltration

Previous reports revealed that the MMAE molecule of ADC drugs is a toxin molecule that results in immunogenicity¹⁸. To figure it out, E0771-hHER2 cells were s.c. inoculated into PD-1 mice, and tumor growth was confirmed. When mice with E0771-hHER2 tumors were i.v. treated with vehicle, 10 mg/kg of RC48, or 10 mg/kg of its parental anti-hHER2 antibody on day 5, the mean tumor volumes on day 9 of vehicle-, anti-hHER2 antibody-, and RC48-treated groups were 345.07 ± 78.00 , 358.83 ± 107.69 , and 244.82 ± 78.90 mm³, respectively (Fig. 2a). Compared with the other two groups, RC48 showed a better antitumor effect, indicating that the antitumor effect of RC48 in this model depends mainly on the payload delivered into the tumor by anti-hHER2 antibody.

Next, the tumor was excised on day 9, and immunofluorescence staining was performed. It was found that the RC48 group exhibited increased infiltrated T cells in the tumor tissue compared with that of the parental monoclonal antibody (Fig. 2b), which may have induced an immune response. This indicates the potential for treatment combinations with immune checkpoint inhibitors.

RC48 and PD-1/PD-L1 combination treatment shows improved tumor inhibition

As immune cell infiltration was observed in mouse tumors after RC48 therapy, we speculated that adding an immune checkpoint inhibitor can lead to synergistic therapeutic effects. Therefore, we assessed the combination of RC48 with anti-PD-1 or anti-PD-L1 antibodies in an immunocompetent mouse model with E0771-hHER2 (s.c. inoculation). When mice were treated with vehicle (PBS), RC48 (5 mg/kg, once a week, 2 times, i.v.), anti-PD-L1 antibody (15 mg/kg, twice a week, 2 cycles, i.v.), or a combination of the two, tumor growth after monotherapy was slower than that of the PBS group; meanwhile, tumors in the combination group disappeared within two or three weeks, showing a very significant combined outcome (Fig. 3a). In the RC48+anti-PD-1 group (10 mg/kg, twice a week, 2 cycles, i.v.), the same result of significant combined outcome was also obtained (Fig. 3b). Thus, the combination of PD-1 or PD-L1 antibody with ADC achieved a combinatorial effect. Individual tumor growth curves for each group are shown in Fig. 3c, and the standard error in the RC48 + anti-PD-L1 treatment group was negligible.

Characterization of intratumoral immune cells after RC48 + PD-1/PD-L1 combination therapy

To elucidate the rapid shrinkage of tumors in the combination therapy group, we analyzed the infiltrated immune cells in tumors of mice injected with ADC and/or PD-1/PD-L1 antibodies. After 7 days of treatment, the proportion of CD45 in bulk tumors from the two combination groups was significantly higher than that in the monotherapy group (Fig. 4a, Fig.S2). The proportion of CD3⁺, CD4⁺, CD8⁺ and NK cells in the combination groups was also higher than in the vehicle control and monotherapy group (Fig. 4b–e).

To examine whether RC48, PD-1/PD-L1, or a combination of the two modulates the expression of immune-associated markers on tumor cells, PD-L1 expression levels were determined by FACS (Fig. 4f). Interestingly, the median fluorescence intensity of PD-L1 on hHER2⁺ cells as well as total cells from the excised tumors was higher in the anti-PD-1 therapy (both mono- and combo-) group than other groups (Fig. 5g). Otherwise, both anti-PD-1 and anti-PD-L1 therapy decreased the PD-L1 expression on the surface of the infiltrating CD45⁺ immune cells. Therefore, our data reveal an immune-based mechanism of action for combining RC48 with immune checkpoint inhibition.

Tumor immunostaining after RC48 + PD-1/PD-L1 combination therapy

Histomorphological evaluation of tumors after 7 days of RC48 monotherapy and RC48 + immune checkpoint

inhibitors revealed no histologically evident treatment effect in the tumor cell compartment. Quantitative changes indicated that expression of Ki67—a tumor proliferation marker—was decreased (Fig. 5a-b). These findings demonstrate the direct anti-tumor microenvironment of combining RC48 with immune checkpoint inhibitors.

Contribution of RC48+PD-1/PD-L1 combination treatment to immune memory formation

A complete response was observed in 100% of E0771-hHER2 (s.c. inoculation)-bearing mice treated with RC48+PD-1 and RC48+PD-L1. These cured mice were randomly divided into different groups and rechallenged with E0771-hHER2 or E0771-WT cells (s.c. inoculation). Naive mice were used as a control cohort (Fig. 6a).

E0771-hHER2 cells were completely rejected in rechallenged mice. Furthermore, E0771-WT cells were also rejected, albeit to a lesser extent, in mice cured of the E0771-hHER2 tumor (Fig. 6b, Fig. S3). Meanwhile, E0771-hHER2 and E0771-WT cells grew normally in naive mice. These results suggest that multiple antigens other than hHER2 are recognized by the immune system in mice cured of E0771-hHER2 tumors upon RC48 and PD-1/PD-L1 treatments. Thus, the combination treatment induces T cells that recognize not only hHER2 but also other antigens of tumor cells.

Discussion

To investigate the direct effect of different agents on humanized targets within tumor cells, several studies have been conducted on immunocompromised mouse models (xenograft mouse models)²¹⁻²⁵. In such experiments, the importance of the immune system, especially T and B cells, was tended to be overlooked. In our study, we examined the immunologic effect of RC48 using an immunocompetent mouse model by introducing a hHER2 expressed murine cancer cell line. Furthermore, to study PD-1/PD-L1 signal blockade, humanized antibodies such as pembrolizumab and atezolizumab instead of murine antibodies were utilized in this study. Our PD-1 humanized transgenic mouse is a good model for achieving this goal. Transplanting human peripheral blood mononuclear cells into nude mice is another available model that used to evaluate drug combinations in human cancer cell lines *in vivo* (data not shown)^{26,27}. Taking advantage of our transgenic model, we aimed to specifically dissect the immune-modulatory capacities of RC48, and subsequently its interaction with immunotherapy.

Previous studies have indicated that ADCs can induce antitumor immune activity upon intracellular release of the cytotoxic payload⁶⁻¹⁰. In other words, when ADC drug molecules arrive at the target site, they are enriched on the tumor surface, exerting a direct killing effect on the tumor or bystanders, greatly improving immune responses. For example, ado-trastuzumab emtansine (T-DM1) increased the number of tumor-infiltrating lymphocytes in human primary breast cancers and induced infiltration by effector T cells in murine breast tumors²⁸. In addition, RC48 reduces the number of tumor cells, thereby minimizing the chronic exposure of T cells to tumor-derived antigens, which may render them nonfunctional. We therefore propose that the ADC with its cytotoxic payload reinstates immunosurveillance by driving a distinct DC maturation program, and subsequently T cell infiltration, whereas PD-1-blocking therapy potentially reinvigorates exhausted T cells^{20,29,30}.

Furthermore, monotherapies of PD-1/PD-L1 antibodies have demonstrated substantial clinical activity in different tumors³¹⁻³⁸. However, only a limited number of patients respond to PD-(L)1 blockade, likely those whose tumors are pre-infiltrated by T cells. Others have reported that PD-L1 is upregulated in cancer cells and

tumors after treatment with immunotherapy-based agents. Whereas DS-8201a demonstrates immune-modulating activity, the expression of PD-L1, an immune-inhibiting molecule, is increased during DS-8201a treatment; therefore, new treatments are needed. One solution to this problem is combination therapy^{20,30}, such as with HER2-targeting therapy and blockade of the PD-1/PD-L1 inhibitory pathway, which has been tested in mouse models and are currently being evaluated in clinical trials (NCT02318901, NCT02605915, NCT01896999, NCT02581631, NCT02684292, NCT02572167). For combinations with immune checkpoint inhibitors, ADCs are considered superior to other chemotherapeutic agents because ADCs work selectively at the tumor site with reduced side effects in other organs.

In our model, combining RC48 treatment with PD-1/PD-L1 antibodies resulted in complete inhibition and greatly enhanced T cell responses, leading to complete tumor rejection and memory formation^{28,39}. Our data strongly support T cell dependence and the efficacy of combination therapy, which is achieved through non-redundant but complementary mechanisms, i.e., RC48 augments T cell infiltration into the tumor by inducing tumor-specific, adaptive antitumor immunity, whereas PD-1 blockade reinvigorates exhausted T cells. Similar therapeutic benefits have been observed with other chemotherapies^{40,41}. Notably, we used suboptimal yet therapeutically active doses of RC48, indicating that immune checkpoint inhibitors can boost the antitumor activity of ADCs, leading to a better tolerability of ADCs at sub-curative doses. More importantly, the synergistic effects of RC48 ADC with immune checkpoint inhibitors may lead to a reduction in the dosing regimen of both therapeutic modalities below their required effective doses during monotherapy. This can be expected to lead to a reduction in dose-limiting toxicities of individual treatments.

Mechanisms underlying the immunologic memory effect may rely on a series of processes including cell death, activation of DCs, T-cell activation, and possibly others. We found that the RC48 and PD-1/PD-L1 combination treatment contributed to immune memory formation. This study provides insights into the mechanisms underlying RC48 therapeutic activity and a rationale for potential therapeutic combination strategies with immunotherapy. Our data suggest that the PD-1/PD-L1 pathway inhibits activation of the immune system by RC48, where the anti-PD-1 antibody blocks the PD-1/PD-L1 inhibitory signal, increasing the efficacy of RC48. Therefore, the benefits of a combination therapy of RC48 with anti-PD-1/PD-L1 antibodies are strongly supported and this treatment modality is expected to be a new effective treatment for HER2-positive tumors.

Acknowledgements

The authors thank RemeGen, Ltd. for RC48 preparation.

Disclosure

Jianmin Fang reports being a shareholder of RemeGen Ltd., outside the submitted work. The authors report no other potential conflicts of interest in this work.

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Figures and legends

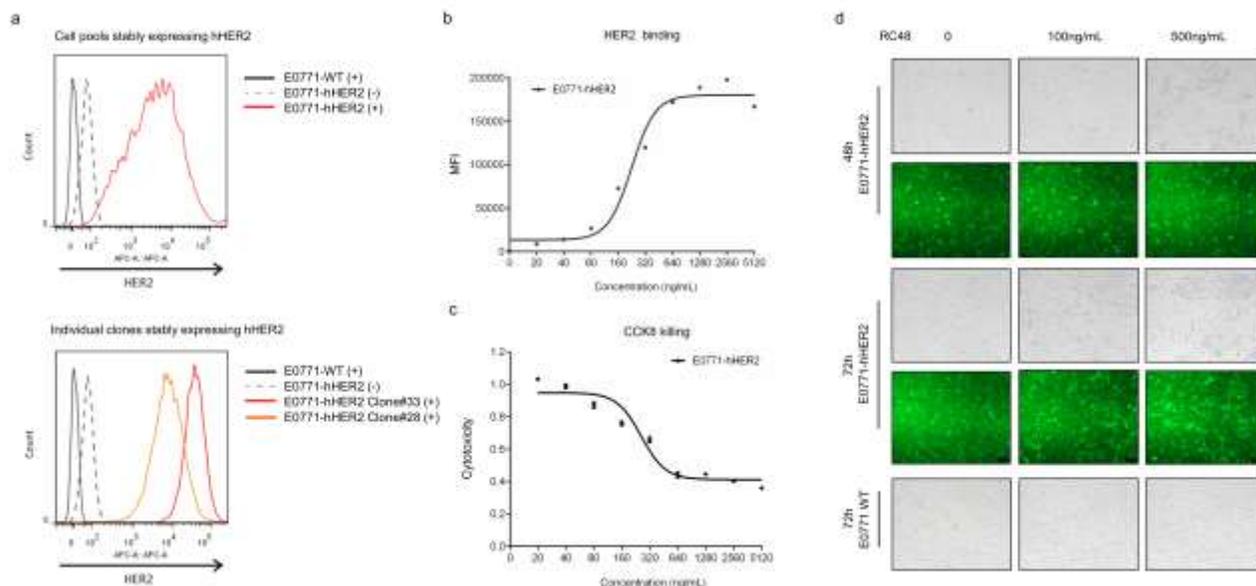


Fig. 1. Antitumor effect of RC48 in a hHER2+ mouse cancer cell.

a) Expression of exogenously introduced human *her2* gene was confirmed in E0771 cells. Individual clones were isolated by single-cell sorting using flow cytometry.

b) Her2 binding ability of E0771-hHER2 (clone#33) using disitamab (1 μ g/mL).

c) CCK-8 assay for determining cell viability of E0771-hHER2 after RC48 treatment (clone#33).

d) RC48 displays target-specific cell death properties. Images of E0771-hHER2 and E0771-WT cells after treatment with RC48 (0, 100, 500 ng/mL) for 48 and 72 h. Because the zsGreen1 fluorescence gene is also carried in the human *her2* gene-containing vector, the tumor cells are shown in green.

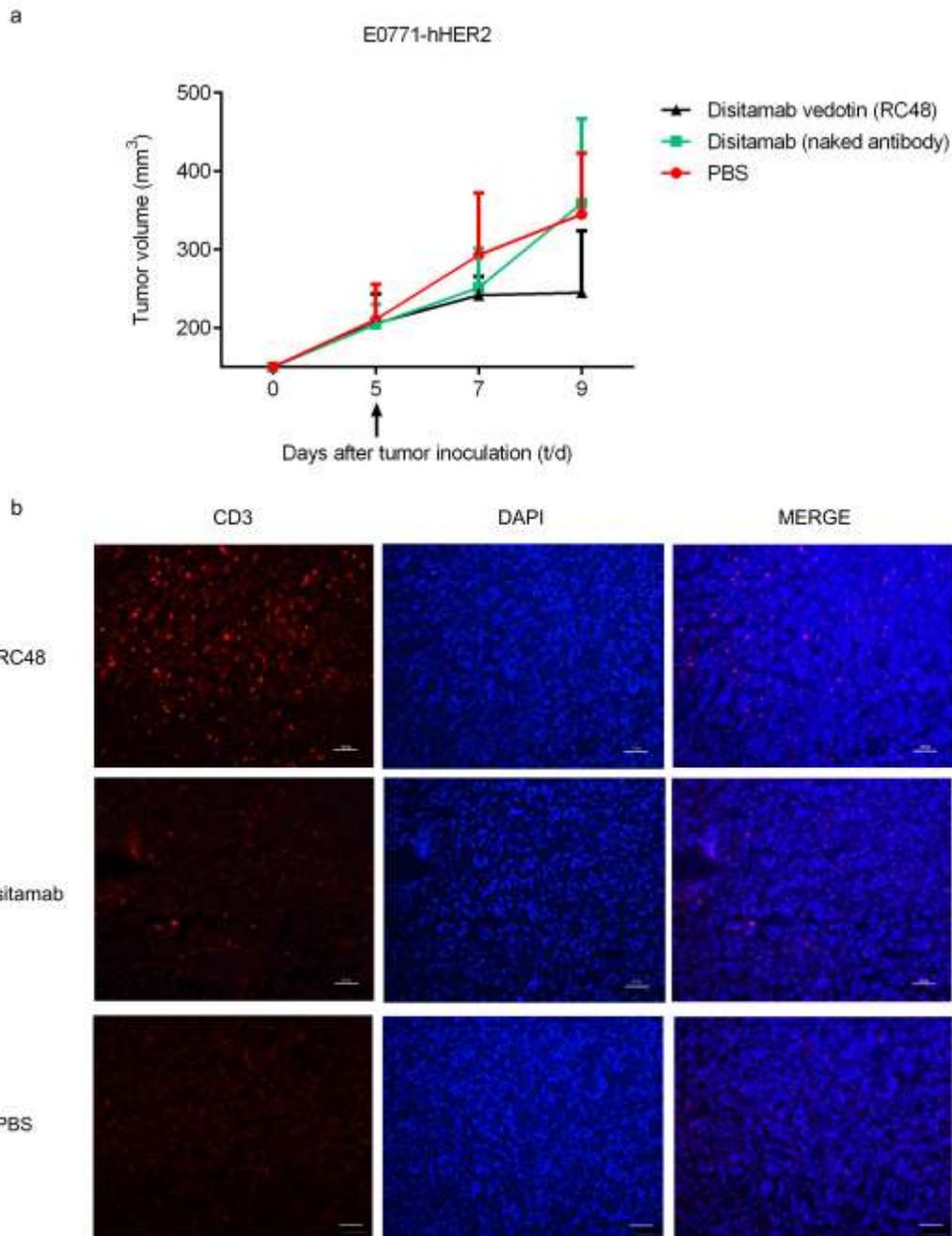


Fig. 2. RC48 strongly affects tumor growth in hHER2 xenograft breast cancer model and increases T cell infiltration.

a) Antitumor effect was examined in a syngeneic mouse model with E0771-hHER2 cells (s.c. inoculation) upon treatment with anti-hHER2 antibody or RC48 (10 mg/kg, once, i.v.) at the time points indicated by arrow. The graph shows mean tumor volumes and standard errors (n = 5).

b) On day 9, mice were euthanized and isolated tumors were stained with CD3 antibody for T cells (red) and DAPI. Bar=50 μ m.

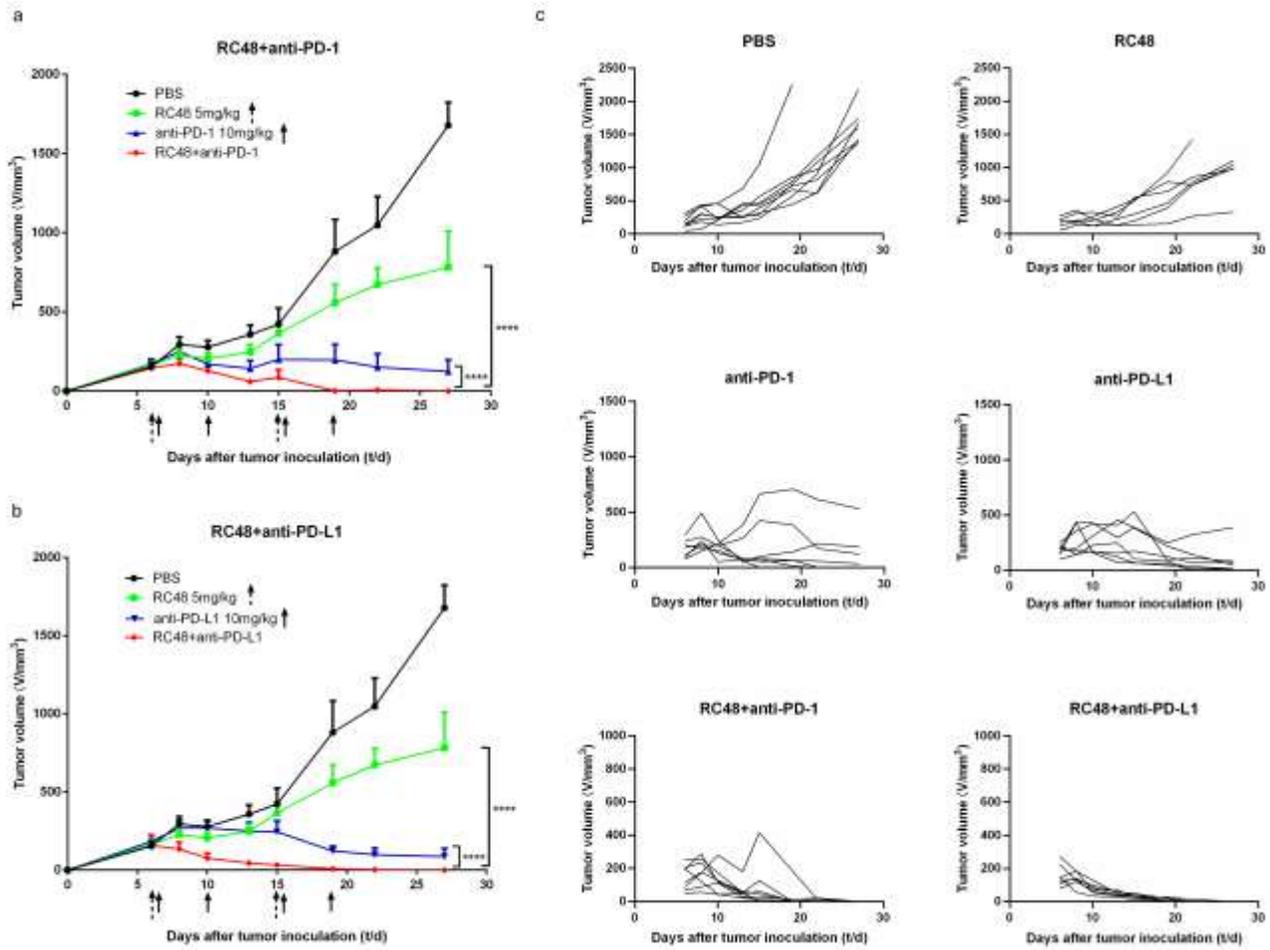


Fig. 3. Antitumor activity of immune checkpoint inhibitors and RC48 combination therapy.

a) Therapeutic response of E0771-hHER2 xenografted mice with a tumor volume of 150–200 mm³ following treatment with vehicle (PBS), RC48 alone (5 mg/kg, once a week, twice, i.v.), anti-PD-L1 alone (15 mg/kg, twice a week, 2 cycles, i.v.), or their combination (RC48 + anti-PD-L1). Compounds were administrated at the time points indicated by arrows.

b) Therapeutic response of of E0771-hHER2 animals with a tumor volume of 150–200 mm³ following treatment with vehicle (PBS), RC48 alone (5 mg/kg, once a week, twice, i.v.), anti-PD-1 alone (10 mg/kg, twice a week, 2 cycles, i.v.), or their combination (RC48 + anti-PD-1). Statistical significance was analyzed by two-way ANOVA, ****p < 0.0001.

c) Individual tumor growth curve of the indicated treatments in A and B.

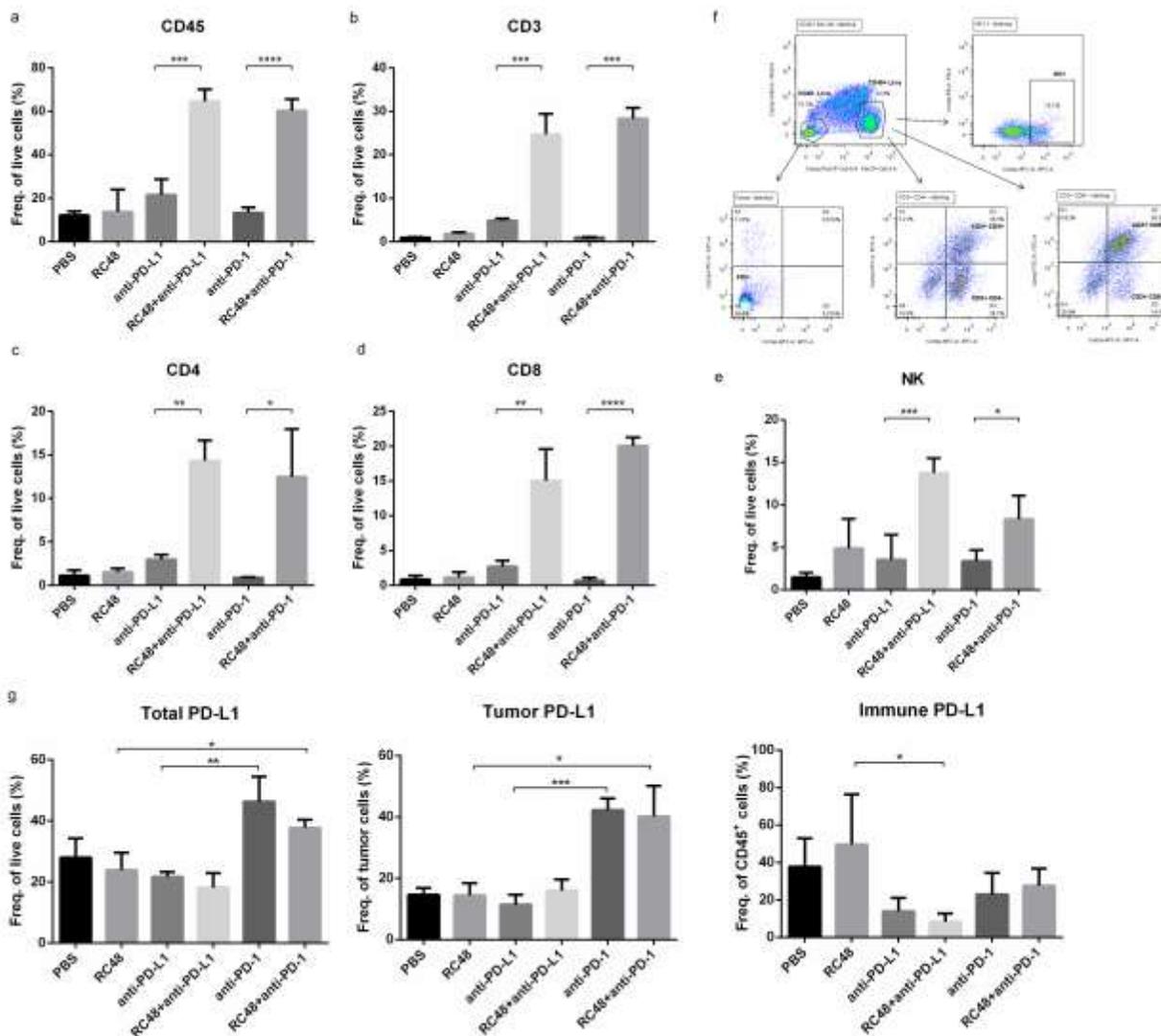


Fig. 4. Proliferation and activation of CD4⁺ and CD8⁺ T cells infiltrating murine breast tumors. a–e) Single-cell suspensions from the excised E0771 tumors treated as indicated were stained for immune cell markers CD45, CD3, CD4, CD8 for T cells and NK1.1 for NK cells according to the analysis method shown in f). g) Single-cell suspensions from the excised E0771 tumors were stained for the immune checkpoint marker PD-L1. Analysis of the PD-L1 expression on tumor cells, immune cells, and total live cells, respectively. Statistical significance was analyzed by an unpaired two-tailed *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

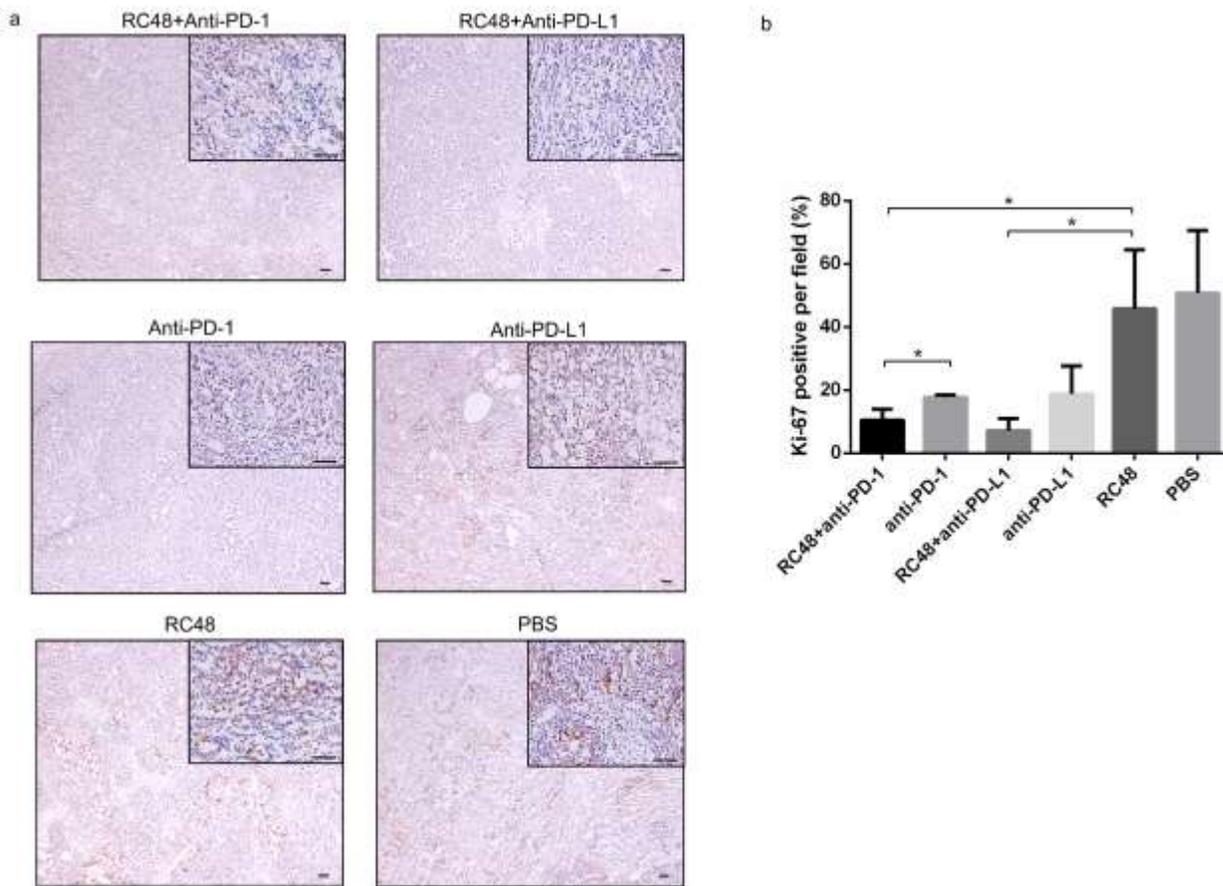


Fig. 5. Tumor immunostaining analysis after RC48 + PD-1/PD-L1 combined therapy.

a) Representative tumor sections were immunohistochemical stained of Ki67 for the indicated treatments. Scale bar=100 μ m.

b) Analysis of Ki67 positive field for the indicated treatments. Statistical significance was analyzed by an unpaired two-tailed *t*-test, **p* < 0.05.

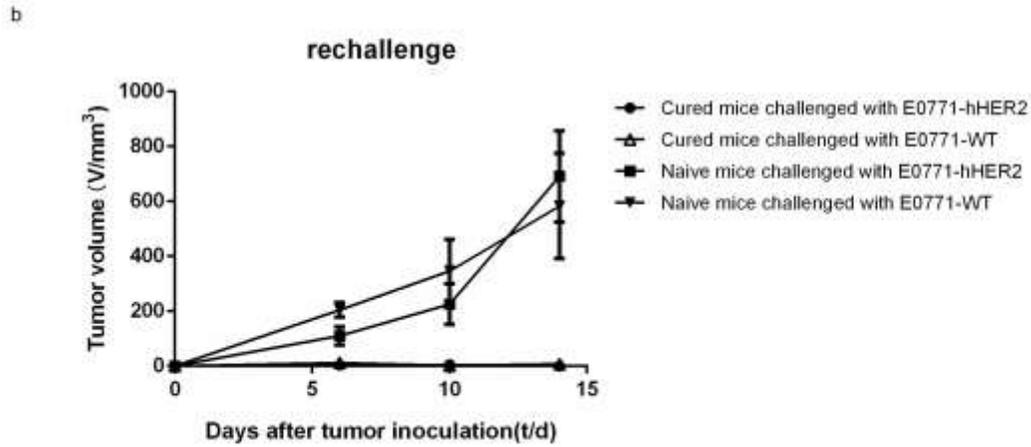
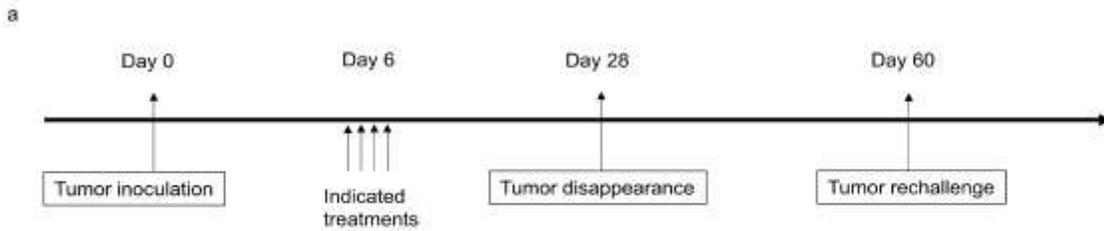


Fig. 6. RC48 and PD-1/PD-L1 combination therapy affords long-lasting immune protection.

a) Outline of a representative rechallenge experiment.

b) E0771-hHER2 tumor-free animals after ADC and PD-1/PD-L1 combination treatment were rechallenged with E0771-hHER2 and E0771-WT cancer cells. Tumor growth curve of the indicated groups.

