Blockade of ErbB2 and PD-L1 using a bispecific antibody to improve targeted anti-ErbB2 therapy

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ABSTRACT
A significant proportion of human epidermal growth factor receptor 2 (Her2/ErbB2)-positive metastatic breast cancer patients are refractory to Her2-targeted trastuzumab-like therapy. Some of this resistance has been attributed to the upregulation of immune checkpoints such as programmed cell death-1 (PD-1) and its ligand, PD-L1 in Her2-positive breast cancer patients. Therefore, therapies targeting both the PD-1/PD-L1 interaction and oncogenic Her2 signaling are of significant clinical interest. Here, we constructed a mouse bispecific antibody targeting PD-L1 and rat Her2 (referred to as BsPD-L1xrErbB2) aiming to redirect the anti-PD-L1 response toward Her2-expressing tumor cells. BsPD-L1xrErbB2 demonstrated additive binding to interferon (IFN)-γ treated Her2⁺ TUBO tumor cells, but it did not affect the proliferation of tumor cells in-vitro. BsPD-L1xrErbB2 also blocked the PD-1/PD-L1 interaction. This bispecific antibody was constructed with a mouse IgG2a Fc backbone and interacted with Fcγ receptors and resulted in complement deposition (C3). ADCC and complement action could be potential mechanisms of action of this molecule. BsPD-L1xrErbB2 successfully reduced TUBO tumor growth and increased tumor rejection rate compared to the monovalent anti-PD-L1, monovalent anti-ErbB2 or the combination of anti-PD-L1 and anti-ErbB2 monotherapies. The enhanced anti-tumor effect of BsPD-L1xrErbB2 was dependent on CD8⁺ T lymphocytes and IFN-γ, as depletion of CD8⁺ T lymphocytes and neutralization of IFN-γ completely abolished the antitumor activity of the bispecific antibody. Consistently, BsPD-L1xrErbB2 treatment also increased the frequency of intratumor CD8⁺ T lymphocytes. Taken together, our data support a bispecific antibody approach to enhance the anti-tumor efficacy of PD-1/PD-L1 checkpoint blockade in Her2-positive metastatic breast cancers.

Introduction
Trastuzumab therapy is a standard monoclonal antibody-based therapy targeting oncogenic Her2 (Human epidermal growth factor receptor 2, also called ErbB2) signaling in Her2-positive breast cancers. Apart from its role in blocking tumor promoting oncogenic Her2 signaling, the anti-tumor efficacy of trastuzumab is dependent on antibody-dependent cellular cytotoxicity (ADCC). Its mechanism of action also builds on several innate and adaptive immune factors such as type I and II IFNs, IL-21, CD11b⁺ and F4/80⁺ myeloid cells, NK cells, CD4⁺ and CD8⁺ T lymphocytes in ErbB2⁺ mouse models of breast cancers.¹⁻⁴ Some patients are refractory to trastuzumab treatment.⁵ In addition to tumor cell-intrinsic factors such as downregulation of cell surface Her2 or mutations in the Her2-mediated PI3-kinase/AKT signaling pathways,⁵ upregulation of immunosuppressive molecules such as PD-1 ligand, PD-L1 and adenosine generating CD73 are associated with trastuzumab resistance.⁶‑⁸ Intratumor expression of PD-L1 has been identified in up to 72% Her2⁺ breast cancers,⁸⁻¹⁰ and PD-L1 expression along with CD8⁺ T cell infiltration is associated with favorable prognosis and increased survival.¹⁰ Consistently, recent studies have shown that neoadjuvant trastuzumab therapy significantly upregulates immunosuppressive markers such as PD-L1 and indoleamine 2, 3 dioxygenase (IDO) in the tumor-associated macrophages (TAMs) of HER2⁺ breast cancer patients and this correlates with poor trastuzumab response.¹¹ Therefore, therapies targeting oncogenic Her2 along with these immunosuppressive molecules are of significant clinical interest. Our previous study has also shown that anti-PD-1 monoclonal antibody (mAb) therapy significantly improved anti-ErbB2 therapy,⁴ and we also initiated a clinical trial examining the therapeutic potential of anti-PD-1 mAb in advanced, trastuzumab-resistant, Her2-positive breast cancers.¹²

Bispecific antibodies carry two antigen specificities in one antibody molecule and so might provide significant opportunities over monospecific antibodies (reviewed in¹³,¹⁴). Firstly, bispecific antibodies targeting both an immune checkpoint and a tumor antigen, may direct specific effectors of the immune system towards tumor cells.
system to target tumor cells, anticipating to enhance their cytotoxicity and reducing potential immune-mediated toxicities. Secondly, in contrast to monospecific Abs, bispecific antibodies, when acting in cis, may provide higher binding specificity as they interact with two different surface antigens. Thirdly, it is hypothesized that bispecific antibodies may help to avoid the development of therapy resistance. Disease modulators when present on a single tumor cell are involved in complex signaling pathways and often targeting one pathway may lead to unexpected changes in other pathways that leads to therapy resistance. The use of bispecific antibodies may help to avoid the development of resistance as targeting two disease modulators on a single tumor cell may increase the anticancer effect and thus, avoid the development of resistance.\textsuperscript{13}

Bispecific antibody approaches also have their limitations compared to monotherapies or combinations thereof. They do not allow for sequential therapies and it is not possible to modify the dose of target-binding protein in each arm. Most bispecific antibodies in preclinical and clinical development are engaging T cells, via the CD3 binding arm, for tumor cell killing. Bispecific antibodies targeting Her2 along with CD3 or Her3 have shown enhanced anti-tumor activity in preclinical models of Her2\textsuperscript{+} breast cancers.\textsuperscript{15–19}

Here, we generated a mouse IgG2a BsPD-L1xrErbB2 bispecific antibody (referred to as BsPD-L1xrErbB2) targeting oncogenic rat ErbB2 and mouse PD-L1 as proof of concept for Her2-directed blockade of the PD-1/PD-L1 interaction. We characterized BsPD-L1xrErbB2 \textit{in vitro} and successfully demonstrated its enhanced anti-tumor activity \textit{in vivo}, thereby justifying the rationale that a bispecific antibody approach targeting ErbB2 and blocking the PD-1/PD-L1 interaction might be promising in Her2\textsuperscript{+} breast cancer patients.

**Results**

**Anti-PD-L1 and anti-ErbB2 monoclonal antibody therapy in ErbB2\textsuperscript{+} mouse tumors**

PD-L1 is expressed in a wide range of cancer types and high PD-L1 expression is associated with improved clinical outcome in triple-negative breast cancers and Her2\textsuperscript{+} breast cancers likely as an indication of pre-existing immune responses triggering local adaptive resistance.\textsuperscript{20,21} To understand the therapeutic potential of anti-PD-L1 antibody in Her2\textsuperscript{+} breast cancers, we tested PD-L1 expression on the rat ErbB2\textsuperscript{+} mouse TUBO cell line. Untreated TUBO cells did not express any significant level of PD-L1 expression (Figure 1a). IFN-γ is a known regulator of PD-L1 expression and has been shown to contribute toward enhanced immune suppression.\textsuperscript{22} Upon stimulation with mouse IFN-γ, PD-L1 expression was induced on TUBO cells in a concentration-dependent manner and reached a plateau at around 48 h, while the ErbB2 expression remained constant with around 15,000 antibody binding sites irrespective of the concentration or duration of the IFN-γ exposure (Figure 1a). We confirmed increased PD-L1 expression at the mRNA level after IFN-γ stimulation in several other ErbB2\textsuperscript{+} tumor cell lines (Supplementary Figure 1A). Next, we evaluated the anti-tumor activity of anti-PD-L1 and anti-ErbB2 mAb in two different mouse models of Her2\textsuperscript{+} mammary cancers. Consistent with our previous observations,\textsuperscript{1} H2N100 tumors were completely rejected at higher doses of anti-ErbB2 mAb therapy (Figure 1b, c; 10/10 rejections at 50 μg dose) while TUBO tumors showed significant resistance to anti-ErbB2 mAb therapy at the same dose (Figure 1d, e; 1/10 rejections). In contrast, anti-PD-L1 mAb therapy exhibited slightly better therapeutic activity against TUBO tumors compared to H2N100 tumors. Therefore, in subsequent studies, we used the TUBO tumor model that showed moderate resistance to anti-ErbB2 mAb therapy.

**Characterization of bispecific mmlgG2a-PD-L1 x ErbB2 antibody**

Given the reduced tumor growth with anti-PD-L1 therapy in the moderately resistant Her2\textsuperscript{+} tumors, we next evaluated the combination potential of dual targeting of PD-L1 and ErbB2 in TUBO tumors by generating bispecific antibodies targeting PD-L1 and ErbB2 on mouse IgG2a background (referred as BsPD-L1xrErbB2). Appropriate bispecific controls were also produced as described in detail in Materials and Methods. We first tested the binding affinity of BsPD-L1xrErbB2 on TUBO cells.

In the untreated condition, clear binding of the bivalent anti-rat ErbB2 (clone 7.16.4, expressed as mlgG2a) was shown, as well as binding of the b12xrErbB2 (monovalent control for bispecific) and BsPD-L1xrErbB2 bispecific antibody (Figure 2a). As expected, the potency of the monovalent and bispecific antibodies were lower than the bivalent anti-rat ErbB2 antibody because of loss of avidity to Her2 (Figure 2a). Neither bivalent anti-PD-L1 antibody (MPDL3280A) nor monovalent anti-PD-L1 control antibody (B12xPD-L1), showed any binding to TUBO cells (Figure 2a, b) which might be explained by the minimal amount of PD-L1 expression that is present on these cells in the untreated condition.

Treatment of TUBO cells with mouse IFN-γ increased PD-L1 expression which reached even higher levels compared to the Her-2 expression (Figure 1a). Binding of anti-ErbB2 and b12xrErbB2 (monovalent bispecific antibody control for rErbB2) to IFN-γ-treated TUBO cells was comparable to the binding on untreated cells (Figure 2c, d) as IFN-γ treatment did not change ErbB2 expression. Binding of bivalent anti-PD-L1 (MPDL3280A) and monovalent anti-PD-L1 control (B12xPD-L1) on treated cells was more efficacious compared to the anti-rErbB2 bivalent antibody and monovalent antibodies due to the higher PD-L1 expression compared to ErbB2 expression after mouse IFN-γ stimulation. The BsPD-L1xrErbB2 however reached the highest efficacy in binding to IFN-γ-treated TUBO cells with a ~ 20-fold improved E50 compared to untreated TUBO cells (Figure 2), indicating that this bispecific antibody might be highly potent in binding to ErbB2\textsuperscript{+} tumors with high PD-L1 expression. These data also suggest that the binding of the BsPD-L1xrErbB2 antibodies to mouse IFN-γ treated TUBO cells was mainly driven by the PD-L1 expression.

To determine the effect of BsPD-L1xrErbB2 on the proliferation of TUBO cells, in the presence or absence of mouse IFN-γ, TUBO cells were incubated with BsPD-L1xrErbB2 and control antibodies for 72 h. Inhibition of proliferation of TUBO cells was observed after treatment with bivalent anti-ErbB2 antibody. This effect was markedly reduced when a monovalent variant of the
Figure 1. Anti-ErbB2 and anti-PD-L1 mAb therapy in rat ErbB2-positive mouse tumors. (a) Her2 expression and PD-L1 upregulation on TUBO cells upon IFN-γ stimulation. TUBO cells were stimulated with recombinant mouse IFN-γ for 72 h. PD-L1 and Her-2 expression were quantified on the stimulated cells at 24, 48 and 72 h using a QIFI kit and MPDL3280A and 7.16.4 antibodies, respectively. H2N100 (b, c) and TUBO (d, e) tumor cells (5 × 10^5 cells) were injected subcutaneously into Balb/c wild type mice, and treated with 10 µg or 50 µg of anti-ErbB2 mAb (7.16.4), 200 µg anti-PD-L1 mAb or control Ig (200 µg) injected intraperitoneally on days 17, 21, 24 and 28. Mice were monitored for tumor growth and results are expressed as mean tumor area ± SEM. (c, e) Data is shown for the percentage of mice rejecting tumors/group (gray bars, tumor-bearing mice; black bars, tumor-free mice for at least 60 d). Arrow represents the day treatment was started. Statistical analysis was performed using two-way ANOVA Turkey’s multiple comparisons test at day 30 for B and day 39 for D (***, P < 0.0005; ****, P < 0.0001; ns: not significant).
same antibody B12xErbb2 was used (Figure 3a). BsPD-L1xErbb2 targeting both PD-L1 and ErbB2 had minimal effect on the TUBO cell proliferation (Figure 3a). Despite the finding that pretreatment with IFN-γ strongly enhanced its binding, the BsPD-L1xErbb2 bispecific antibody did not affect TUBO cell proliferation (Figure 3b).

BsPD-L1xErbb2 antibody was capable of blocking the PD-1/PD-L1 interaction and might have direct anti-tumor killing efficacy
To validate if the PD-L1 arm in the BsPD-L1xErbb2 blocked the PD-1/PD-L1 interaction, the bispecific antibody was tested using a PD-1+ Jurkat cell line expressing an NFAT-reporter cocultured with a PD-L1 expressing CHO-K1 cell line. As expected, introduction of the anti-PD-L1 (MPDL3280A) blocked the interaction in a dose-dependent fashion. Surprisingly, the monovalent anti-PD-L1 control antibody (B12xPD-L1) and the BsPD-L1xErbb2 bispecific antibody had similar potency as the bivalent anti-PD-L1 antibody (Figure 3c). Bivalent anti-ErbB2, monovalent anti-ErbB2 or isotype control (B12) antibody did not block the PD-1/PD-L1 interaction confirming the specificity of the assay (Figure 3c).

Interestingly, although all antibodies used in these experiments were produced with a mouse IgG2a Fc tail, not all demonstrated the same potency in the interaction with FcγRIV. Bivalent anti-PD-L1 antibodies, but not bivalent anti-ErbB2 antibodies, induced activation of the effector cells, and the combination of both bivalent antibodies showed reduced activity. Importantly, the bispecific BsPD-L1xErbb2 antibody showed activity in FcγRIV reporter assay albeit at a lower level than the bivalent PD-L1 antibody probably due to loss of avidity for PD-L1 (Supplementary Figure 2A). The control bispecific antibody with Fc regions carrying the L234A and L235A mutations that abrogated FcγR binding (‘inert’) showed no activation confirming the specificity of this assay (Supplementary Figure 2A). These results prove that BsPD-L1xErbb2 has an active FC portion. To further understand the possible effector mechanisms induced by the bispecific antibody, in vitro binding of C3 was determined as a surrogate for putative complement-dependent cytotoxicity (CDC). C3 deposition was observed only on TUBO cells that were treated with anti-ErbB2 antibody. Though TUBO tumor cells were pretreated with IFN-γ to induce PD-L1 expression, no signal was observed in cells treated with anti-PD-L1 antibody. The bispecific BsPD-L1xErbb2 antibody was less efficacious at low concentrations but demonstrated comparable or even higher C3 deposition at higher concentrations likely due to increased number of bound antibodies per cell (‘single-arm’ binding). As expected, none of the control antibodies with an inert Fc were able to deposit C3 on the TUBO cells. These data suggest that complement activation might be one of the mechanisms of the bispecific and bivalent anti-ErbB2 antibodies (Supplementary Figure 2B).

BsPD-L1xErbb2 antibody improves therapeutic efficacy of anti-ErbB2 mAb therapy
Next, we evaluated the therapeutic efficacy of the BsPD-L1xErbb2 in the TUBO mouse tumor model of ErbB2+ mammary cancer. We treated established TUBO tumors (mean tumor size 60–80 mm3) with different doses of anti-ErbB2...
were critical for the anti-tumor in the We have previously also required for BsPD-L1xrErbB2 blocks PD-1/PD-L1 interaction but does not affect the viability of tumor cells in vitro. TUBO tumor cells were cultured in the absence (a) or presence (b) of 10ng/ml of mouse IFN-γ overnight and cells were incubated with different concentrations of control Ig, anti-ErbB2, anti-PD-L1, monovalent anti-rErbB2 (B12xErbB2) or BsPD-L1xrErbB2 for 72 h. Viability of TUBO tumor cells is shown as determined by using the CellTiter-Glo system. (c) NFAT Reporter Activity (RLU) as a measure of PD-1/PD-L1 interaction are plotted on the y-axes against the antibody concentrations in log nM on the x-axes.

**Discussion**

The potency of an anti-tumor drug is largely dependent on its tumor selectivity. Bispecific antibodies are novel tools in drug armory that can target two different antigens on same or different cell types and can potentially enhance drug potency by dual targeting, or increasing tumor selectivity and reducing immune-mediated systemic toxicities. A few bispecific drugs have been recently been approved and several are under investigation in clinical trials for various malignancies. We and others showed that CD8+ T cells and IFN-γ were required for the anti-ErbB2 mAb therapy. In order to test whether CD8+ T cells and IFN-γ are also required for BsPD-L1xrErbB2 therapy, we depleted either CD8+ T cells or CD4+ and CD8+ T cells together, or neutralized mouse IFN-γ in the groups of mice treated with bispecific control Ig (B12), anti-PD-L1 and anti-ErbB2 combination therapy and BsPD-L1xrErbB2 therapy. As shown before, bispecific antibody therapy induced more tumor rejections compared to the anti-PD-L1 and anti-ErbB2 combination therapy group. Both the BsPD-L1xrErbB2 and anti-PD-L1 and anti-ErbB2 combination therapy groups completely lost their anti-tumor activity in the presence of anti-CD8β, anti-CD4/anti-CD8β, or anti-IFN-γ suggesting that CD8+ T cells and IFN-γ were critical for the anti-tumor efficacy of the BsPD-L1xrErbB2 antibody and anti-PD-L1/anti-ErbB2 combination therapy (Figure 7 and Supplementary Figure 3).
Figure 4. Treatment with BsPD-L1 x ErbB2 bispecific antibody reduces tumor growth and increase tumor rejections. TUBO tumor cells (5 × 10^5 cells) were injected subcutaneously into Balb/c wild type mice, and treated with different concentrations of control Ig, anti-ErbB2 mAb (7.16.4), anti-PD-L1 mAb or BsPD-L1 x ErbB2 bispecific antibody injected intraperitoneally on days 19, 22, 26 and 29 (a, b) or on days 16, 19, 23 and 26 (c, d). Mice were monitored for tumor growth and results are expressed as mean tumor area ± SEM. (b, d) Data is shown for the percentage of mice rejecting tumors/group (gray bars, tumor-bearing mice; black bars, tumor-free mice for at least 60 d). Arrow represents the day treatment was started. Statistical analysis was performed using two-way ANOVA Turkey’s multiple comparisons test at day 44 for A and day 28 for C (*, P < 0.05; **, P < 0.005; ***, P < 0.0005; ****, P < 0.0001; ns: not significant).
Her2-positive breast cancers. Herein we demonstrate in mice the enhanced anti-tumor activity of BsPD-L1xrErbB2 bispecific antibody targeting oncogenic rat ErbB2 and mouse PD-L1. IFN-γ is a pleiotropic cytokine with opposing effects in tumor microenvironment. IFN-γ from T and NK cells has anti-tumor effects, while alternatively increased IFN-γ levels induce immunosuppressive factors such as IDO and PD-L1, that are likely to provide immune escape mechanisms and contribute to enhanced tumor growth. Recently increased PD-L1 expression after trastuzumab therapy was shown to correlate with poor trastuzumab response. We did not observe any significant level of PD-L1 expression in rat ErbB2+ mouse tumor cells. However, treatment with mouse IFN-γ increased PD-L1 expression, but not ErbB2 expression, in a time- and concentration-dependent manner. Anti-PD-L1 mAb induced moderate anti-tumor effects in two different rat ErbB2 mouse models suggesting that therapies targeting PD-L1 can enhance anti-Her2 therapeutic effects. Characterization of a BsPD-L1xrErbB2 antibody revealed that the avidity of BsPD-L1xrErbB2 was similar to the monovalent anti-ErbB2 mAb control. However, its overall binding increased four-fold after treatment of tumor cells with IFN-γ, owing to increased PD-L1 expression on tumor cells, suggesting that bispecific antibody might be highly potent in ErbB2+ tumors with high PD-L1 expression. The BsPD-L1xrErbB2 antibody also blocked PD-1 and PD-L1 interaction and

Figure 5. TUBO re-challenge in mice rejected tumors after bispecific antibody therapy. (a, b) Balb/c mice that rejected TUBO tumors after BsPD-L1xrErbB2, anti-ErbB2 alone, anti-PD-L1 alone or the combination therapy from different experiments were collected and 2 × 10⁶ TUBO tumor cells were injected in the opposite flank of mice in each group and tumor growth was monitored. TUBO tumors did not grow in any of the group of mice that had rejected tumors before. (c) Data is shown for the percentage of mice rejecting tumors/group (gray bars, tumor-bearing mice; black bars, tumor-free mice for at least 60 d). Number represents the tumor-free mice/total number of mice in a group.
downstream signaling pathway without affecting tumor cell proliferation. When compared to bivalent ErbB2 antibody, the BsPD-L1xErB2 showed a strongly reduced effect on tumor cell proliferation. Since PD-L1 is also expressed by immune cells, generally PD-L1/PD-L1 blocking antibodies are engineered to have an IgG background limited to no effector function, to avoid elimination of PD-L1/PD-L1 expressing immune cells.24,25 The BsPD-L1xErB2 antibody with a mouse IgG2a backbone is likely to be capable of eliminating ErbB2+ tumor cells through its active Fc tail that could induce ADCC and/or CDC. Indeed, we demonstrated the enhanced activation of effector T cells toward target ErbB2+ tumor cells upon BsPD-L1xErB2 antibody treatment in a Fc-mediated reporter assay, suggesting a potential for ADCC activity. The therapeutic activity of avelumab, a humanized IgG1 mAb against PD-L1 was dependent on NK cell-mediated ADCC.26 Similarly, the anti-tumor effects of anti-ErbB2 mAb therapy were largely attributed to its Fc-mediated ADCC in mouse models of ErbB2+ mammary tumors.3

Importantly, when used in vivo, the BsPD-L1xErB2 antibody had improved anti-tumor activity compared with the combination of anti-PD-L1 and anti-ErbB2 mAb therapy. Increased infiltration of CD8+ T cells in the tumor is often associated with improved clinical responses in several malignancies including Her2+ breast cancers,27 while the presence of granulocytic MDSC in the tumor is associated with its pro-tumorigenic immunosuppressive phenotype.28 Consistently, we observed an increased proportion of intratumor CD8+ T cells and decreased proportion of granulocytic MDSCs between BsPD-L1xErB2 antibody and the combination of anti-ErbB2 and anti-PDL1 antibodies, suggesting that the observed increased tumor rejection in BsPD-L1xErB2 antibody-treated group may involve additional mechanisms including possibility of increased C3 deposition as observed for the BsPD-L1xErB2 vs bivalent anti-ErbB2 antibodies. Nevertheless, by depleting either CD8+ T cells or CD4+ and CD8+ T cells together, or neutralizing mouse IFN-γ, we further showed that both CD8+ T cells and IFN-γ were critical for the anti-tumor efficacy of the BsPD-L1xErB2 antibody therapy. No macroscopic toxicity was noted and all mice treated with the BsPD-L1xErB2 appeared healthy upon treatment.

Collectively our data demonstrated that the BsPD-L1xErB2 antibody has several mutually reinforcing anti-cancer properties that are not always available in conventional monoclonal antibodies. In particular, BsPD-L1xErB2 antibody was simultaneously able to bind both PD-L1 and rat ErbB2; blocked PD-1/PD-L1 interactions in a Her2-directed manner without affecting tumor cell proliferation in vitro; induced activation of effector cells in FcyRIV reporter assay suggesting ADCC activity; and enhanced the proportion of...
intratumor CD8\(^+\) T cells and improved the therapeutic efficacy of anti-ErbB2 mAb therapy by reducing tumor growth and increasing tumor rejections. Technical progress in the past few years has advanced the development of anti-cancer bispecific antibodies into human clinical trials and our data provide a strong rationale to use this approach to target PD-L1\(^+\)ErbB2\(^+\) breast cancers to improve Her2+ targeting therapies.

Materials and methods

Mice

Balb/c wild type (WT) mice were purchased from the Walter and Eliza Hall Institute for Medical Research or bred in house. All mice were bred and maintained at the QIMR Berghofer Medical Research Institute and used from the age of 8 weeks. All experiments were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee.

Cell culture

Rat ErbB2-positive H2N100 tumor cells were generated from female Balb/c MMTV-ErbB2/neu transgenic mice and cultured as described previously.\(^1\)\(^,\)\(^2\)\(^,\)\(^9\) TUBO cells were cultured in complete DMEM supplemented with 10% heat-inactivated fetal calf serum (Thermo Scientific), 1X glutamax, 50 U/ml penicillin, 100 µg/ml streptomycin and 10 mM HEPES (Sigma-Aldrich), while other cell lines were cultured in RPMI supplemented with 10% heat-inactivated fetal calf serum, 1X glutamax, 50 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate from Gibco-Life Technologies and 10 mM HEPES, 1% non-essential amino acid solution, and incubated at 37°C in 5% CO\(_2\) incubator. The anti-rat ErbB2 mAb (clone 7.16.4) hybridoma was kindly provided by Mark I. Greene (University of Pennsylvania, Philadelphia, PA).

Generation of bispecific antibodies

Bispecific antibodies were made as described previously.\(^3\)\(^0\)\(^–\)\(^3\)\(^2\) Antibody heavy-chain expression vectors were constructed by de novo synthesis (Geneart) of codon-optimized VH coding regions of antibodies rHer2-7.16.4 and PDL1-MDPL3280A, genetically fused to the heavy-chain constant coding regions of mouse mmIgG2a (V00825) and inserted into expression vector pcDNA3.3 (Invitrogen). Likewise, separate light-chain expression vectors were constructed by inserting the appropriate VL coding regions in frame with the CL coding regions of the mouse (V00807) kappa light chain into expression vector pcDNA3.3. A QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce the L234A, L235A, F405L, K409R, T370K and R411T (EU numbering conventions are used throughout the manuscript) point-mutations. All antibodies were produced under serum-free conditions by co-transfecting relevant heavy and light chain expression vectors in FreeStyleTM 293-F or Expi293FTM cells, using 293fectinTM or ExpiFectamineTM 293, respectively.
Antibodies (all mmIgG2a and variants thereof generated for this study) were purified by protein A affinity chromatography (MabSelect SuRe; GE Health Care), dialyzed overnight to PBS, and filter-sterilized over 0.2-μM filters. Alternatively, antibodies were purified by protein G affinity chromatography (GE Health Care). The purity was determined by SDS-PAGE/CE-SDS and the concentration was measured by absorbance at 280 nm (specific extinction coefficients were calculated for each protein). Batches of purified antibody were tested by high-performance size-exclusion chromatography (HP-SEC) to determine the presence of aggregates or degradation products. Purified antibodies were stored at 2–8°C. Endotoxin levels of batches used in vivo were below 0.2 endotoxin units/mg IgG. Batches were visually inspected before use and in case of precipitates the samples were centrifuged and the concentration was determined.

**Controlled Fab-arm exchange**

Equimolar amounts of mmIgG2a-F405L-R411T and mmIgG2a-T370K-K409R (for effector function-silenced (inert) variants mmIgG2a-L234A-L235A-F405L-R411T and mmIgG2a-L234A-L235A-T370K-K409R) antibodies were mixed and incubated with 2-Mercaptoethylamine (2-MEA; Sigma) at a final concentration of 1 mg/mL per antibody. The final concentration of 2-MEA was 75 mM. The mixtures were typically incubated for 5 h at 31°C. To remove 2-MEA, the mixtures were buffer-exchanged against PBS using PD-10 desalting columns (5 kDa molecular weight cut-off; GE Healthcare) or dialysis using Slide-A-Lyzer cassettes (10 kDa molecular weight cut-off). Samples were stored overnight at 4°C to allow for the re-oxidation of the disulfide bonds. Quality of the batches were verified by biochemical characterization in the assays shown in supplementary table 1.

**In-vitro proliferation assay**

Cells were plated in 96 well flat bottom plates at a density of 2500 cells per well in serum containing medium supplemented with 10 ng/ml mouse IFN-γ (R&D Systems) or control medium. The cells were incubated overnight in an incubator at 37°C and 5% CO₂. After removal of the medium, fresh serum-free medium containing a titration series of the test antibodies was added to the cells. As positive control, cells were treated with 5 µM staurosporine to induce maximal killing. The cells were incubated for additional 72 h and cell viabilities were determined using the CellTiter-Glo system. After addition of 50 µl of CellTiter-Glo reagent, the cells were incubated for 90 min at 37°C and 5% CO₂. The samples were transferred to white optiplates and luminescence was measured using an EnVision*Envision 2104 multilabel counter Microplate reader (PerkinElmer, Inc.). Viability was calculated as follows: Viable cells (% of control) = (signal-background)/(no mAb control-background)*100%, in which the background was defined as the signal measured in the wells treated with staurosporine and cells incubated in medium without antibodies as negative control sample.

**NFAT reporter assay**

To validate if the PD-L1 arm in the BsPD-L1xrErbB2 antibody blocked the PD-1/PD-L1 interaction, the bispecific antibody was tested in an NFAT reporter assay. PD-1/PD-L1 reporter assay (Promega) uses a PD-L1+ aAPC/CHO-K1 cell line and a PD-1+ Jurkat reporter cell line (effector cells). In short, concentrated antibody dilution series were plated and subsequently both the PD-1+ effector cells and PD-L1+ aAPC/CHO-K1, both in thaw-and-use format, were added. After a 6 h incubation period at 37°C and 5% CO₂, plates were equilibrated to room temperature and after addition of Bio-Glo Luciferase Assay substrate (Promega), plates were measured for luminescence on the Spectramax iDS (Molecular Devices).

**ADCC reporter assay**

The experiment was performed in accordance with the protocol supplied with the Bio-Glo assay kit (Bio-Glo luciferase assay system; Promega). Prior to the experiment, the Bio-Glo luciferase substrate was added to the Bio-Glo Buffer (1 vial in 10 mL buffer). Targets cells (TUBO) were plated in a flat bottom 96 well plate at a density of 12,500 cells/well in the presence of 1 ng/ml IFN-γ. After overnight incubation, the medium was replaced by assay buffer and effector Jurkat T cells genetically engineered with mouse FcyRIV and an NFAT-response element that drives luciferase expression (NFAT-RE-luc2) (75000/well). Antibody dilutions were added and the plates were incubated at 37°C and 5% CO₂ for 6 h. Then, Bio-Glo Luciferase assay reagent was added to the wells and after 15-min incubation luminescence was measured using Envision reader. In parallel, the expression of both ErbB2 and PD-L1 on the target cells was confirmed using flow cytometry.

**Mouse C3 deposition assay**

TUBO cells were trypsinized after O/N treatment with 1 ng/ml IFN-γ and plated in 96-well round bottom plate in serum-free medium at 40 × 10⁴ cells/well. Antibody titrations were added and the cells were incubated for 15 min at room temperature. Mouse serum (1%) was added to stop the reaction and samples were incubated at 37°C and 5% CO₂ for 15 min. C3 deposition was determined using flow cytometry with anti-mouse C3 antibody.

**Receptor expression on tumor cells**

The TUBO cell line expressing rat Erbb2 was cultured up to 80% confluency. The cells were stimulated with a mouse IFN-γ (R&D Systems) at a concentration range 1 to 10 to 100 ng/ml and subsequently, the cells were incubated for 24h-72h for receptor expression analysis. TUBO cells were washed with PBS, harvested using trypsin and stained with antibodies detecting Rat ErbB2 (IgG2amm-rHer2-7164-T370K-K409R) and mPD-L1 (IgG2amm-PDL1-MDPL3280A-F405-R411T). Quantification was performed using a QIFIKIT (DAKO) and the samples were analyzed by flow cytometry.
**In vivo treatments**

H2N100 (5 × 10^5) and TUBO (5 × 10^5) tumor cells were injected subcutaneously into Balb/c WT mice or IFN-γ-deficient Balb/c mice. Mice were treated with four intraperitoneal injections of anti-ErbB2 (7.16.4) anti-PD-L1 (MPDL3280A), bispecific antibodies or control immunoglobulin (cIg) twice a week for 2 weeks after the tumors reached a size of 60–80 mm³. A subset of mice additionally received α-CD4 (GK1.5), α-CD8β (53.5.8) to deplete T cell subsets and α-asialoGM1 to deplete NK cells, and neutralizing anti-IFN-γ (H22) antibody as indicated. Tumor growth was monitored and tumor size was recorded with a digital caliper every 2–3 d as the product of two perpendicular diameters.

**Flow cytometry analysis**

TUBO tumors were cut into small pieces and digested in medium containing RPMI with collagenase II (1 mg/mL) and DNase (20 μg/mL) for 45 min. Tumors and spleen samples were filtered through 70 μm filter and washed in PBS. Samples were lysed for red blood cells by ACK lysis buffer, washed with PBS and single-cell suspensions were incubated for 20 min in Fc blocking buffer (2.4G2 antibody). Thereafter, samples were stained with viability stain Zombie Yellow (Biologend) and fluorescence-conjugated mAbs, all diluted in 2% FCS containing PBS: anti-mouse-CD45.2 (104), TCR-β (H57-597), CD4 (RM4-5), CD8 (53.6.7), CD11b (M1/70) and Ly6G (1A8) for 30 min in ice. Samples were fixed and permeabilized using fixation permeabilization buffer from ebioscience for 15 min in ice and stained with anti-mouse FoxP3 (FJK-16S). For staining of cells in culture, tumors cells were stained with anti-mouse-PD-L1 (MH5) and anti-ErbB2 (7.16.4) for 30 min in ice. All mAbs were purchased either from Biologend or ebioscience. Samples were acquired on LSR Fortessa IV Flow Cytometer (BD Biosciences) and data were analyzed on FlowJo V10 (Treestar).

**Statistical analysis**

Statistical analysis was performed using Graphpad Prism Software. Data were compared using a Mann–Whitney U test or one-way or two-way ANOVA as indicated. Data were considered statistically significant where the p values were equal to or less than 0.05.

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**Conflict of interest**

MJS has research agreements with Bristol Myers Squibb, Aduro Biotech and Tizona Pharmaceuticals. The following authors have a financial interest in Genmab: J.N. have stock and/or warrants. The following authors have a financial interest in Aduro Biotech Inc.: J.K., M.H., H.v. E. and A.v.E. have stocks and/or warrants. All other authors have no conflict of interest to declare.

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