Combining CD47 blockade with trastuzumab eliminates HER2-positive breast cancer cells and overcomes trastuzumab tolerance

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Trastuzumab, a targeted anti-human epidermal-growth-factor receptor-2 (HER2) monoclonal antibody, represents a mainstay in the treatment of HER2-positive (HER2$^+$) breast cancer. Although trastuzumab treatment is highly efficacious for early-stage HER2$^+$ breast cancer, the majority of advanced-stage HER2$^+$ breast cancer patients who initially respond to trastuzumab acquire resistance to treatment and relapse, despite persistence of HER2 gene amplification/overexpression. Here, we sought to leverage HER2 overexpression to engage antibody-dependent cellular phagocytosis (ADCP) through a combination of trastuzumab and anti-CD47 macrophage checkpoint immunotherapy. We have previously shown that blockade of CD47, a surface protein expressed by many malignancies (including HER2$^+$ breast cancer), is an effective anticancer therapy. CD47 functions as a "don't eat me" signal through its interaction with signal regulatory protein-α (SIRPα) on macrophages to inhibit phagocytosis. Hu5F9-G4 (magrolimab), a humanized monoclonal antibody against CD47, blocks CD47's "don't eat me" signal, thereby facilitating macrophage-mediated phagocytosis. Preclinical studies have shown that combining Hu5F9-G4 with tumor-targeting antibodies, such as rituximab, further enhances Hu5F9-G4's anticancer effects via ADCP. Clinical trials have additionally demonstrated that Hu5F9-G4, in combination with rituximab, produced objective responses in patients whose diffuse large B cell lymphomas had developed resistance to rituximab and chemotherapy. These studies led us to hypothesize that combining Hu5F9-G4 with trastuzumab would produce an anticancer effect in antibody-dependent cellular cytotoxicity (ADCC)-tolerant HER2$^+$ breast cancer. This combination significantly suppressed the growth of ADCC-tolerant HER2$^+$ breast cancers via Fc-dependent ADCP. Our study demonstrates that combining trastuzumab and Hu5F9-G4 represents a potential new treatment option for HER2$^+$ breast cancer patients, even for patients whose tumors have progressed after trastuzumab.

antibody therapy | breast cancer | CD47 | macrophage checkpoint immunotherapy | trastuzumab

Overexpression of human epidermal-growth-factor receptor-2 (HER2) occurs in ∼16% of breast cancers in the United States (1–3) and has been associated with a number of adverse prognostic factors (summarized in ref. 4). Prior to the advent of HER2-targeted therapeutics, HER2 overexpression was associated with increased risk of recurrence and poor survival rates (1, 2). Trastuzumab is a humanized monoclonal antibody that selectively binds HER2. Clinical use of trastuzumab has dramatically improved the outcomes of patients with HER2$^+$ breast cancer and remains the foundational component of modern standard of care treatment regimens for HER2$^+$ breast cancer in the neoadjuvant, adjuvant, and metastatic settings (5, 6). Early studies of trastuzumab's mechanism of action focused on trastuzumab's inhibition of protumor growth HER2 signaling pathways (7–9). Subsequent research revealed that trastuzumab also coopts a patient's immune system to promote an antitumor response (7–11). This later body of research initially elucidated trastuzumab's ability to engage Fc-receptors on natural killer cells (NKs) to promote antibody-dependent cellular cytotoxicity (ADCC) (12, 13). Recent reports have further illuminated trastuzumab's ability to engage Fc-γ receptors (FcγR) on macrophages and promote antibody-dependent cellular phagocytosis (ADCP) (14).

Administering trastuzumab to early-stage HER2$^+$ breast cancer patients significantly increases disease-free survival and overall survival rates (15–17). Treating advanced-stage HER2$^+$ breast cancer patients with the most efficacious trastuzumab-based regimens, however, produces less hopeful outcomes. For example, the Food and Drug Administration (FDA)-approved regimen studied in the CLEOPATRA clinical trial for HER2$^+$ metastatic breast cancer in the first line of treatment utilized trastuzumab in combination with docetaxel and pertuzumab; this regimen resulted in

Significance

This study demonstrates the efficacy of combining macrophage-checkpoint inhibition with tumor-specific antibodies for cancer immunotherapy. The combination of anti-CD47 (magrolimab) and anti-HER2 (trastuzumab) antibodies eliminated HER2$^+$ breast cancer cells with increased efficacy due to the enhancement of antibody-dependent cellular phagocytosis by macrophages, even when the cancer cells were tolerant to trastuzumab-induced antibody-dependent cellular cytotoxicity by natural killer cells. We believe these findings present a promising therapeutic approach for treating HER2$^+$ breast cancer patients whose tumors are either sensitive or resistant to trastuzumab treatment, as long as the cells harbor the HER2 trastuzumab-binding epitope. This study supports the notion that combining CD47 blockade with existing macrophage FcR-engaging tumor-specific antibodies may be an effective approach for treating a wide range of cancers.

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a median progression-free survival of 18.7 mo (18, 19). In the same study, 19.8% of patients did not achieve an objective clinical response to trastuzumab-based treatment (20). And, of the advanced-stage $HER2^+$ breast cancer patients who initially responded to trastuzumab, pertuzumab, and docetaxel, the median duration of response was 20.2 mo; thereafter, the majority of patients experienced objective disease-progression, defining acquired clinical resistance to trastuzumab-based therapy (18).

A myriad of potential mechanisms of trastuzumab resistance have been reported, such as: 1) perturbation of HER family receptors or binding of therapeutic antibodies to HER2 (e.g., shedding of the HER2 extracellular domain, expression of the Δ16HER2 splice isoform, overexpression of MUC4/MUC1 resulting in steric hindrance to trastuzumab binding to the HER2 extracellular domain, and increased phosphorylation of HER3); 2) parallel receptor pathway activation (e.g., overexpression of other HER family members, up-regulation of IGF1 receptor, erythropoietin receptor, AXL receptor, or MET receptor); and 3) activation of downstream signaling events distal to HER2 receptor (e.g., hyperactivation of the PI3 kinase/Akt pathway by loss of PTEN or PIK3CA mutational activation, cyclin E amplification/overexpression, up-regulation of miR-21, and expression of the estrogen receptor) (21). Impairments in trastuzumab-mediated ADCC may also lead to relative resistance to trastuzumab (22, 23). Interestingly, it has been shown that even after $HER2^+$ breast cancers relapse or progress after trastuzumab, resistant cells most often still overexpress HER2 (24). Given the many ways in which trastuzumab resistance develops, there is an urgent clinical need for novel treatment approaches that provide HER2 specificity without eliciting the same mechanisms of trastuzumab resistance that are currently seen in the clinic (25). This led us to hypothesize that engagement of macrophages and activation of ADCP could still be effective even in the face of various resistance mechanisms—as long as the HER2 ectodomain target epitope is present.

Our laboratory has previously shown that many different cancers overexpress the CD47 surface protein to convey a "don't eat me" signal to macrophages (26–29) and to counteract "eat me" signals (30–32), thereby resulting in immune evasion through inhibition of macrophage phagocytosis. This led to the development of a new type of immunotherapy based on macrophage checkpoint inhibition through blockade of CD47. Hu5F9-G4 (magrolimab) is a humanized monoclonal antibody against CD47 that blocks CD47's interaction with signal regulatory protein-α (SIRPα), thereby diminishing the inhibition of macrophages by cancer cells (33). As a monotherapy, Hu5F9-G4's anticancer activity works by blocking CD47's antiphagocytic signaling. The combination of Hu5F9-G4 and tumor-targeting antibodies, moreover, promotes ADCP by increasing the amount of "eat me" signals provided by the interaction of cancer-targeting antibody Fc domains and macrophage Fc-receptors (33–36). We have previously demonstrated this principle of ADCP enhancement in the context of rituximab-resistant CD20-expressing diffuse large B cell non-Hodgkin's lymphoma. These studies showed that combining Hu5F9-G4 with rituximab (an anti-CD20 tumor-targeting antibody) in human xenograft models produced an anticancer ADCP response (34). In the clinical trials that followed, about half of the patients who are relapsed and refractory to rituximab plus or minus chemotherapy nevertheless responded to magrolimab plus rituximab (35). This clinical study suggested that combining Hu5F9-G4 with rituximab may resensitize refractory lymphoma cells to rituximab via an ADCP mechanism (35, 37).

Based on these previous studies, we hypothesized that administering a combination of trastuzumab and Hu5F9-G4 to ADCC-tolerant $HER2^+$ breast cancer cells would resensitize these cells to trastuzumab. We found that this combination was more efficacious than either treatment alone. We also found that this combinatorial treatment augments ADCP via Fc-receptor-mediated phagocytosis. Taken together, our study suggests that combining Hu5F9-G4 and trastuzumab may represent an alternative or complementary approach to the current standard of care for advanced $HER2^+$ breast cancer that expands trastuzumab's efficacy through engaging ADCP while preserving and utilizing trastuzumab's HER2-targeting capabilities.

Results

Selection of $HER2^+$ Breast Cancer Cell Lines Tolerant to Trastuzumab-Mediated ADCC. In this study, we sought to determine the contribution of macrophage-mediated ADCP to trastuzumab efficacy, separate from NK-mediated ADCC. To that end, we first took $HER2^+$ breast cancer cells that are sensitive to both ADCP and ADCC and generated cell lines that are tolerant to ADCC. ADCC-tolerant breast cancer cell lines were derived from two parental $HER2^+$ breast cancer cell lines, SKBR3 and BT474, through repeated exposure (10 rounds of selection) to opsonizing concentrations of trastuzumab in the presence of human peripheral blood mononuclear cells (PBMCs) with an effector:target ratio of 100:1. This selection and enrichment process yielded sublines that, unlike the parental lines, demonstrated relative (though not absolute) resistance to trastuzumab-mediated ADCC. To test whether trastuzumab could still bind to the cells, we stained the parental as well as the resistant sublines with trastuzumab and found comparable levels (*SI Appendix*, Fig. S1). This confirmed that the mechanism of ADCC resistance is not necessarily the loss of the epitope recognized by trastuzumab, thereby suggesting that ADCC and ADCP are two independent mechanisms impacting trastuzumab efficacy. To confirm that relative resistance to ADCC was stable upon passaging, the cell lines were subjected again to an ADCC assay. Parental and ADCC-selected SKBR3/BT474 breast cancer cells were stained with the fluorescent dye 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) and preopsonized with either trastuzumab or PBS. Next, the breast cancer cells were cocultured with primary human PBMCs or with purified primary human NKs at various effector:target cell ratios. Cells were then stained with DAPI and subjected to flow cytometry analysis to determine the percentage of $DAPI^+$ and $CFSE^+$ cells remaining in the PBS or trastuzumab-treated conditions. As can be seen in Fig. 1, the parental lines BT474 and SKBR3 were killed more efficiently by both human PBMCs and NK cells compared to ADCC-tolerant sublines. The tolerance to ADCC was most pronounced when purified NK cells were used as effector cells.

$HER2^+$ ADCC-Tolerant Cell Lines Are Susceptible to Macrophage-Mediated ADCP upon Trastuzumab and Hu5F9-G4 Combination Treatment In Vitro. After confirming that the SKBR3 and BT474 ADCC-selected sublines demonstrated relative resistance to trastuzumab-mediated ADCC, we next asked whether these cell lines retained their sensitivity to ADCP. SKBR3 and BT474 parental and the ADCC-selected sublines derived from them were treated with either PBS, IgG4, trastuzumab, Hu5F9-G4 (anti-CD47 monoclonal antibody), or trastuzumab and Hu5F9-G4 (combination). We conducted the in vitro phagocytosis assays using macrophages from two different sources: 1) human peripheral blood monocyte-derived macrophages (Fig. 2 *C*–*E*); and 2) nonobese diabetic *scid*-γ (NSG) bone marrow-derived macrophages (BMDMs) (Fig. 2 *A* and *B*). We chose NSG BMDMs for our in vitro phagocytosis assays to be consistent with our subsequent use of NSG mice in our in vivo xenograft assays. We observed similar results using both of these macrophage preparations. Breast cancer cells were labeled with CFSE and incubated with differentiated macrophages for 2 h. After the incubation, mouse BMDM were stained with a fluorescent F4/80 antibody and cells were subjected to flow cytometry. Phagocytosis was determined by the percentage of cells that were both $F4/80^+$ and $CFSE^+$. SKBR3 and BT474 parental and ADCC-tolerant lines demonstrated increased susceptibility to ADCP when opsonized with combination treatment of trastuzumab and Hu5F9-G4 compared to treatment with trastuzumab or Hu5F9-G4 alone (Fig. 2 *A* and *B*). Similarly, phagocytosis assays with SKBR3 and BT474 parental

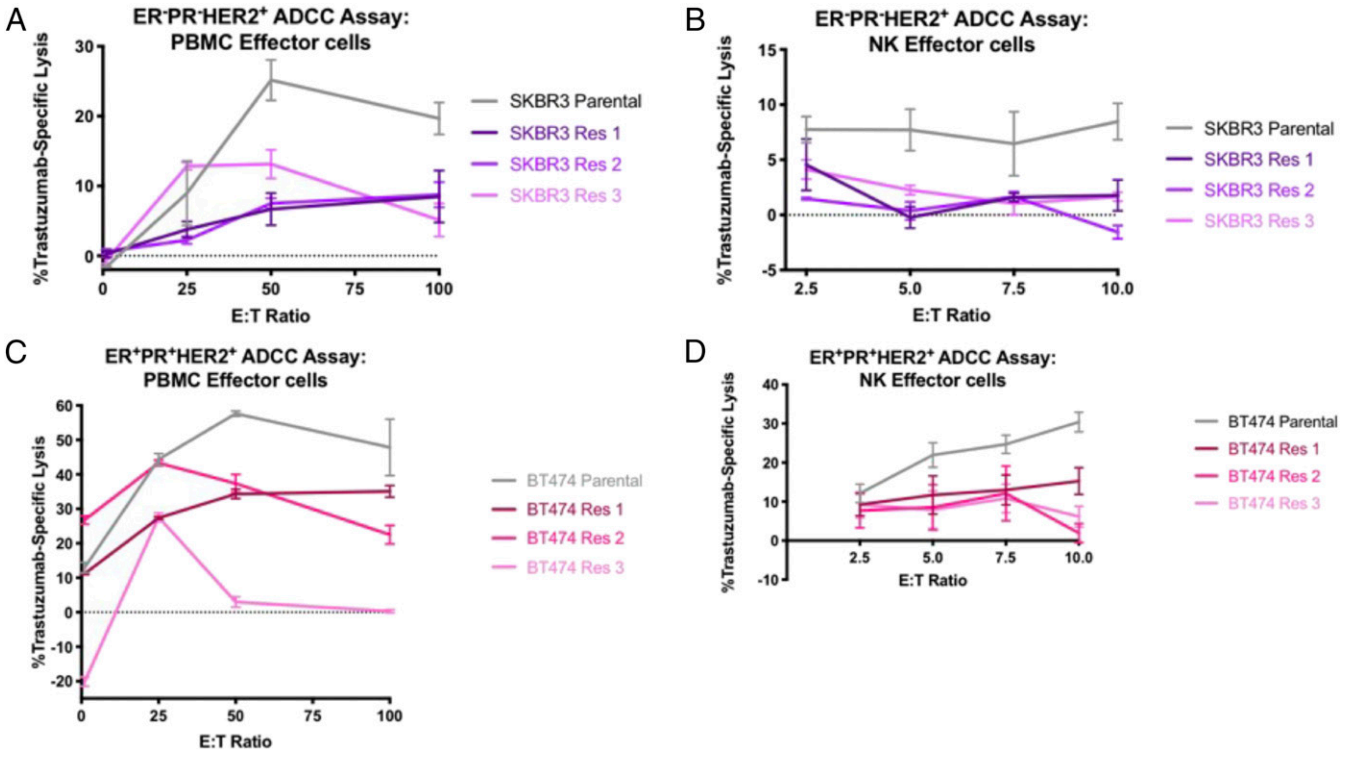

Fig. 1. ADCC-selected HER2$^+$ breast cancer cell lines display relative resistance to trastuzumab-mediated ADCC. (*A* and *B*) SKBR3 (ER$^-$PR$^-$HER2$^+$ [ER, estrogen receptor; PR, progesterone receptor]) parental and ADCC-tolerant breast cancer cell lines were opsonized with trastuzumab, labeled with CFSE, and incubated with (*A*) human PBMCs or (*B*) primary human NK cells. BT474 (ER$^+$PR$^+$HER2$^+$) parental and ADCC-tolerant breast cancer cell lines were opsonized with trastuzumab and incubated with (*C*) human PBMC effector cells or (*D*) human NKs. ADCC was measured by percentage of DAPI$^+$ CFSE$^+$ target cells determined by flow cytometry analysis.

and ADCC-tolerant cell lines as target cells were incubated with human PBMC-derived macrophages for 2 h. Target cells were labeled with CFSE and human macrophages were stained with fluorescent anti-CD45. Phagocytosis was determined by the percentage of cells that were both CD45$^+$ and CFSE$^+$. Breast cancer cells that were treated with the combination treatment demonstrated a significant increase in their susceptibility to macrophage-mediated phagocytosis compared to those treated with a single intervention (Fig. 2 *C* and *D*). To assess the levels of surface CD47 across all cell lines used, we stained cells with anti-CD47 and used flow cytometry to compare the two parental lines to the ADCC-tolerant sublines derived from them; we found no significant differences in CD47 levels (*SI Appendix*, Fig. S1).

Given the differing susceptibilities of SKBR3 and BT474 ADCC-tolerant sublines to ADCP versus ADCC, we next asked whether the increased ADCP was due to an Fc-dependent mechanism. To test this, we generated a trastuzumab F(ab′)$_2$ fragment and found that the Fc portion of trastuzumab is necessary to generate the enhanced ADCP in the combinatorial treatment. In the absence of trastuzumab's Fc domain, the combination of Hu5F9-G4 and trastuzumab F(ab′)$_2$ fragment produced significantly less ADCP than the combination of Hu5F9-G4 and trastuzumab with its Fc portion intact (Fig. 2*E*). Taken together, these results demonstrate that the susceptibility of BT474/SKBR3 ADCC-tolerant sublines to ADCP depends, at least in part, on engagement of Fc-receptors on macrophages.

Trastuzumab and Hu5F9-G4 Combination Treatment Restricts the Growth of HER2$^+$ ADCC-Tolerant Tumors In Vivo. To determine the impact of anti-CD47 combination with trastuzumab in vivo, we chose to use NSG mice that are devoid of T and B lymphocytes, have no NK cells, and cannot generate adaptive responses—but are macrophage-replete. This experimental xenograft model allowed us to evaluate the effect of macrophage innate immunity on human tumor cells growing in an animal that only has innate effector cells. Importantly, although CD47–SIRPα interactions are generally highly species-specific, we know based on previous studies that the NSG mouse version of SIRPα binds human CD47 with high affinity, so we had an opportunity to test human-relevant treatment efficacy with human HER2$^+$ breast cancer xenografts. We first sought to establish how the parental SKBR3/BT474 breast cancer cell lines respond to the different treatment combinations. GFP-Luciferase–labeled BT474 parental cells were engrafted into the left mammary fat pads of female NSG mice. Baseline tumor burden was established using in vivo bioluminescence imaging ("starting range"). Twenty-five days after engraftment, mice were treated with the aforementioned single treatments or combinatorial treatment and tumor growth was monitored for a period of 17 wk. At week 7, treatments were stopped to determine whether the tumors would progress in the absence of treatment. Treatment with Hu5F9-G4 maintained tumor burden within the starting range that further progressed upon stopping treatment (Fig. 3*A*). Trastuzumab treatment resulted in lower tumor burden at starting range but also demonstrated progression once treatment was stopped. In the combinatorial treatment however, tumors were significantly below the starting range and did not show signs of tumor progression within the 10-wk nontreatment period following the last dose.

Using a similar approach, we next tested the susceptibility of BT474 trastuzumab ADCC-tolerant cell line xenografts (Res 1 and Res 2) in vivo. Treatment with Hu5F9-G4 alone resulted in maintenance of tumor burden within the starting range and treatment with trastuzumab alone had significant efficacy that was enhanced by the addition of the anti-CD47 Hu5F9-G4

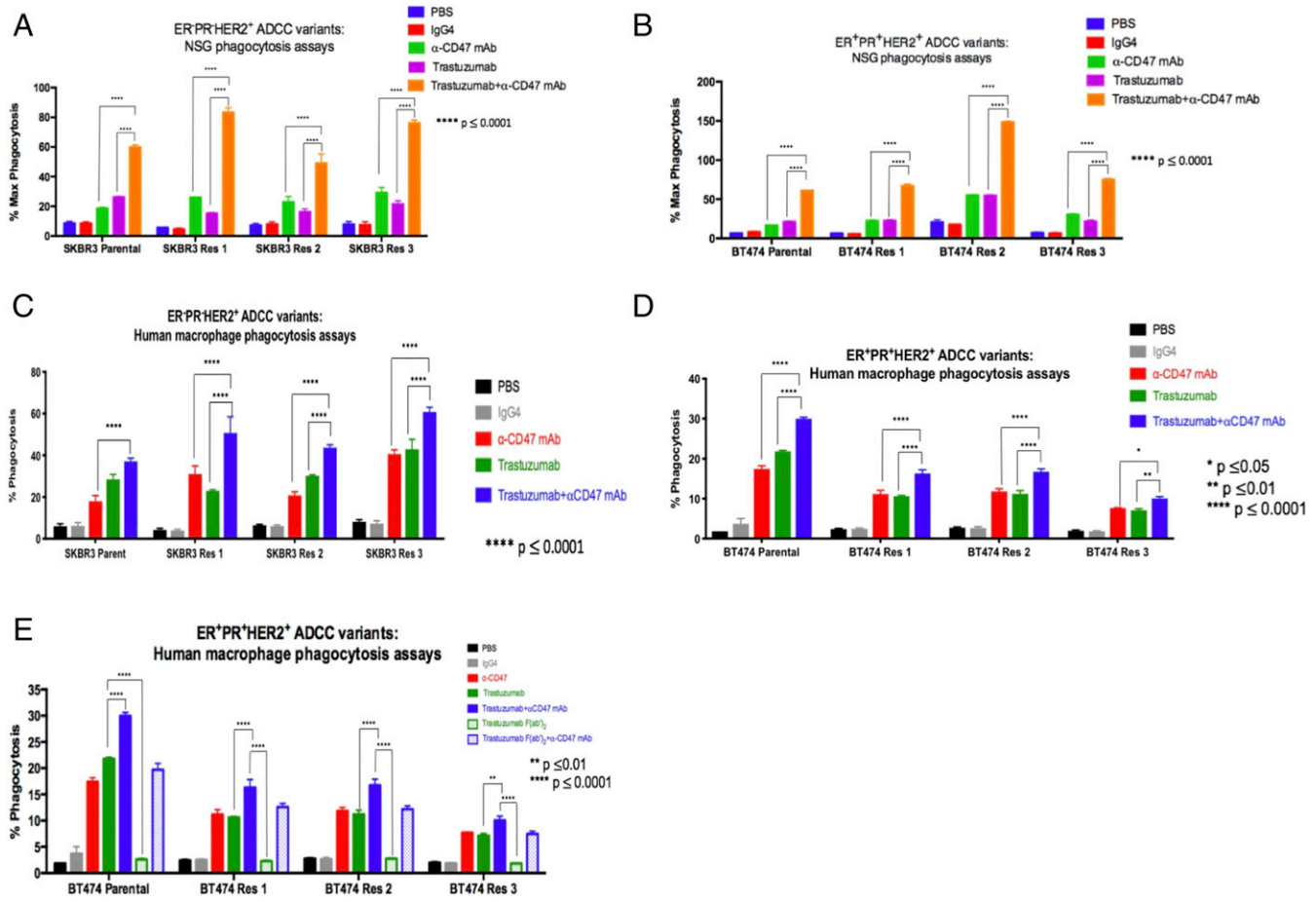

Fig. 2. Trastuzumab ADCC-tolerant cell lines are susceptible to macrophage-mediated phagocytosis through an Fc-dependent mechanism. A phagocytosis assay with (*A*) SKBR3 or (*B*) BT474 parental or ADCC-tolerant breast cancer lines as target cells. Target cells were stained with CFSE and treated with PBS, IgG4, anti-CD47, trastuzumab, or trastuzumab plus anti-CD47, and cocultured with BMDMs from NSG mice. A phagocytosis assay with (*C*) SKBR3 or (*D*) BT474 parental or ADCC-tolerant breast cancer lines as target cells. Tumor target cells were treated with PBS, IgG4, anti-CD47, trastuzumab, or trastuzumab plus anti-CD47, and were cocultured with human PBMC-derived macrophages. (*E*) A phagocytosis assay with BT474 parental or ADCC-tolerant breast cancer lines as target cells cocultured with human PBMC-derived macrophages. Tumor target cells were treated with PBS, IgG4, anti-CD47, trastuzumab, trastuzumab plus anti-CD47, trastuzumab $F(ab')_2$, or trastuzumab $F(ab')_2$ plus anti-CD7. Phagocytosis was determined by the percentage of $F4/80^+$ (for NSG macrophages)/$CD45^+$ (for human macrophages) and $CFSE^+$ cells. Each experiment was repeated at least twice, with at least six replicates for each treatment condition.

(Fig. 3 *B* and *C*). Notably, the cancer cell line derivatives were selected in the presence of trastuzumab and PMBCs. They are therefore relatively resistant to trastuzumab-mediated ADCC as seen in Fig. 1, but remain sensitive to trastuzumab-mediated ADCP as evidenced by the in vitro phagocytosis assays as well as the in vivo treatment.

Taken together, these results indicate that the combination treatment inhibits tumor growth more significantly than either treatment alone in the parental BT474 cell lines. And, the combination treatment's inhibition of tumorigenesis is maintained well after the combination treatment is stopped. Although the combination treatment's benefit was less pronounced in the ADCC-selected BT474 Res 1 and Res 2 lines, Fig. 3 *D* and *E* demonstrates that the combination treatment provided a survival benefit over the single-agent antibody-alone control treatments. The greater combined in vivo efficacy with anti-CD47 plus trastuzumab is therefore consistent with our hypothesis—based upon our in vitro models—that ADCP in the combination-treated group is significantly greater than in the mice receiving either PBS or single-agent anti-CD47 or anti-HER2 antibody treatments. Given the combination treatment's ability to inhibit tumorigenesis as well as increase survival rates, these results support the scientific rationale for using such combination therapy in future translational studies.

Trastuzumab and Hu5F9-G4 Combination Treatment Leads to Increased Clearance of HER2$^+$ Trastuzumab ADCC-Tolerant Tumors In Vivo. The growth inhibition we observed in our in vivo xenograft assays could be due to macrophage-mediated cell removal. Alternatively, it could be caused by reduced cellular proliferation. To tease out these possibilities, we conducted an in vivo innate clearance assay (depicted in Fig. 4*A*). Notably, we designed this assay to be performed within 4 h. This timeframe is long enough to allow us to test whether the combination treatment would lead to increased cellular removal of parental and ADCC-tolerant cells in an in vivo setting where both ADCC and ADCP were possible. This timeframe, however, is not long enough to allow for any meaningful cellular proliferation. This assay thus allowed us to focus on the former inquiry relating to whether treatment-induced tumor growth inhibition resulted from cellular removal, rather than the latter question concerning cellular proliferation. Briefly, the BT474 parental, BT474 Res 1, BT474 Res 2, and BT474 Res 3 were stained with CellTrace yellow, CFSE, CellTrace Far red, and CellTrace violet, respectively. The cell lines were then opsonized with IgG1+IgG4 (a control), Hu5F9-G4, trastuzmab, or the combination treatment. The parental and three ADCC-tolerant cell lines were then combined (based on their respective antibody treatments), intraperitoneally injected in equal starting numbers into immunocompetent

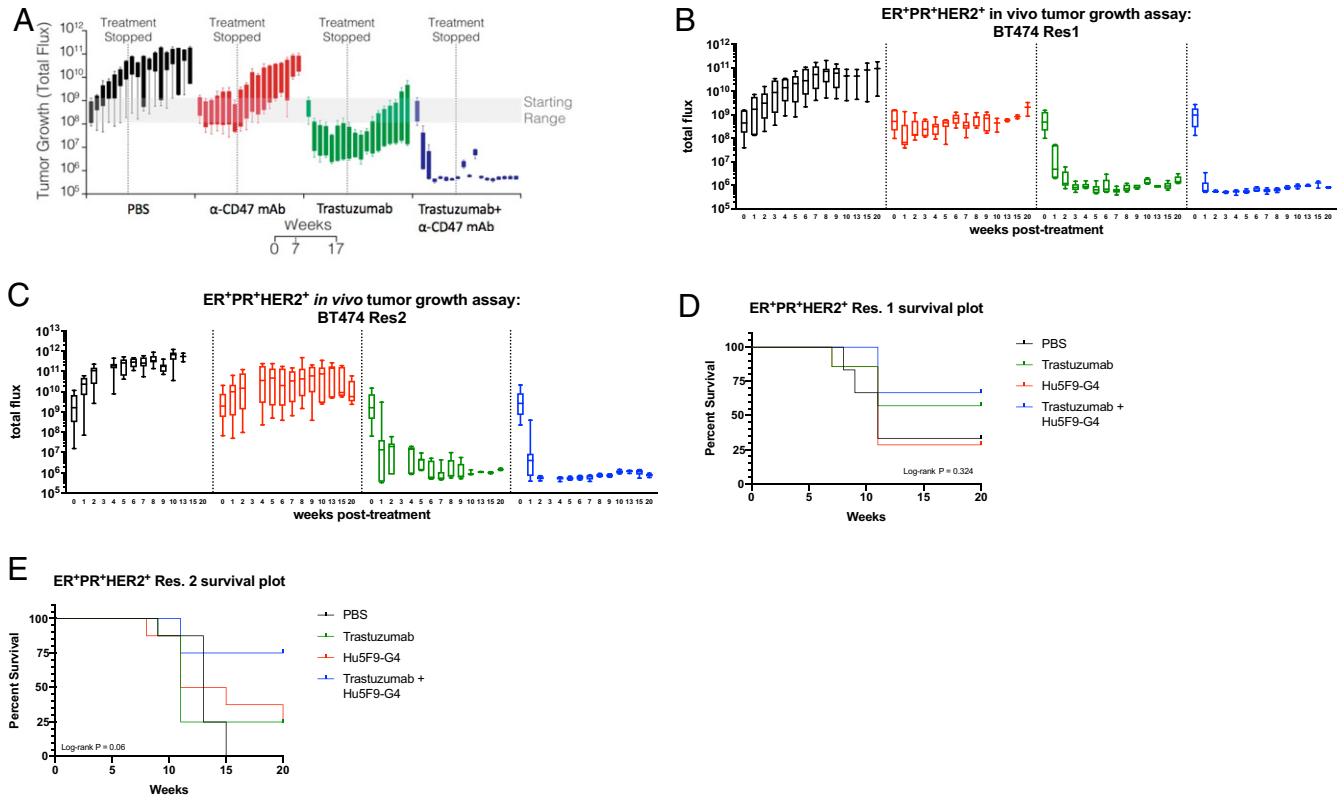

Fig. 3. HER2$^+$ BT474 trastuzumab ADCC-tolerant breast cancer cells respond to trastuzumab and CD47 blockade in vivo. (*A–C*) GFP-Luciferase BT474 parental cells or BT474 ADCC-selected lines were injected into the left mammary fat pad of female NSG mice. Twenty-five days after engraftment, mice were treated with PBS, anti-CD47, trastuzumab, or trastuzumab plus anti-CD47 for 7 wk. Tumor burden was measured using in vivo bioluminescence imaging. (*D* and *E*) Survival plots for mice harboring BT474 ADCC-tolerant tumors. In the study design for *A*, the parental cell line included 7 wk of treatment and an additional 10 wk of follow-up after the cessation of therapy (marked by the vertical line). The same experimental protocol was conducted for the evaluation of the cell lines that underwent ADCC selection.

C57BL/6 mouse, and incubated for 4 h. The cells were retrieved using an intraperitoneal lavage technique and analyzed with flow cytometry to determine the fraction of cells recovered from each of the cell lines.

As can be seen in Fig. 4*B*, the combination treatment led to a statistically significant decrease in the percentage of recovered cells versus either Hu5F9-G4 or trastuzumab treatment alone. This was true across all cell lines, parental and ADCC-tolerant (except for BT474 Res 2). This short-term in vivo Winn assay thus demonstrates that the combination of trastuzumab and magrolimab leads to rapid clearance of intraperitoneally injected cancer cells, thereby suggesting that the enhanced efficacy is due to cell removal—likely by peritoneal macrophages. The combination treatment leads to indiscriminate clearance of parental and ADCC-tolerant cell types alike, despite the relative resistance of the sublines to ADCC and other effects of trastuzumab. These results further underscore the potential utility of using the combination therapy in clinical trials, especially in the context of advanced-stage HER2$^+$ breast cancer patients whose tumors have become clinically resistant to trastuzumab. Our results suggest the combination of anti-CD47 with anti-HER2 antibody trastuzumab treatment may help resensitize refractory HER2$^+$ breast cancers to trastuzumab treatment.

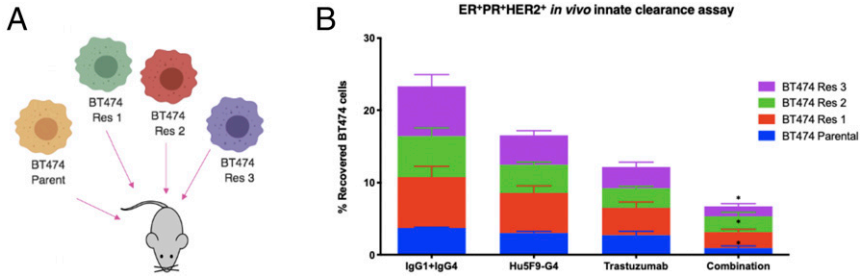

Fig. 4. Trastuzumab and Hu5F9-G4 treatment leads to increased ER$^+$PR$^+$HER2$^+$ clearance in vivo. (*A*) Schematic of experiment. BT474 parental, BT474 Res 1, BT474 Res 2, and BT474 Res 3 were stained with CellTrace yellow, CFSE, CellTrace Far red, and CellTrace violet, respectively, and opsonized with the following treatments: IgG1+IgG4; trastuzumab; Hu5F9-G4; Combination (trastuzumab plus Hu5F9-G4). The cells were intraperitoneally injected in equal proportions into an immunocompetent C57BL/6 mouse and incubated for 4 h. The cells were retrieved using an intraperitoneal lavage (Winn assay) and analyzed by flow cytometry to determine the fraction of cells remaining posttreatment. (*B*) An in vivo innate clearance assay with BT474 parental and ADCC-tolerant breast cancer lines as targets cells. The combination treatment led to fewer recovered parental and ADCC-tolerant BT474 cells than either treatment alone.

Discussion

Since the discovery of trastuzumab's therapeutic potential, attempts to improve its antitumor effects have focused on elucidating its mechanisms of action. It has become increasingly evident, however, that in addition to the effect trastuzumab has on HER2 receptor function, the immune system plays a critical role in contributing to trastuzumab's anticancer effects. Several studies have identified ADCC as a dominant immune-based mechanism of action of trastuzumab (10); we and other groups now additionally document trastuzumab's ability to mediate ADCP as a contributing mechanism of action (12–15, 17).

Despite our growing understanding of trastuzumab's mechanisms of action, most patients with advanced-stage $HER2^+$ breast cancer ultimately exhibit de novo or acquired resistance to trastuzumab therapy (38, 39). To date, the only treatment options for these patients involve therapy with other existing HER2-targeted monoclonal antibodies (e.g., pertuzumab and margetuximab), small-molecule HER2 kinase inhibitors (e.g., tucatinib, neratinib), or antibody–drug conjugates (e.g., ado-trastuzumab emtansine, trastuzumab deruxtecan) (22, 40, 41). While second- and third-line treatment regimens in some cases offer modest survival gains, most patients continue to experience disease progression leading to cancer-related mortality (23). Therefore, alternative treatment strategies are sorely needed to achieve a longer lasting antitumor response while overcoming the limitation of drug resistance. We therefore conducted the experiments presented herein to test whether we can engage an ADCP modality to eliminate breast cancer cells through trastuzumab-mediated macrophage phagocytosis, even when the cells are tolerant to trastuzumab-mediated ADCC.

Both macrophages and NK cells employ cell surface high-affinity activating FcγRs to interact with the Fc domains of anti-HER2 antibodies for their respective effector functions. Following this interaction, NK cells release killer vesicles containing at least perforin and granzymes A and B, and macrophages target cell phagocytosis and intracellular death (phagoptosis). To evaluate macrophage-mediated ADCP's role in targeting ADCC-sensitive and -tolerant breast cancer sublines following CD47 blockade, we used in vitro and in vivo studies to compare the anticancer effects of antibodies targeting HER2 alone or in combination with magrolimab. This allowed us to test and demonstrate the cooperativity of magrolimab and trastuzumab in ADCP in vitro and in vivo to enhance macrophage-mediated innate immune responses to human $HER2^+$ breast cancers.

We found that macrophage-mediated ADCP is an effective mechanism through which trastuzumab operates to eliminate $HER2^+$ breast cancer cells. Even when breast cancer cells become tolerant to ADCC killing mechanisms, there is no cross-resistance to ADCP mediated by the same antibody, as long as trastuzumab can still bind to its target HER2 epitope at the cell surface. Moreover, informed by our extensive work on the regulation of macrophage phagocytosis and the development of CD47 blockade as a cancer immunotherapy, we tested the efficacy of adding anti-CD47 to trastuzumab. We found that: 1) cells that were selected for HER2-resistance ADCC tolerance in the presence of PMBCs were still susceptible to ADCP by macrophages; 2) the addition of anti-CD47 to trastuzumab potentiated ADCP of human $HER2^+$ breast cancer cells; and 3) in a xenograft model, $HER2^+$ human xenografts generated from ADCC-selected cells responded to trastuzumab therapy in an Fc-dependent manner and in the absence of lymphocytes and NK cells (recall these studies were conducted in NSG mice). Thus, trastuzumab-sensitive and ADCC-tolerant $HER2^+$ tumors with relative resistance to ADCC ex vivo are highly susceptible to macrophage-mediated phagocytosis when treated with the combination of trastuzumab and Hu5F9-G4.

Based on our results, we propose that the addition of CD47 blockade to an anti-HER2 treatment of $HER2^+$ breast cancer may be efficacious in patients whose cancers have progressed after anti-HER2 therapy, as long as HER2 is still on the cell surface and able to bind therapeutic anti-HER2 antibodies. The combination treatment can enhance antitumor efficacy in $HER2^+$ breast cancer, regardless of whether the patient has developed clinical resistance to trastuzumab. The rationale is to add the phagocytic ADCP modality to the treatment plan to reduce the likelihood of a rapid relapse due to trastuzumab resistance. Our experience with rituximab targeting CD20 and now trastuzumab targeting HER2 suggests that for other surface proteins against which a targeted monoclonal antibody already exists, combining CD47 blockade with antitumor antibodies enhances ADCP by decreasing the "don't eat me" signals from cancer cells and increasing the "eat me" signals from tumor-targeting monoclonal antibody Fc domains. Enhancing ADCP is also important from a cancer immunotherapy vantage point, in that it has the potential to augment cross-presentation and thus activate tumor-specific anticancer responses from the adaptive immune system. The combination treatment, therefore, holds promise of both increasing tumor immunosurveillance, while decreasing cancer cell immune evasion.

Consistent with our findings, others have found that up-regulation of CD47 is an important mechanism for trastuzumab resistance in $HER2^+$ breast cancers (42). This further supports our conclusion that clinical use of the combination treatment may enhance antitumor efficacy for resistant variants as long as the tumors still express HER2 target antibody-binding epitopes.

Our results additionally provide insight into the utility of combining different treatment modalities to exploit macrophages within the tumor microenvironment for ADCP. Tumor-associated macrophages (TAMs) are a major component of the breast tumor microenvironment, often indicative of unfavorable prognosis and reduced survival (16). TAMs are well-recognized for their immunosuppressive functions and their role in promoting tumor progression. We and others have shown that blockade of the CD47-SIRPα signaling axis converts TAMs into an antitumor state, leading to enhanced phagocytosis and suppressed tumor growth (43–45). $HER2^+$ breast cancer cell overexpression of CD47, along with the high density of TAMs within the $HER2^+$ breast cancer tumor microenvironment, strongly suggests that Hu5F9-G4 enhance trastuzumab-mediated innate immune responses in $HER2^+$ breast cancer patients (46–48).

As a first step toward clinical translation of Hu5F9-G4 plus trastuzumab, it will be necessary to investigate safety studies. We would propose evaluating the pharmacokinetics and potential clinical efficacy of this combination therapy in the refractory metastatic disease setting in patients who have already been treated with FDA-approved drugs shown to have a progression-free survival or overall survival benefit (e.g., trastuzumab, pertuzumab, T-DM1, tucatinib, margetuximab, and trastuzumab deruxtecan). Once safety and proof-of-concept efficacy data are established in a refractory metastatic disease setting, if successful, we would next propose administering the combination of trastuzumab and Hu5F9-G4 to patients in earlier lines of metastatic disease treatment, then ultimately to patients with early-stage nonmetastatic $HER2^+$ breast cancer with curative intent in the neoadjuvant or adjuvant settings. The rationale behind these proposed therapeutic approaches is that engaging ADCP leads to both increased efficacy through cancer cell removal and potentially also to increased immunosurveillance of $HER2^+$ breast cancer cells via cross-presentation of tumor-specific antigens, which reduces the likelihood of tumor cell immune evasion due to trastuzumab resdistance (49). Also possible is the use of this combination in patients with metastatic disease just following cancer-free hematopoietic stem cell rescue from high-dose chemotherapy (50).

In terms of future directions, early-phase clinical trials have shown that treatment with anti–PD-1 immune checkpoint antibody pembrolizumab plus trastuzumab was effective, including durable clinical benefit, in patients with $PD\text{-}L1^+$, trastuzumab-resistant, advanced, $HER2^+$ breast cancer (51, 52). Our group has

previously shown that in addition to its role in down-regulating T cells, PD-1 also functions as an inhibitory receptor on macrophages and that PD-1 blockade can unleash macrophage phagocytosis of cancer cells (53). Therefore, the clinical response to the combination of pembrolizumab (anti–PD-1) and trastuzumab may, at least in part, be attributed to ADCP. We hypothesize that the combination treatment we propose herein may further augment ADCP when combined with immune checkpoint inhibitors targeting the PD-(L)1 axis. Also possible might be the use of this combination in patients with metastatic disease who achieve clinical complete responses just following cancer-free hematopoietic stem cell rescue from high-dose chemotherapy, when micrometastatic disease burden is at a minimum (50). In any stage of $HER2^+$ breast cancer, it may be beneficial to add CD47 blockade to anti-HER2 therapy to enhance the innate antitumor immune response. Recently published data from Deuse et al. (54) demonstrate that NK cells with up-regulated SIRPα can also be inhibited by CD47 on the surface of their target cells, further strengthening the likelihood that adding CD47 blockade can benefit patients undergoing anti–HER2 therapy, either after a relapse or as a front-line therapy. That possibility will need to be examined in the future to determine the magnitude of NK, macrophage, and if antigen cross-presentation occurs, adaptive immune T cells, as well as de novo endogenous polyclonal antibody responses to tumor cells.

Collectively, our findings present a promising therapeutic approach for the treatment of $HER2^+$ breast cancer patients whose tumors are either trastuzumab-sensitive or -resistant, as long as the epitope recognized by the therapeutic anti-HER2 antibody remains intact. Although our study uses trastuzumab as the tumor-targeting antibody, we posit that other human-engineered IgG1 tumor-targeting antibodies might yield similar therapeutic results when combined with CD47 blockade (and/or other macrophage checkpoint inhibitors), thereby offering a useful adjunct to antitumor antibody treatment.

Materials and Methods

Cell Lines. BT474 and SKBR3 parental cell lines were purchased from ATCC. BT474 and SKBR3 ADCC-resistant lines 1, 2, and 3 were generated in the laboratory of M.D.P. Briefly, cells were separately cultured in the presence of a clinically therapeutic dose (100 μg/mL) of trastuzumab. Next, these trastuzumab-treated breast cancer cell lines were cocultured with human PBMCs. Upon coculture with human PBMCs, the majority of SKBR3/BT474 breast cancer cells were eliminated. Those breast cancer cells that survived were expanded to cellular confluency. Afterward, this process of trastuzumab treatment followed by human PBMC coculture was repeated 10 times.

Mice. NSG and C57BL/6 mice were bred and maintained at the Stanford University Research Animal Facility in accordance with the Administrative Panel on Laboratory Animal Care.

Reagents, Antibodies, and Flow Cytometry Analysis. Trastuzumab was obtained from Stanford University Hospital (Genentech). Trastuzumab $F(ab')_2$ fragments were generated using standard protocols as previously described. IgG1was obtained from BioXCell. IgG4 was obtained from BioLegend. Hu5F9-G4 was obtained from Lonza. Data were acquired using a BD LSR Fortessa Analyzer and analyzed using FlowJo software.

Generation of Human PBMCs and NK Cells. Human PBMCs were isolated using a Ficoll-Paque gradient (GE Healthcare Bio-Sciences) from human blood obtained from the Stanford University Medical Center. Human NK cells were isolated using an EasySep Human NK Cell Isolation Kit (Stem Cell Technologies).

Generation of NSG BMDMs. Whole bone marrow cells were isolated from NSG mice. Monocytes were terminally differentiated into macrophages by incubating whole bone marrow in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS supplemented with 10 ng/mL recombinant murine macrophage colony stimulating factor (M-CSF; Peprotech) for 7 to 10 d and harvesting the adherent fraction.

Generation of Human PBMC-Derived Macrophages. To prepare human macrophages from PBMCs, normal human blood was obtained from the Stanford University Medical Center and PBMCs were isolated therefrom using a Ficoll-Paque gradient (GE Healthcare Biosciences). Monocytes were further isolated from the PBMC fraction by incubating PBMCs in cell culture plates for 1 h at 37 °C. Nonadherent cells were discarded by washing the cell culture plates with PBS. Adherent cells were incubated for 7 to 10 d in IMDM containing 10% human serum AB (Gemini Bio-Products) to allow for terminal differentiation of monocytes to macrophages.

ADCC Assays. BT474 and SKBR3 cells were separately labeled with 5 μM CellTrace CFSE according to the manufacturer's protocol (Thermo Fisher Scientific). Flow cytometry-based cytotoxicity assay was predicated on Edri-Brami et al. (55). For the ADCC assay, 2.5×10^3 CFSE-labeled SKBR3/BT474 cells were plated in individual wells of a 96-well ultralow attachment plate (Corning) in RPMI containing 10% heat-inactivated FBS (heat inactivation was achieved by incubating FBS at 56 °C for 30 min).

In Vitro Phagocytosis Assays. BT474 and SKBR3 cells were separately labeled with 5 μM CellTrace CFSE according to the manufacturer's protocol (Thermo Fisher Scientific). For the phagocytosis assay, 1×10^5 CFSE-labeled SKBR3/BT474 cells were then plated in each well of a 96-well ultralow attachment plate (Corning) in serum-free IMDM. Isotype antibody control, trastuzumab, anti-CD47 monoclonal antibody (Hu5F9-G4), and trastuzumab plus Hu5F9-G4 combination treatment were added to the CFSE-labeled SKBR3/BT474 cells at a concentration of 10 μg/mL. The cells and antibody treatments were incubated at 37 °C for 30 min. During the incubation period of the cells and antibody treatments, NSG and human-derived macrophages were harvested via incubation in TrypLE (ThermoFisher Scientific) for 5 min followed by gentle scraping. After the cells and antibody treatments had incubated for 30 min, 5×10^4 macrophages were added each experimental well. The macrophages and SKBR3/BT474 cells were cocultured at 37 °C for 2 h. Following the 2-h coculture, macrophages were stained with either APC-conjugated F4/80 (for NSG mouse macrophages; BioLegend) or APC-conjugated CD45 (for human macrophages; BioLegend). The SKBR3/BT474 plus macrophage cocultures were subsequently analyzed using a BD LSR Fortessa Analyzer. The percent phagocytosis was calculated as the percentage of GFP^+ SKBR3/BT474 cells contained within APC^+ macrophages (i.e., double-positive cells).

In Vivo Tumor Growth Assays. Lentiviral production of a pCDH-CMV-EF1-puro construct (Systems Bioscience) containing a ubiquitin promoter driving the expression of a fusion protein containing the Luc2 (pgl4) luciferase gene (Promega) and the eGFP gene (Becton Dickenson) was carried out using standard protocols. BT474 parental and ADCC-resistant cells were transduced with lentivirus. GFP^+/$Luciferase^+$ BT474 cells were suspended in RPMI containing 25% Matrigel (BD Biosciences) and 1×10^5 cells were implanted into the left mammary fat pad of 4- to 8-wk-old NSG female mice. Bioluminescent activity was visualized in vivo after D-luciferin injection (Biosynth) on an IVIS Spectrum (Caliper Life Sciences) instrument and quantified using Image 4.0 software. Total flux (photons/second) values were obtained from the mammary fat pad region. Mice were matched based on total flux 25 d after cell engraftment and subsequently treated weekly via intraperitoneal injections of the following treatments: trastuzumab was administered via intraperitoneal injection at 100 μg, once a week; Hu5F9-G4 was administered via intraperitoneal injection at 250 μg, every other day; PBS was administered via intraperitoneal injection at 100 μL, once a week. Treatments were stopped after 7 wk. Mice were imaged every week and differences in tumor growth were assessed using Student's *t* test.

In Vivo Innate Clearance Assay. BT474 cells were separately labeled with the following according to the manufacturer's protocol (Thermo Fisher Scientific): BT474 parental cells were labeled with 5 μM CellTrace yellow, BT474 Res 1 cells were labeled with 5 μM CellTrace CFSE, BT474 Res 2 cells were labeled with 1 μM CellTrace Far red, BT474 Res 3 cells were labeled with 5 μM CellTrace violet. The four differently labeled cell lines were subsequently mixed in a 1:1 ratio and incubated with the following antibody treatments for 20 min at 37 °C: PBS, IgG1+IgG4 isotype control (10 μg/mL, each), trastuzumab (10 μg/mL), Hu-5F9-G4 (10 μg/mL), trastuzumab plus Hu-5F9-G4 (10 μg/mL, each). The labeled cells were subsequently injected into the intraperitoneal space of C57BL/6 female mice. All injections were performed in 0.5 mL in PBS, 1×10^6 of each BT474 cell line per mouse. Four hours after the injections, mice were killed and the peritoneums were washed out with PBS. The peritoneal lavage was subsequently analyzed by flow cytometry.

Data Availability. All study data are included in the article and *SI Appendix*.

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