The HER2-specific monoclonal antibody (mAb), Trastuzumab, has been the mainstay of therapy for HER2+ breast cancers (BC) for ~20 years. However, its therapeutic mechanism of action (MOA) remains unclear, with antitumor responses to Trastuzumab remaining heterologous and metastatic HER2+ BC remaining incurable. Consequently, understanding its MOA could enable rational strategies to enhance its efficacy. Using both novel murine and human versions of Trastuzumab, we found its antitumor activity dependent on Fcγ-Receptor stimulation of tumor-associated-macrophages (TAM) and Antibody-Dependent-Cellular-Phagocytosis (ADCP), but not cytotoxicity (ADCC). Trastuzumab also stimulated TAM activation and expansion, but did not require adaptive immunity, natural killer cells, and/or neutrophils. Moreover, inhibition of the innate immune ADCP checkpoint, CD47, significantly enhanced Trastuzumab-mediated ADCP, TAM expansion and activation, resulting in the emergence of a unique hyper-phagocytic macrophage population, improved antitumor responses and prolonged survival. In addition, we found tumor-associated CD47 expression was inversely associated with survival in HER2+ BC patients and that human HER2+ BC xenografts treated with Trastuzumab+CD47 inhibition underwent complete tumor regression. Collectively, our study identifies Trastuzumab-mediated ADCP as a significant antitumor MOA that may be clinically enabled by CD47 blockade to augment therapeutic efficacy.
CD47 blockade augmentation of Trastuzumab antitumor efficacy dependent upon antibody-dependent-cellular-phagocytosis

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Abstract

The HER2-specific monoclonal antibody (mAb), Trastuzumab, has been the mainstay of therapy for HER2+ breast cancers (BC) for ~20 years. However, its therapeutic mechanism of action (MOA) remains unclear, with antitumor responses to Trastuzumab remaining heterologous and metastatic HER2+ BC remaining incurable. Consequently, understanding its MOA could enable rational strategies to enhance its efficacy. Using both novel murine and human versions of Trastuzumab, we found its antitumor activity dependent on Fcγ-Receptor stimulation of tumor-associated-macrophages (TAM) and Antibody-Dependent-Cellular-Phagocytosis (ADCP), but not cytotoxicity (ADCC). Trastuzumab also stimulated TAM activation and expansion, but did not require adaptive immunity, natural killer cells, and/or neutrophils. Moreover, inhibition of the innate immune ADCP checkpoint, CD47, significantly enhanced Trastuzumab-mediated ADCP, TAM expansion and activation, resulting in the emergence of a unique hyper-phagocytic macrophage population, improved antitumor responses and prolonged survival. In addition, we found tumor-associated CD47 expression was inversely associated with survival in HER2+ BC patients and that human HER2+ BC xenografts treated with Trastuzumab+CD47 inhibition underwent complete tumor regression. Collectively, our study identifies Trastuzumab-mediated ADCP as a significant antitumor MOA that may be clinically enabled by CD47 blockade to augment therapeutic efficacy.
Introduction:

Approximately 20% of Breast Cancer (BC) overexpress HER2, recognized as an oncogenic driver of an aggressive cancer phenotype with a poor prognosis (1, 2). Monoclonal antibodies (mAbs) targeting HER2 were developed in the 1980s to inhibit HER2 oncogenic signaling, leading to the clinical development and regulatory approval of Trastuzumab in 1998 for metastatic HER2 overexpressed BC, followed by clinical trials of Trastuzumab use in the adjuvant setting. Following its approval, additional HER2 targeting mAbs have also been generated to improve outcomes (3, 4). However, the clinical benefit associated with HER2 mAb therapies in patients with HER2 overexpressing BC remains heterologous and metastatic HER2+ BC remains incurable (5, 6). Consequently, mechanistic studies of the antitumor mechanism(s) of action (MOA) of Trastuzumab and its resistance remain critical, not only to improve outcomes in patients with HER2+ BC, but also to gain insight into mechanisms that would extend mAb therapies to other types of cancers.

While suppression of HER2 signaling was a primary focus of early mechanistic studies, subsequent studies also focused on the role of immunity in mediating the antitumor effects of Trastuzumab (7). In particular, studies have shown the interaction of anti-HER2 antibodies with Fcγ-receptors (FCGR) expressed on innate immune cells such as macrophages, monocytes, natural killer (NK) cells and dendritic cells may be involved in its therapeutic activity (8, 9). The consequences of crosstalk with FCGR-bearing immune cells (8-10) are supported by the clinical observation that some host FCGR polymorphisms are associated with improved clinical outcome in HER2+ BC patients treated with Trastuzumab (11). Specifically, several studies have suggested the importance of these receptors in mediating Antibody-Dependent-Cellular-Cytotoxicity (ADCC), through NK cells or neutrophils for Trastuzumab efficacy (8, 9, 12-14). However, other studies have suggested the importance of adaptive immunity in mediating Trastuzumab efficacy, indicating that T cells may be critical for its antitumor MOA (8, 15).
While multiple MOAs involving either innate or adaptive immunity are possible, an underexplored mechanism is through mAb engagement of FCGRs to stimulate macrophage-mediated Antibody-Dependent-Cellular-Phagocytosis (ADCP). Inconsistent reports about the role of ADCP exist, with a recent study demonstrating the ability of Trastuzumab to elicit ADCP (16), while another study suggests that Trastuzumab-mediated ADCP triggers macrophage immunosuppression in HER2+ BC (17). These disparate results may be partially attributed to the use of a wide range of tumor models (many not specifically driven by active HER2-signaling), as well as the use of different HER2-specific mAb clones of varied isotypes, which can elicit a range of different responses from various FCGRs (18, 19). Thus, the immunologic basis for the activity of Trastuzumab remains inconclusive, but could be effectively investigated through the development and use of appropriate HER2 targeting mAbs and model systems.

In this study, we developed and utilized fully murinized Trastuzumab mAbs (clone 4D5) with isotypes of different activating-to-inhibitory ratio (A/I ratio, calculated by dividing the affinity of a specific IgG isotype for an activating receptor by the affinity for the inhibitory receptor) (19), as well as clinical-grade Trastuzumab, to determine the MOA for Trastuzumab antitumor efficacy. These mAbs were tested in multiple settings to interrogate ADCC and ADCP, as well as the impact on HER2 signaling and complement-dependent cytotoxicity (CDC). To determine the antitumor efficacy of these HER2 mAbs, we employed orthotopic implantation of HER2+ murine BC cells (transformed using a constitutively active isoform of human HER2) in immunocompetent models, as well as Fcgr−, immune-deficient backgrounds, and human HER2+ BC xenograft models. In addition, we utilized a novel transgenic HER2+ BC model driven by an oncogenic isoform of human HER2 to simulate an endogenous mammary tumor immune microenvironment (20, 21). Collectively, these studies revealed an essential role for tumor-associated macrophages (TAMs) in mediating the therapeutic activity of Trastuzumab through promoting ADCP of HER2+ tumor cells without evidence for significant induction of
adaptive T cell responses against HER2. We also observed that this effect was subverted by innate mechanisms of immunosuppression in the tumor microenvironment that limit macrophage ADCP.

Previous studies have demonstrated that ADCP is principally regulated by anti-phagocytic “don’t eat me” signals that are amplified in many cancers (22, 23). Chief among these is CD47, which has been shown to be highly expressed in different cancers and functions to suppress phagocytosis through binding to and triggering signaling of macrophage SIRPα (23, 24). Notably, CD47 expression is also upregulated in BC (25). As a potential means to subvert innate immune regulation and enhance ADCP and possibly alter the macrophage phenotype in HER2+ BC, we also targeted the CD47-SIRPα innate immune checkpoint. In this study, we demonstrate that TAM ADCP can be significantly enhanced by blocking the CD47-SIRPα checkpoint to enable Trastuzumab-mediated macrophage phagocytosis of HER2+ tumor cells. Collectively, these findings support the importance of the ADCP MOA, as well as suggest the therapeutic potential of utilizing CD47-SIRPα checkpoint blockade in combination with Trastuzumab in HER2+ BC and potentially in other resistant HER2+ cancers (i.e. gastric, bladder, etc) (26).

Results:

Generation of murine Trastuzumab (4D5) and its antitumor dependence on ADCP by tumor-associated macrophages (TAMs).

Trastuzumab was based on a HER2-specific mouse IgG1 monoclonal antibody (4D5-IgG1, low A/I ratio), which was subsequently ‘humanized’ to a human IgG1 isotype (high A/I ratio) that allows for superior activation of Fc receptors (27). Thus, to accurately study the function of Trastuzumab in an immunocompetent mouse model, we constructed a murine 4D5 monoclonal
antibody, but using the IgG2A isotype (4D5-IgG2A, high A/I ratio, Figure 1A) to better
approximate an Fc-receptor activating ‘murine version’ of Trastuzumab (18, 19, 28).

Unsurprisingly, we found that 4D5-IgG2A HER2 binding is equivalent to Trastuzumab (Figure
S1A). This allowed us to interrogate the importance of the HER2-antibody Fc region as well as
minimize the humoral immune responses against Trastuzumab, a human antibody, when
administered into a murine host (Figure S1B).

To test the antitumor efficacy of 4D5-IgG2A, we began by interrogating its impact on
oncogenic HER2 signaling. As HER2 is weakly transformative in most cell lines, we employed a
highly oncogenic isoform of human HER2 (HER2Δ16) that constitutively dimerizes to create a
transformed BALB/c mammary cell line dependent upon HER2 signaling (21). In studies using
HER2Δ16, we observed that both 4D5-IgG2A and Trastuzumab could suppress HER2 signaling
(although not as potent as Lapatinib (Figure S1C-D), but not significantly enough to prevent
tumor cell growth in vitro (Figure S1E). This is in line with several recent studies, suggesting that
the impact of Trastuzumab is mediated through immune based mechanisms (29, 30). Using
transformed MM3MG-HER2Δ16 as a model for HER2-driven BC growth in vivo, we next
implanted these cells in the mammary fat pad of immunocompetent BALB/c mice. Tumor
bearing mice were treated weekly with 4D5-IgG2A or clinical-grade Trastuzumab to determine if
they could suppress tumor growth in an immunocompetent context. We found that both 4D5-
IgG2A and Trastuzumab significantly suppressed HER2+ BC growth demonstrating that murine
IgG2A was capable of significant antitumor activity (Figure 1B). Notably, we observed that 4D5-
IgG2A and Trastuzumab significantly increased the levels of tumor-associated macrophages
(TAMs) (Figure 1C), but did not increase other immune infiltrates such as NK cells and T cells
(Figure S2A). Furthermore, using IFN-γ ELISPOT assays we found 4D5-IgG2A and
Trastuzumab treatment had no effect on systemic adaptive T cells responses against human
HER2 epitopes (Figure S2B-C). In agreement with published reports (12), we observed NK cell-
mediated ADCC was increased by 4D5 or Trastuzumab treatment in co-culture systems (Figure S2D). To determine if NK cells and/or adaptive immune cells mediate antitumor immunity in vivo, we next tested HER2 mAb ability to suppress HER2+ BC growth in T cell, B cell, and NK cell deficient SCID-Beige mice. Contrary to published reports (8), we found surprisingly no change in its antitumor efficacy (Figure 1D), suggesting the roles of adaptive immune and NK cells are minimal in Trastuzumab/4D5 action in our in vivo model system. As neutrophil levels (LY6G+ CD11b+) were suppressed (Figure S2A) and previous studies have also implicated neutrophils in Trastuzumab-mediated immunity (14), we next depleted neutrophils using anti-LY6G in SCID-Beige studies (Figure S3D-E), but did not observe any difference in antitumor efficacy (Figure 1E). To investigate the possible role of complement dependent cytotoxicity (CDC), we performed CDC assays in vitro and found that neither 4D5-IgG2A nor Trastuzumab were able to induce CDC in comparison to polyclonal HER2 Abs, in line with other studies of Trastuzumab (31) (Figure S2E).

The increase of TAMs levels after treatment suggested a functional role in Trastuzumab antitumor immunity. We therefore implemented several strategies to deplete macrophages in our SCID-beige HER2+ BC model. Using a prolonged anti-CSF1R antibody injection strategy (32), we achieved significant reduction of TAMs and which also limited TAM increase in 4D5-treated tumors (Figure 1F). Importantly, the reduction of TAM levels resulted in a significant decrease of HER2 mAb therapeutic efficacy (Figure 1G). We also utilized clodronate liposome injection to deplete macrophages in this model, but found we could only readily deplete macrophages in systemic circulation and not those in the tumor (Figure S3A). Interestingly, this depletion had no effect on HER2 mAb therapy (Figure S3B), suggesting that macrophages in the mammary tumor are the major antitumor effectors. To explore the efficacy of macrophage-mediated HER2-specific antitumor activity, we established a BMDM co-culture system to investigate the relative ADCC and ADCP activity mediated by 4D5-IgG2A and Trastuzumab.
Using Latrunculin A, an inhibitor of actin polymerization and therefore blocking phagocytosis of immune complexes (34), we revealed the dominant antitumor activity of HER2 mAbs mediated by macrophages is through ADCP (Figure 1H). Concanamycin A, an V-ATPase inhibitor reported to also inhibit perforin and cytotoxicity (35), had no effect on HER2 mAb activities. Collectively, these results suggested that Trastuzumab therapy modifies the tumor microenvironment by promoting TAM expansion, and that the dominant mechanism of action by Trastuzumab is mediated by ADCP of HER2+ tumor cells by macrophages.

The ADCP activity of 4D5 requires the engagement with Fcγ-Receptors and is isotype dependent

To further validate the mechanism of ADCP by 4D5-IgG2A treatment, we utilized Fcer1g−/− animals to test the requirement for Fcγ-receptor (FCGR) engagement on phagocytic immune cells. Using macrophages cultured from Fcer1g−/− and control mice, in vitro ADCP assays revealed that FCGRs on macrophages are critical for 4D5-induced ADCP of HER2+ BC (Figure 2A). Accordingly, we found the in vivo antitumor efficacy of 4D5-IgG2A therapy are mostly ablated in Fcer1g−/− mice (Figure 2B). Importantly, FCGR expression was also required for macrophage expansion by 4D5-IgG2A in the tumor microenvironment (Figure 2C).

These data demonstrated that HER2 mAb engagement with macrophage FCGRs is required for ADCP activity. Among the four mouse FCGRs, FCGR4 is the predominant FCGR mediating macrophage ADCP, plays a central role for mouse IgG2A activity, and has also been shown to exhibit the strongest binding affinity for Trastuzumab (16, 36-38). To determine the impact of HER2-mAb isotype on FCGR4 engagement and antitumor efficacy, we compared the efficacy of 4D5-IgG1 (low A/I ratio) and compared its antitumor efficacy with 4D5-IgG2A (Figure 1A). We found that unlike 4D5-IgG2A which elicited significant antitumor effects in vivo and ADCP in
vitro, 4D5-IgG1 has no effect against HER2+ BC in vivo (Figure 2D) and was inferior in promoting tumor ADCP by BMDM (Figure 2E). To determine their impact on FCGR4 and other activating FCGRs directly, we developed a mouse FCGR activation and signaling to NFAT-luciferase reporter system based on published methods (39). In agreement with established literatures on mouse IgG subclasses and FCGR biology (18, 19, 40), we found that 4D5-IgG2A engages with all three activating FCGRs, whereas 4D5-IgG1 only weakly activates FCGR3 (Figure 2F-2H). Additionally, mouse FCGR1 and FCGR4 have strong human-murine cross-reactivity with clinical grade human Trastuzumab (human IgG1 isotype) as reported before (40), thus potentially explaining its in vivo efficacy in mice. Collectively, these results illustrate that HER2 mAb’s antitumor activity requires the successful engagement and activation of Fcγ-receptors on macrophages to induce ADCP.

**CD47 Blockade increased therapeutic efficacy of 4D5 and augments tumor-associated macrophage expansion and phagocytosis.**

Our findings strongly supported an ADCP MOA for Trastuzumab antitumor efficacy, which suggests strategies to enhance ADCP may be synergistic with Trastuzumab therapies. As previous studies have demonstrated that blockade of CD47-SIRPα can enhance mAb therapeutic efficacy, we investigated if targeting this ADCP-specific axis would enhance HER2 mAb ADCP without affecting ADCC activity. To begin our investigation, we documented the elevated expression of Cd47 in our MM3MG-HER2Δ16 tumors and generated CD47-KO cells (Figure S4A) to determine the contribution of this axis to ADCP and ADCC in vitro. We observed that CD47-KO tumor cells exhibited generally enhanced ADCP that was significantly enhanced by HER2 mAbs, but had no effect on ADCC (Figure 3A). Additionally, we found that 4D5-mediated ADCP of CD47-KO tumors elicited the expression of pro-inflammatory cytokines and chemokines by macrophages (e.g. IL6, TNFα, CCL3, CCL4 etc.), presumably due to enhanced...
ADCP activity (Figure 3B and Figure S5). This demonstrates that 4D5-IgG2A alone triggers ADCP but was insufficient to stimulate significant pro-inflammatory activation within macrophages. However, upon blockade of the CD47 negative regulatory axis, ADCP and an associated pro-inflammatory phenotype was significantly enhanced in macrophages.

As CD47 directly altered ADCP and macrophage activation in vitro, we next evaluated the impact of CD47-KO expression on tumor growth and HER2 mAb therapy in vivo. We found that CD47-KO HER2+ BC cells showed a delayed growth when implanted into mice, and were significantly more susceptible to 4D5-IgG2A inhibition (Figure 3C). Furthermore, we found significantly elevated TAM levels in CD47-KO tumors compared to the control tumors after 4D5-IgG2A treatment (Figure 3D). In a reciprocal approach, we overexpressed Cd47 in the tumor cells (Figure S4B) and found this increased tumor resistance to 4D5-IgG2A therapy (Figure 3E) and prevented TAMs increase (Figure 3F). These two genetic approaches validated the role of CD47 in suppressing Trastuzumab ADCP-mediated antitumor activity, and suggest blockade of CD47 could unleash the full potential of Trastuzumab therapeutic efficacy by altering macrophage activation and expansion.

As recent studies have suggested CD47 blockade antibodies can elicit clinical responses (41), we next wanted to determine if CD47 blockade may enhance Trastuzumab efficacy. Thus, we combined 4D5-IgG2A mAb with CD47 blockade antibody MIAP410 in immunocompetent mice bearing the MM3MG-HER2Δ16 tumors. While 4D5-IgG2A and CD47 blockade monotherapies both showed therapeutic efficacy, their combination significantly suppressed tumor growth more effectively than either 4D5-IgG2A or CD47 alone and also further increased TAM levels (Figure 4A and 4B and S7). In contrast, we observed that levels of other infiltrating immune cell types, except for regulatory T cells, were not significantly increased by weekly treatment of 4D5-IgG2A with CD47 blockade (Figure S6A). As regulatory T cells were altered, we speculated that adaptive immune responses could also play a role in these enhanced
responses. To explore the impact of adaptive immunity in the context of CD47 blockade, we repeated our in vivo experiments in adaptive immune-deficient SCID-Beige mice (Figure 4C). As before, we observed a strong combinatorial effect between HER2 mAb and CD47 blockade, suggesting adaptive immunity and NK cells were not essential to the enhanced response with this combination therapy. Also as before, we found that CD47 blockade with 4D5-IgG2A further increased TAM levels (Figure 4D), suggesting that relieving the CD47 checkpoint specifically promotes macrophage expansion and phagocytosis in tumors.

In order to directly demonstrate tumor ADCP by endogenous macrophages in the tumor microenvironment, we labeled MM3MG-HER2Δ16 tumor cells with DiD dye (a carbocyanine membrane-binding probe) prior to implantation, a strategy to detect phagocytosis of labeled target cells in vivo (42). When the tumors reached a volume of ~1000 mm³, we treated the animals with 4D5-IgG2A antibody or in combination with CD47 blockade (Figure 4E). FACS analysis showed increased phagocytosis of labeled tumor cells by TAMs in 4D5-IgG2A treated animals (Figure 4F), directly demonstrating 4D5-IgG2A treatment promotes ADCP of HER2+ tumor cells in vivo. Furthermore, we found the addition of CD47 blockade further increased ADCP of labeled tumor cells by TAMs (Figure 4F). As expected, this therapeutic mechanism requires the engagement with FCGRs on macrophages, since 4D5+αCD47 induced ADCP of tumor cells in vivo was completely abolished in Fcer1g-KO mice (Figure 4G). In sum, these studies demonstrate that HER2 mAb stimulates ADCP from endogenous TAMs against HER2+ BC, which can be boosted via combination with CD47 blockade therapy.

CD47 blockade synergizes 4D5 therapeutic activity in a transgenic HER2+ breast cancer mouse model
Having demonstrated efficacy in an orthotopic model of HER2+ BC, we wanted to extend our study using a spontaneous model of HER2+ BC that approximates a late stage HER2+ BC (where HER2 mAbs are not highly effective) (43). Analogous to a clinical trial (Figure 5A), the individual animals with palpable breast tumors (~200 mm³) were enrolled in a specific treatment group. We found that mice in the 4D5-IgG2A monotherapy treatment group had a significant increase in survival time and delayed tumor growth, whereas CD47 blockade monotherapy had no significant effect compared to the control group (Figure 5B and 5C). Strikingly, combination therapy of 4D5-IgG2A with CD47 blockade resulted in a further prolonged survival rate and delayed tumor growth compared to 4D5 monotherapy, suggesting that this combination may be efficacious in advanced HER2+ BCs. To determine if these therapies again alter the immune infiltrates, we analyzed the composition of the tumor microenvironment by flow cytometry. As before, we found an increase in TAMs within the 4D5-IgG2A monotherapy group, whereas the combination therapy group showed an even higher increase (Figure 5D). Additionally, we also observed a slight reduction of T cell infiltration and neutrophil levels (Figure S6B).

**Single-cell transcriptome analysis of TAMs in HER2+ BC after 4D5 with CD47 blockade combination therapy**

To further determine if macrophages were differentially activated, we performed single-cell RNA sequencing on dissociated tumors from the HER2 transgenic mice. These studies confirmed the increase of macrophages upon 4D5-IgG2A plus αCD47 treatment and also revealed the emergence of a distinct group of macrophages (that we termed “Phag MΦ” cluster) that are phenotypically distinct from the resident macrophage clusters (i.e. “M1 MΦ” and “M2 MΦ” clusters) (Figure 6A and 6B). Notably, we found that this Phag MΦ cluster contained large quantities of human HER2 RNA and other tumor specific transcripts (such as Epcam and Cyto-keratins), indicating that they have actively phagocytosed tumor cells. This cluster was
expanded by 4D5-IgG2A treatment and increased further by combination 4D5+CD47 mAbs
treatment (Figure 6B and Table 1). In agreement with our FACS analysis, the level of total
macrophages were significantly increased while T cell and neutrophil levels were reduced after
4D5 or combination therapy (Figure 6B and Table 1). Interestingly, the frequency of cytotoxic
gene expression (Ifng and Gzmb) among CD8+ T cells were increased following treatments
(Figure 6 and Table S1).

Using differential gene expression analysis, we first assessed the impact of our treatments on
the M1-like and M2-like macrophage clusters in comparison to control (Figure 7A and 7B). Of
note, these two macrophage clusters do not demonstrate evidence for hyper-phagocytosis of
tumor cells at this time point of analysis, as evidenced by their lack of tumor marker uptake
(Figure 6B). Gene expression data revealed our treatments promoted macrophage polarization
into a pro-inflammatory antitumor phenotype, as evidenced by an increase in genes involved in
interferon, inflammatory cytokines, chemokines and TLR pathways (Figure 7A and 7B).
Accordingly, these changes were the most significant with combination therapy, and also more
strongly observed in the M1-like MΦ cluster.

In contrast, the Phag MΦ cluster (predominantly presence in the combination treatment group)
have surprisingly increased expression of gene signatures for wound-repair (e.g.
Thrombospondins and Tenascins), ECM remodeling (e.g. Collagens and MMPs), growth factors
(e.g. IGF1, TGFβ and EGF) and anti-inflammatory genes (e.g. IL4, IL13, IL1r) compared to the
other two MΦ clusters (Figure 7C). This is also accompanied by decreased expression of genes
for pro-inflammatory cytokines/chemokines, phagocytosis/opsonization, and antigen
presentation (Figure 7C).

These scRNAseq analyses revealed that while Trastuzumab with CD47 blockade polarizes
macrophages into an antitumor phenotype and greatly increases tumor phagocytosis, prolonged
treatment and continuous tumor hyper-phagocytosis may also trigger a transcriptional switch in
TAMs for repair of ADCP-induced tissue damage. Thus, while these studies demonstrate the antitumor efficacy of Trastuzumab+CD47 blockade, it also suggest that prolonging this process can trigger a wound healing response in macrophages that could have pro-tumor and/or immunosuppressive functions (44-46).

**Human CD47 gene expression is a prognostic factor in HER2+ breast cancer and limits the therapeutic activity of Trastuzumab.**

As all of our investigations had been performed on different murine HER2+ BC models, we also wanted to determine if ADCP activity of Trastuzumab can be seen in human HER2+ BC and if CD47 could likewise limit its antitumor efficacy. Based on our findings, we hypothesized that CD47 expression may allow for resistance and reduced survival of HER2+ BC patients undergoing Trastuzumab therapies. To investigate this hypothesis, we utilized the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) gene expression dataset (47) and stratified breast cancer patients of different molecular subtypes into “CD47 high” and “CD47 low” groups based on optimum threshold. This analysis revealed that CD47 gene expression associates with lower patient overall survival (Figure 8A) and was most significant in the HER2+ molecular subtype compared to TNBC or ER+ subtypes (Figure 8B). This suggests that CD47 signaling may be an important resistance mechanism for HER2+ breast cancer and Trastuzumab therapy.

We next investigated whether human CD47 limits the ADCP effect of Trastuzumab against amplified HER2+ human BC cells. To address this in vitro, we first generated CD47-KO KPL-4 (HER2+ BC) cells (Figure S4C) and compared them to controls after Trastuzumab treatment in ADCP experiments using human PBMC derived macrophages. As in mouse studies, we found loss of human CD47 in tumor cells increased their susceptibility to ADCP elicited by
Trastuzumab (Figure 8C). To determine if this antitumor effect also occurs in vivo against human HER2+ BC cells, we implanted KPL-4 control and CD47-KO cell lines into SCID-beige mice (which contain a mouse SIRPα that can bind to human CD47 (48)) and treated with clinical grade Trastuzumab. As before, we saw a strong effect from Trastuzumab treatment that was significantly enhanced with CD47-KO, resulting in tumors being completely eliminated (Figure 8D). In Trastuzumab-treated mice, we again found a significant increase of TAMs (Figure 8E) and an upregulation of pro-inflammatory genes (Figure 8F) as seen in the murine tumor model. Unfortunately, the complete regression of CD47KO+Trastuzumab tumors precluded any further analysis of these tumors. Collectively, these studies suggest that the dominant antitumor mechanism of Trastuzumab therapy is through ADCP of HER2+ tumor cells, which can be substantially impaired through the CD47-SIRPα axis. This suggests that combinatorial therapy with CD47 blockade could be beneficial in patients with Trastuzumab resistance.

Discussion:

Even since the demonstration of clinical benefit provided by therapeutic HER2 specific mAbs to patients with HER2 overexpressing BC, the mechanism of action for the therapeutic HER2 mAb, Trastuzumab has been the subject of numerous studies. Some reports suggest that Trastuzumab may both block oncogenic HER2 signaling as well as inducing ADCC (7, 49, 50). Using reflective murine versions of clinically approved HER2 specific mAb Trastuzumab, our in vitro studies confirmed these reported MOAs, specifically blockade of HER2 signaling and Trastuzumab-mediated ADCC by NK cells. In contrast, the in vivo antitumor mechanisms of Trastuzumab/4D5 remain less conclusive, with early studies suggesting the importance of signal blockade (51, 52), and subsequent studies demonstrating the direct involvement of ADCC eliciting FcR-expressing cells (10) (such as neutrophils and NK cells), and more recent studies highlighting the importance of adaptive immunity) (8, 15). Notably, few studies have examined
Trastuzumab-mediated ADCP with a single study documenting the ability of Trastuzumab to elicit ADCP in vivo (16), while another study suggested that Trastuzumab-mediated ADCP from tumor-associated macrophages (TAMs) is immunosuppressive (17). Consequently, our novel models and agents provided a reliable platform and opportunity to interrogate the in vivo antitumor mechanism of HER2 specific mAbs against HER2 driven BC.

In this study using multiple models of human HER2 expressing BC, i.e. MM3MG-HER2Δ16, KPL-4 and an endogenous transgenic HER2+ BC model that is tolerant to human HER2, and using the murine version of Trastuzumab with the functionally equivalent mouse isotype (4D5-IgG2A), we demonstrate that macrophages are the major effectors carrying out the antitumor immunity of Trastuzumab therapy through antibody-dependent-cellular-phagocytosis (ADCP).

Although TAMs have been shown to promote tumor progression, it is known that they also retain their Fc-dependent antitumor function when induced by targeted therapies (i.e. monoclonal antibodies) (53, 54). Our conclusion about the therapeutic impact of TAMs is supported by the following findings: (1) the therapeutic effect of Trastuzumab is equivalent in wild type and in SCID-beige mice and does not alter systemic HER2-specific adaptive immunity and T cell/NK cell infiltration in tumors, indicating adaptive immunity and NK cells are not necessary immune cells to mediate antitumor effects; (2) The depletion of macrophages but not neutrophils had a significant negative effect on Trastuzumab efficacy, (3) Trastuzumab treatment greatly and consistently increased TAMs frequency; (4) Trastuzumab treatment induced ADCP of HER2+ tumor cells in vitro and in vivo in a Fc-receptor dependent fashion; (5) Blocking of the innate immune ADCP CD47-SIRP1α regulatory axis significantly enhanced Trastuzumab therapeutic outcomes and also increased ADCP of tumor cells; (6) Trastuzumab combination with CD47 blockade induced TAMs into a highly phagocytic, immune-stimulatory and antitumor phenotype but also produced a wound-healing, immune-regulatory group of TAMs after prolonged tumor phagocytosis.
Our study provides insight on the potential of utilizing TAMs as a potent mediator of innate antitumor immunity that can be further exploited. It was initially believed that macrophages were present in high numbers in solid tumors as a mechanism of rejection. However, it soon became clear that TAMs are typically unable to induce an effective antitumor response in the immunosuppressive tumor microenvironment (55). Furthermore, high TAMs infiltration levels are often associated with poor patient prognosis in breast, lung, prostate, liver, thyroid, pancreas, kidney and many other solid cancer malignancies (56). Indeed, studies have shown that immunosuppressive TAMs can support tumor development by promoting angiogenesis, tissue invasion, metastasis and suppressing tumor attack by NK and CTL cells (57). In contrast, TAMs in colorectal cancer have a more activated, immune-stimulatory phenotype and interestingly, high TAM density in colorectal cancer correlates with increased patient survival, (54, 58).

Nonetheless, TAMs in multiple histologic types of tumors retain their expression of Fcγ-receptors and increasing evidence suggests mAbs can phenotypically modify immunosuppressive TAMs towards an antitumor phenotype (53, 54, 59). As such, the manipulation of TAMs, potentially through a tumor targeting mAb (e.g. Trastuzumab) or targeting of regulatory axis receptors (e.g. CD47/SIRPα), are promising therapeutic approaches for multiple types of cancer.

While previous studies (8, 9) have documented the involvement of T cell immunity in mediating HER2 mAbs efficacy, we were unable to detect a significant enhancement of adaptive T cell responses with Trastuzumab monotherapy in either our orthotopic or HER2-tolerant endogenous models of HER2+ BC. This may be due to the nature of our tumor models, the timing of our analysis, or the specific mAb utilized. In our immunocompetent in vivo studies, we utilized both murine and human HER2 mAbs similar to Trastuzumab (isotypes with a high A/I ratio), as well as both human HER2 transformed cells and an endogenous mouse model of HER2+ BC. Previous studies (8, 9, 17) have utilized rat neu expressing ErbB2 models, non-
HER2 transformed cells, and/or alternate Ab isotypes (mouse IgG1 with a low A/I ratio), which may account for a lack of ADCP activity and alteration of immunogenicity. Of note, a recent study using 4D5 antibody containing mouse IgG1 isotype reported that HER2 mAb elicited macrophage ADCP is an immunosuppressive mechanism (17). Given that the mouse IgG1 subclass strongly activates inhibitory FCGR signaling on effector cells (low A/I ratio) and therefore being very different from Trastuzumab (human IgG1, high A/I ratio) (18, 19, 40), this emphasizes the need of using functionally equivalent mouse isotypes in translational studies to accurately model human antibody therapy. Nevertheless, clinical studies have demonstrated significant associations between adaptive immune responses and Trastuzumab + chemotherapy efficacy (60). Phagocytosis of tumor cells by macrophages has been documented to boost the priming of tumor specific adaptive CD4+ and CD8+ T cells (36, 61), while different types of chemotherapy have been documented to enhance phagocytosis and augment immunogenic tumor cell death (62). Taken together, we believe that the clinical use of immunogenic chemotherapy combinations could stimulate adaptive immunity that would be potentially enhanced by Trastuzumab-mediated ADCP. However, in the absence of strong immune-stimulation (potentially through chemotherapy or immunogenic cell lines), Trastuzumab does not appear highly effective at eliciting adaptive immunity and functions mainly through the stimulation of ADCP.

In identifying ADCP as a critical mechanism for Trastuzumab efficacy, we also explored if it could be further enhanced through the blockade of the CD47 innate immune checkpoint. CD47 is highly expressed in BC and functions to suppress phagocytosis through binding with SIRPα on macrophages (23, 24). Interestingly, we found CD47 gene expression is a negative prognostic factor in human BC, most significantly in HER2+ BC. As treatment of HER2 overexpressing tumors with Trastuzumab has been available for many years, this observation suggests that CD47 may be functioning in Trastuzumab-treated patients to mediate
ADCP/therapeutic resistance. This conclusion is supported by the enhanced effects observed between Trastuzumab and CD47 blockade in augmenting ADCP and antitumor effects in our study. Moreover, single cell transcriptome analysis of the tumor microenvironment demonstrates that Trastuzumab therapy stimulates TAMs into a pro-inflammatory antitumor phenotype, which is further boosted by CD47 blockade (Figure 7A and 7B). Such changes in macrophage phenotypes were also observed in co-cultured ADCP experiments. This suggests combination of targeted mAbs therapy with CD47-SIRPα blockade could be beneficial in HER2+ BC and potentially other solid tumors. Proof-of-concept studies using tumor-targeting mAbs and CD47 blockade have been demonstrated in preclinical lymphoma models, as well as a recent phase I study of anti-CD20 mAbs (Rituximab) and CD47 blockade, in Rituximab-refractory Non-Hodgkins Lymphoma patients (41, 63).

Additionally by implementing different methods, such as multi-color FACS analysis and single-cell transcriptome analysis, we are the first to demonstrate in vivo tumor phagocytosis by macrophages upon combination of Trastuzumab with CD47 blockade therapies. Moreover, we were able to identify a distinct cluster of hyper-phagocytic TAMs within the TME. The identification of this population of TAMs may also serve as a predictive biomarker of this form of therapy. Gene expression analysis suggested that after profound phagocytosis of tumor cells, these macrophages switched to a tissue repair phenotype, as evidenced by their upregulation of gene signatures for wound-healing, growth factors, ECM remodeling, and anti-inflammatory markers compared to resident macrophages (Figure 7C). Indeed, several studies have demonstrated that cellular phagocytosis over time influences macrophage phenotype, causing a switch from pro-inflammatory to a growth promoting, reparative phenotype (44-46). Interestingly, while the total number of CD8+ TILs were reduced by prolonged combination therapy, the relative percentage of cytotoxic T cells was greatly increased (Figure S7), possibly suggesting a boost in overall tumor-specific T cells frequency. In this manner, this combination therapy may
allow for enhanced tumor antigen presentation at the earlier time points of treatment through increasing tumor phagocytosis and antigen uptake, while prolonged treatment limits general T cell infiltration after progression to a wound-healing TAM phenotype. Future experiments using Trastuzumab+αCD47 mAbs analyzing multiple treatment time points, reducing the length of treatment, or combining with other immune checkpoint blockades could potentially improve the infiltration of tumor-specific CTLs.

While this is an area in need of additional study, our results suggest that strategies to specifically enhance ADCP activity may be critical in overcoming resistance to HER2 mAb therapies by inhibiting tumor growth and potentially enhancing antigen presentation. While only a single clinical trial using combination of a therapeutic mAb (anti-CD20) and CD47 mAbs has been reported, this study demonstrated a ~50% response rate (11 of 22 patients) and ~36% complete response rate (8 of 22 patients) in resistant/refractory non-Hodgkins’ lymphoma (41). These clinical findings, in conjunction with our recent preclinical studies, strongly suggest combination therapy approach of Trastuzumab with CD47-SIPRα checkpoint blockade could potentially show more benefits and insights of Trastuzumab therapy in HER2+ BC patients. However, the transcriptional switch seen in macrophages after prolonged ADCP also requires attention in future studies that utilize CD47 blockade in combination with targeted mAbs.

In sum, our study suggests that the dominant therapeutic MOA for Trastuzumab is through its elicitation of TAM mediated ADCP, which can be enhanced by strategies to specifically augment ADCP. This has potential implications for the use of Trastuzumab in HER2+ cancers, as well as the utilization of other targeted therapies (such as EGFR, CD20, etc.), where efforts to enhance and control ADCP have not been prioritized.

Methods:
Cell lines and genetic modifications strategies.

Mouse mammary gland cell lines MM3MG and EPH4 were obtained from ATCC and cultured as described by ATCC protocol. The cDNA of a naturally occurring splice variant of human $HER2$ ($HER2\Delta 16$), or wild type $HER2$, were transduced into MM3MG and NMUMG cells using lentiviral transduction. Human HER2+ breast cancer cell line KPL4 was a kind gift from Dr. Kurebayashi (University of Kawasaki Medical School, Kurashiki, Japan) (64) and SKBR3 were purchased from ATCC and cultured as described by ATCC protocol. Jurkat-NFAT-LUC line were obtained from Invivogen (jktl-nfat). CRISPR-Cas9 approached were used to knockout mouse $Cd47$ in MM3MG-HER2Δ16 cells or human $CD47$ in KPL4 cells. Gene targeting of mouse $Cd47$, human $CD47$ and control gene $GFP$ by CRISPR/Cas9 was accomplished through the use of pLentiCRISPRv2 (Addgene plasmid # 52961) using published protocols (65). Genes were targeted using the guide sequences (cccttgcatcgtccgtaatg and ggataagcgcgatgccatgg) for mouse $Cd47$, (atcgagctaaaatatcgtgt and ctactgaagtatacgtaaag) for human $CD47$, and (GGGCGAGGAGCTGTTCACCG) for the $GFP$ control. Successful targeting of CD47 was determined by flow cytometry screening after single cell clonal selection. The overexpression vector of mouse $Cd47$ was generated by synthesizing the $Cd47$ gene and cloning it into pENTR1a (using NEB Gibson Isothermal Assembly Mix) and then using L/R clonase to generate expression lentiviruses (pLenti-CMV-Puro) and cells were selected using puromycin.

Mice

Female Balb/c (Jackson Labs, Bar Harbor, MA), SCID-beige (C.B-1gh-1b/GbmsTac-Prkdc<sup>scid</sup> Lyst<sup>bg</sup> N7; Taconic Biosciences, Model# CBSCBG), $Fcer1g^\sim$ (C.129P2(B6)-$Fcer1g^{tm1Rav}$ N12; Taconic Biosciences, Model# 584) mice between the ages of 6 and 10 weeks old were used for all experiments. The $HER2\Delta 16$ transgenic model was generated by crossing $MMTV$-$rtTA$ strain
(a kind gift by Dr. Lewis Chodosh, UPenn, Philadelphia, USA) with TetO-HER2d16-IRES-EGFP strain (a kind gift by Dr. William Muller, McGill University, Montreal, Canada). 6-weeks old mice were put on doxycycline diet and enrolled for experiments when they develop palpable breast tumor (usually in 4-6 weeks post dox diet).

**Therapeutic antibodies and other experimental reagents**

Clinical Grade Trastuzumab (human IgG1) were obtained from Duke Medical Center. 4D5, the murine version of Trastuzumab (with the IgG2A and IgG1 mouse isotypes) were produced by GenScript through special request. CD47 Blockade antibody MIAP410 (BE0283) and control mouse IgG2A (BE0085) were purchased from BIOXCELL. Neutrophil depletion anti-LY6G antibody (IA8, BP0075-1) and macrophage depletion antibody anti-CSF1R (AS598, BE0213) were purchased from BIOXCELL. Clodronate liposomes were purchased from [www.clodronateliposomes.org](http://www.clodronateliposomes.org).

**Orthotopic implanted HER2+ breast cancer mouse models and therapeutic antibody treatments**

MM3MG cells expressing human HER2Δ16 were implanted into their mammary fat pads (1x10⁶ cells) of Balb/c mice. For the human xenograft model, KPL-4 cells (1x10⁶ cells) were implanted into mammary fat pads (MFP) of SCID-Beige Balb/c mice. Tumor growth were measured with caliper-based tumor volume measurement (length x width x depth) over time. For therapeutic treatments, Trastuzumab or 4D5 were administered weekly (200 µg per mice intraperitoneally) around 4-5 days post tumor implantation. CD47 blockade (MIAP410) were administered weekly when indicated (300 µg per mice intraperitoneally) around 4-5 weeks post tumor implantation. For macrophage depletion, anti-CSF1R antibody were administered triweekly (300 µg per mice intraperitoneally).
intraperitoneally), starting at two weeks before tumor implantation and with treatment maintained over the course of the experiment. Clodronate liposomes were administered biweekly (100 µL per mice, intraperitoneally). For neutrophil depletion, anti-LY6G antibody were administered biweekly (300 µg per mice intraperitoneally) for the first two weeks post tumor implantation.

Transgenic HER2Δ16 mouse model and therapeutic antibody treatments

The HER2Δ16 transgenic mouse model was generated by crossing two strains of mice, TetO-HER2Δ16-IRES-EGFP and MMTV-rtTA. This system was described previously (20), but utilizes a TET-ON system (with MTV-rtTA) to drive expression of HER2Δ16 to generate HER2+ BC. For experiments, one-month old mice were put on Doxycycline diet (200mg/kg, Bio-Serv, Flemington, NJ) to induce spontaneous HER2-driven breast cancer. Individual animals were randomly enrolled into a specific treatment group as soon as palpable breast tumors were detected (~200mm³) in any of the eight mammary fat pads. Control and 4D5-IgG2A antibodies were treated 200 µg weekly, whereas MIAP410 were treated 300 µg weekly intraperitoneally. Animals were terminated once their total tumor volume reached >2000 mm³.

Flow Cytometry Analysis of tumor infiltrating immune cells

When tumor growth reached humane end point size (>1000 mm³), whole tumors from mice were harvested and cut into <1 mm small pieces, and incubated for 1 hour in digestion buffer (DMEM + 100 µg/mL collagenase + 0.2 U/mL DNAse + 1 µg/mL hyalurodinase). Single cell suspensions were spin down through a 70 µm filter and washed with medium. Approximately 5 million cells were used for staining and flow cytometry analysis. The following panel of immune cell markers (Biolegend) were used: CD45 BV650, CD11b PE-Cy7, LY6G APC, LY6C BV410,
F4/80 PerCP-CY5.5, CD8B APC-CY7, CD4 PE-TR, CD49b FITC and viability dye (Aqua or Red). Tumor-associated macrophages (TAM) were identified by F4/80+ LY6G- LY6C- CD11b+ CD45+ gating. LY6G+ neutrophils were identified by LY6G+ CD11b+ CD45+ gating, whereas LY6C+ monocytes were gated on LY6C+ CD11b+ CD45+ cells.

**In vivo ADCP assay**

MM3MG-HER2Δ16 cells were labeled with Vybrant DiD labeling solution (Thermo V22887) according to manufacturer’s protocol, and labeled cells were implanted (1x10^6) into MFP of Balb/c mice. Once tumor reaches around 1000 mm^3 in sizes, mice were treated with either control antibody (200 µg), 4D5 (200 µg), or 4D5 in combination with MIAP410 (300 µg) per day for two consecutive days. Tumor associated macrophages were analyzed by FACS (CD11b+, F4/80+, LY6G-, LY6C-) and the percentage of TAMs that have taken up DiD-labeled tumor cells were quantified for in vivo ADCP analysis.

**In vitro ADCP and ADCC assays**

ADCP and ADCC by macrophages – Bone marrow derived macrophages (BMDM) were generated from mouse tibia, differentiated for 10 days with 50 ng/mL mouse MCSF (Peprotech 315-02). Briefly, 50 million bone marrow cells were plated in 10 cm^2 tissue culture dish with MCSF on day 0. Unattached cells in supernatant were removed and fresh media + MCSF were added on day 3, day 6 and day 9. BMDM were used for ADCP/ADCC assays on day 10. Tumor cells MM3MG-HER2Δ16 were labeled with Brilliant Violet 450 Dye (BD 562158) according to manufacturer’s protocol, and incubated with control or anti-HER2 antibodies (10 µg/mL) in 96-wells (100,000 cells/well) for 30 minutes at 37 °C. BMDM were then added for co-culture at a 3:1 ratio of Tumor vs BMDM. After 2 hours co-culture, phagocytosis of BV450-labeled tumor
cells by BMDM were analyzed by FACS with CD45-APC staining and Live-death (Red) staining. When indicated, ADCP inhibitor Latrunculin A (120 nM, Thermo L12370) and ADCC inhibitor Concanamycin A (1 µM, Sigma C9705) were added as assay controls. For human macrophages ADCP assay, human monocytes-derived macrophages (hMDM) were generated from three donors’ PBMCs. hMDM were generated with 50 ng/mL human MCSF (Peprotech 300-25) and 50 ng/mL human GM-CSF (Peprotech 300-03). KPL-4 cells were used as human HER2+ tumor targets and labeled and co-cultured similarly as with mouse ADCP assay.

**FCGR binding/activation assay**

Jurkat cells expressing mouse Fcgr1, Fcgr2b, Fcgr3 or Fcgr4 with NFAT-Luciferase reporter were generated with lentiviral transduction and selected with puromycin (validated in Figure S4D-F). For the assay, MM3MG breast cancer lines expressing HER2 were first plated and treated with Trastuzumab or 4D5 antibodies or control IgG for 1 hour. Jurkat-FCGR-NFAT-LUC effector cells were added and co-cultured for 4 hours. FCGR signaling activation were assessed by luciferase activity quantification.

**Multiplex cytokine and chemokine assay.**

BMDM were co-cultured with MM3MG-HER2Δ16 cells for 24 hours, and supernatants were harvested for analysis of cytokines/chemokines levels. The 26-Plex Mouse ProcartaPlex™ Panel1 kit (Thermo) was used and analyzed using the Luminex MAGPIX system.

**METABRIC analysis of CD47 expression in breast cancer patients**
Pre-processing METABRIC data: Previously normalized gene expression and clinical data were obtained from the European Genome-Phenome Archive (EGA) under the accession id EGAS00000000098 after appropriate permissions from the authors (47). The discovery dataset was composed of 997 primary breast tumors and a second validation set was composed of 995 primary breast tumors. The expression data were arrayed on Illumina HT12 Bead Chip composed of 48,803 transcripts. Multiple exon-level probe sets from a transcript cluster grouping were aggregated to a single gene-level probe set using maximum values across all the probes for a given gene. The resulting gene expression matrix consists of 28,503 genes.

In order to assess the prognostic significance of CD47 in METABRIC data we generated Kaplan-Meier survival curves on patients stratified by the average expression of CD47 (in to low and high groups) using R package ‘survminer’ (version 0.4.3). Distributions of Kaplan-Meier survival curves for progression-free and overall survival were compared using log-rank test, and a log-rank test p-value \( \leq 0.05 \) is considered to be statistically significant.

**Single-Cell RNA-Seq Analysis**

Fastq files from 10X library sequencing were processed using the CellRanger pipeline available from 10X genomics. As part of the processing the assembled sequencing reads were mapped to the mm10 mouse genome. In order to obtain the transcript counts of human ERBB2 (HER2) the sequencing reads were separately aligned to the current version of the human genome, GRCh38.

The gene expression files consisting of raw counts at the gene level for each cell which was analyzed using version 2.3.4 of the Seurat package. The human ERBB2 counts were combined with the mm10 based counts into one expression matrix for each sample. Briefly, the data analysis steps using Seurat consisted of combining the gene counts for all the cells in the
different conditions into one matrix, filtering low quality cells, normalizing, and adjusting for cell cycle and batch effects. Unsupervised clustering was done to separate the cell types and markers for the cell types were identified using differential gene expression. These markers were then used for identifying the cell subpopulations within the tumor microenvironment, namely the Immune cells, Tumor cells and Fibroblasts. The normalized gene counts were used to generate tSNE maps for visualization of the cell types and heatmaps for the cell type specific gene expression. Expression of predefined gene sets representing pathways of interest where obtained from previous publications and summarized in Table S2. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE139492


Statistical methods

All statistical analysis of tumor growth comparisons and tumor immune infiltrates were performed with GraphPad Prism (v8) using two-way ANOVA or one-way ANOVA test with Tukey’s multiple comparisons. Unless otherwise indicated in the figure, tests results were shown between treatment vs control group. Group sizes for animal tumor growth experiments were determined based on preliminary datasets. All subjects in animal experiments were randomized into a treatment or control group. For in vitro experiments, i.e. ADCP/ADCC/CDC assays, ELISPOT assays, FCGR signaling assay and cytokine assays, all data were statistically analyzed by one-way ANOVA test with Tukey’s multiple comparisons, and performed with at least four biological replicates per experiment and repeated at least two times. RT-qPCR data were analyzed by two-sided Student’s t test for each target gene. 95% confidence interval was considered for statistics and p<0.05 was considered significant.
Study Approval:

All animals were maintained, bred, in accordance with Duke Institutional Animal Care and Use Committee–approved protocol (A198-18-08), and supervised by Division of Laboratory Animal Resources (DLAR).

Author Contributions:

Li-Chung Tsao, H. Kim Lyerly and Zachary C. Hartman conceived the project and wrote the manuscript. Li-Chung Tsao and Zachary C. Hartman designed the experiments, analyzed and interpreted the data. Li-Chung Tsao performed the animal experiments, with assistance from Erika J. Crosby, Bin-Jin Hwang, Xiao Yi Yang, Cong-Xiao Liu and Christopher A. Rabiola. Tao Wang provided technical support and suggestions for flow cytometry. Zachary C. Hartman generated various cell lines and CRISPR knock outs with assistance from Junping Wei and Gangjun Lei. Li-Chung Tsao performed the in vitro cell culture assays. Li-Chung Tsao designed single-cell RNA sequencing experiment and generated 10X Genomics cDNA libraries. Pankaj Agawal performed the clustering and statistical analysis and illustration of single-cell RNA-Seq results. Chaitanya Acharya conducted the stratification and statistical analysis of METABRIC datasets. Erika J. Crosby, Timothy N. Trotter and Casey W. Shupertine provided intellectual input and discussions. The MMTV-rtTA mouse strain was provided by Dr. Lewis Chodosh (UPenn) and TetO-HER2d16-IREs-EGFP mouse by Dr. William Muller (McGill University).

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References:


34. Oliveira CA, Kashman Y Fau - Mantovani B, and Mantovani B. Effects of latrunculin A on immunological phagocytosis and macrophage spreading-associated changes in the F-actin/G-actin content of the cells. (0009-2797 (Print)).


Figure and figure legends:

A. Trastuzumab
   Human IgG1
   Mouse IgG1
   Mouse IgG2A
   High ADCC
   High ADCC
   Low ADCC
   Low ADCC
   ADCP
   ADCP

B. MDMG-HER2Δ16 growth in Balb/c mice

C. Control IgG
   4D5-IgG2A
   Trastuzumab
   Tumor-associated macrophages (TAMs)

D. SCID-Beige mice
   Neutrophil depletion
   Depletion of TAMs
   Macrophage depletion

E. Depletion of TAMs

F. Macrophages with dye-labeled HER2+ BC
   ADCP
   ADCC

G. Control IgG
   4D5-IgG2A
   4D5-IgG2A + ADCP inhibitor
   4D5-IgG2A + ADCC inhibitor

H. Control IgG
   4D5-IgG2A
   4D5-IgG2A + ADCP inhibitor

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Figure 1. Generation of murine Trastuzumab and its antitumor dependence on Antibody-Dependent-Cellular-Phagocytosis (ADCP) by tumor-associated macrophages (TAMs). (A) Cartoon presentation of Trastuzumab and 4D5 antibodies used in this study. (B) MM3MG cells expressing human HER2Δ16 were implanted into the mammary fat pads (1x10⁶ cells) of Balb/c mice. Trastuzumab (human IgG1) or 4D5 (mouse IgG2A) were administered weekly (200 µg per mouse). n = 8-10. (C) Tumors (>1000 mm³ volume) were processed into single cell suspensions, and TAMs (%CD11b+ F4/80+ LY6G- LY6C- of CD45+ cells) were analyzed by FACS. n = 8-10. (D) Experiment as in Figure 1B was repeated in SCID-Beige animals. n = 8. (E) Experiment in SCID-Beige was repeated using neutrophils-depleting anti-LY6G antibodies (clone IA8, 300 µg per mice biweekly). (F-G) To deplete macrophages, SCID-Beige mice were pre-treated with anti-CSF1R antibody (clone AFS98, 300 µg, 3 times per week) for two weeks. (F) Macrophage depletion were verified by FACS. (G) 4D5-IgG2A injection were performed, with anti-CSF1R treatment maintained throughout the experiment. n = 8. (H) Trastuzumab/4D5 induced ADCP of HER2+ BC cells by Bone-marrow-derived-macrophages (BMDM). MM3MG-HER2Δ16 cells were labeled with Brilliant Violet 450 Dye, and co-cultured with BMDM (3:1 ratio) with control or anti-HER2 antibodies (10 µg/mL). ADCP rates were measured by percentage of BMDM uptake of labeled tumor cells (CD45+ and BV450+), and Antibody-dependent-cellular-cytotoxicity (ADCC) rates were measured by percentage of dying free tumor cells (CD45- and LIVE/DEAD stain+). ADCP inhibitor (Latrunculin A) or ADCC inhibitor (Concanamycin A) were added as assay controls. n = 3, experiment has been repeated three separate times. (B, D, E and G) Tumor growth were measured with caliper-based tumor measurement over time. Two-way ANOVA test with Tukey’s multiple comparisons (C, F and H) One-way ANOVA test with Tukey’s multiple comparisons. All data represent mean ±SEM, **P<0.01, ***P < 0.001, ****P<0.0001.
Figure 2. The Antibody-dependent-cellular-phagocytosis (ADCP) activity of mouse Trastuzumab (4D5) requires the engagement with Fcγ-receptors (FCGR) and is IgG2A isotype dependent. (A) Fcγ-receptors are required for 4D5-induced ADCP of HER2+ BC cells by Bone-marrow-derived-macrophages (BMDM) in vitro. BMDM were generated from wild type and Fcer1g−/− mice, and ADCP experiment were performed with the conditions described in Figure 1E. n = 3. (B-C) FCGR is required for the antitumor activity of 4D5 therapy. (B) Wild type or Fcer1g−/− Balb/c mice were implanted with MM3MG-HER2Δ16 cells as before (Figure 1B). 4D5-IgG2A or control antibodies were administered weekly (200 µg per mice intraperitoneally) and tumor growth were measured. n = 5. (C) Tumor-associated macrophages (TAMs) from...
tumors in Figure 2B were analyzed by FACS. \( n = 4-5 \). (D-F) The ADCP activity of 4D5 is IgG2A isotype dependent. (D) MM3MG-HER2Δ16 tumor growth in mice were repeated using 4D5 antibodies containing the mouse IgG1 as comparison to previous IgG2A isotype. \( n = 8-10 \). (E) ADCP experiments with BMDM cultures were performed using 4D5-IgG1 versus 4D5-IgG2A antibody isotypes. \( n = 4 \). (F-H) Mouse FCGR signaling activation assay. MM3MG breast cancer cells expressing \( \text{HER2} \) were plated and treated with indicated antibodies concentrations for 1 hour. Jurkat cells containing NFAT-luciferase reporter and expressing mouse FCGR1 (F), FCGR3 (G) or FCGR4 (H) were added to the target cells containing antibodies and co-cultured for 4 hours. FCGR signaling activation were assessed by luciferase activity quantification. \( n = 4 \). (A, C, and E) One-way ANOVA with Tukey’s multiple comparisons. (B, D, F, G and H) Two-way ANOVA test with Tukey’s multiple comparisons to control IgG group. All data represent mean ±SEM, \( *P < 0.05, **P < 0.001, ****P < 0.0001. \)
**Figure 3. CD47 suppresses the anti-tumor activity of mouse Trastuzumab (4D5).** (A) CD47 knockout cells were generated from MM3MG-HER2Δ16 cells using CRISPR-Cas9 technology.
A control GFP knockout line was generated in parallel. Control and CD47-KO MM3MG-HER2Δ16 cells were labeled with Brilliant Violet 450 Dye, and incubated with Bone-marrow-derived-macrophages (BMDM) at 3:1 ratio with control or 4D5 antibodies (10 µg/mL). Antibody-dependent-cellular-phagocytosis (ADCP) and cytotoxicity (ADCC) activity were measured by as described in Figure 1H. n = 3. Experiment has been repeated two separate times using CD47-KO clones containing a different guide RNA. (B) Secreted cytokines and chemokines by macrophages from co-culture experiment with HER2+ BC were analyzed using the Luminex platform. Additional cytokines detected can be found in Figure S5. n = 3. (C-D) Control and CD47-KO MM3MG-HER2Δ16 cells were implanted into mouse mammary fat pads and treated with 4D5-IgG2A or control antibodies as described before. TAMs were analyzed by FACS after tumor volume reached >1000mm³. n = 5. (E-F) Cd47 overexpressing cells (CD47-OE) were generated in MM3MG-HER2Δ16 cells after transduction with Cd47 cDNA under control of the EF1s promoter. CD47-OE tumor cell growth were compared to parental MM3MG-HER2Δ16 cells in mice treated with control antibody or 4D5-IgG2A. TAMs were analyzed by FACS. n = 5. (A, B, D and F) One-way ANOVA with Tukey’s multiple comparisons test. (C and E) Two-way ANOVA test with Tukey’s multiple comparisons. All data represent mean ±SEM, *P < 0.05, ***P < 0.001, ****P < 0.0001.
Figure 4. CD47 Blockade increased therapeutic efficacy of mouse Trastuzumab and augments tumor-associated macrophage (TAMs) expansion and phagocytosis. (A) Tumor growth experiment (as in Figure 1B) were repeated using CD47 blockade antibody (MIAP410, 300 µg per mice) alone or in combination with 4D5-IgG2a. (B) TAM populations were analyzed by FACS after tumor volume reached >1000mm$^3$. Analysis of additional immune cell types are shown in Figure S4D. Mean ± SEM, $n$ = 8-10. (C) Repeat of similar tumor growth experiment and treatments in SCID-Beige mice. (D) TAM populations from SCID-Beige experiment were analyzed by FACS. $n$ = 10. (E) Schematic representation of in vivo Antibody-dependent-cellular-
phagocytosis (ADCP) experiment. MM3MG-HER2Δ16 cells were labeled with Vybrant DiD dye and implanted (1x10^6 cells) into mammary fat pads of Balb/c mice. Once tumor volume reaches ~1000 mm^3, mice were treated with either control antibody, 4D5-IgG2A (200 µg), or in combination with MIAP410 (300 µg). On the next day, tumors were harvested and tumor-phagocytic macrophages were quantified by FACS. (F) Representative FACS plots and graphical summary showing frequency of macrophages (CD11b+, F4/80+, LY6G-, LY6C-) that have phagocytosed DiD-labeled tumor cells. n = 6. (G) Similar in vivo ADCP experiment were repeated in Fcer1g^−/− mice. n = 8. (A and C) Two-way ANOVA test with Tukey’s multiple comparisons. (B, D, G and G) One-way ANOVA test with Tukey’s multiple comparisons. All data represent mean ±SEM *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001
Figure 5. CD47 blockade synergizes with mouse Trastuzumab therapeutic activity in a transgenic human HER2+ breast cancer (BC) mouse model. (A) Schematic representation of experiment using the endogenous human HER2 transgenic mouse model. Spontaneous breast tumors in the transgenic animals were induced with doxycycline diet. Four treatment arms were set up: Control IgG (200 µg weekly, n= 15), CD47 blockade (MIAP410, 300 µg weekly, n= 14), 4D5-IgG2A (200 µg weekly, n=16) and 4D5-IgG2A combined with MIAP410 (n=16). Individual animals were consecutively enrolled into a specific treatment arm as soon as palpable breast tumors were detected (~200mm³). (B) Survival of mice in each treatment arm, time of start is on the day of palpable tumor detection and treatment enrollment. Log-rank
(Mantel-Cox) test for survival analysis, ****P < 0.0001 of treatment vs control group, ## P < 0.01
significant difference observed between “4D5” group vs “4D5+αCD47” group. (C) Tumor burden
in animals from each treatment arm were measured over time after enrollment in treatment arm.
Each individual animal develops 1 to 4 total tumors in their mammary fat pads. The total tumor
burden per mice is shown. Animals were terminated when their total tumor volume
reached >2000 mm³ (D) Tumors in the transgenic mice were harvested, processed into single
cell suspensions, and analyzed by FACS. Each individual tumor were treated as an individual
measurement. Mean ± SEM, Control IgG n=23, αCD47 n=27, 4D5 n=38, 4D5+ αCD47 n=32,
One-way ANOVA with Tukey’s multiple comparisons test, *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 6. Single-cell transcriptome analysis of immune clusters within HER2+ breast cancer after Trastuzumab with CD47 blockade therapy. HER2+ tumors from HER2Δ16 transgenic animals were isolated for Single-Cell RNA-sequencing using 10X Genomics platform. Data from all tumors were pooled for clustering and gene expression analysis. (A) tSNE plots showing distinct clusters of immune cells in tumors from four treatment groups: control IgG, αCD47, 4D5-IgG2A or combination. (B-C) Heat map of relevant gene markers confirmed the various immune cell clusters in control tumors (B), and the expansion of...
macrophage clusters in the combination therapy treated tumors (C). Macrophages that contains
tumor specific transcripts (e.g. hERBB2, Epcam, Krt8) were labeled as tumor phagocytic
macrophages (Phag МΦ, predominantly found in combination treatment group).
Figure 7. Differential gene expression analysis of TAM clusters in HER2+ BC after Trastuzumab with CD47 blockade therapy. (A-B) Differential gene expression analysis of gene signatures for IFN, pro-inflammation, chemotaxis and TLR/MyD88/NFκb pathways in M1-like MΦ clusters (A) and M2-like MΦ clusters (B) revealed how they were affected by the treatment regimens. (C) Differential gene expression analysis of immuno-regulatory gene signatures (wound-healing, ECM remodeling, growth factors, anti-inflammation) versus immuno-stimulatory gene signatures (pro-inflammation, chemotaxis, antigen presentation, phagocytosis/opsonization) among the three distinct macrophage clusters in the combined dataset.
Figure 8. Human CD47 gene expression is a prognostic factor in HER2+ breast cancer and limits the therapeutic activity of Trastuzumab. (A-B) Kaplan-Meier survival curve for breast cancer (BC) patients METABRIC Dataset. (A) Stratified into low and high groups based on average expression of CD47 in all patients. (B) The same patient stratification based on disease subtype (ER+, HER2+ and TNBC). (C) CD47 knockout in human HER2+ BC line KPL-4 was generated using CRISPR-Cas9 approach. Control and CD47-KO KPL-4 cells were labeled with Brilliant Violet 450 Dye, and incubated with human monocytes-derived-macrophages (hMDM) at a 3:1 ratio, in the presence of control or Trastuzumab (10 µg/mL). Antibody-dependent-cellular-phagocytosis (ADCP) activity were measured by percentage of hMDM uptake of labeled KPL-4 cells (CD45+ and BV450+). Mean ± SEM, biological replicates n = 4. Experiment has been repeated using hMDMs generated from three healthy PBMC donors. (D) Control or CD47-KO KPL-4 cells were implanted into mammary fat pads of SCID-Beige Balb/c mice (5x10⁵ cells). Trastuzumab (50 µg) or control human IgG1 were administered weekly and tumor volume were measured. Two-way ANOVA test with Tukey’s multiple comparisons, ****P<0.0001. (E) Tumor infiltrating macrophages (F4/80+ Gr1- CD11b+) populations were analyzed by FACS, except for “CD47-KO + Trastuzumab” group as no tumor growths have
occurred. Mean ± SEM, n = 7. (F) Tumor-associated macrophages from control treated and trastuzumab treated tumors were sorted by FACS (F4/80+ Gr1- CD11b+CD45+) and analyzed with RT-qPCR for the expression of pro- and anti-inflammatory genes. Mean ± SEM, n=7. Multiple two-sided t-test. (C and E) One-way ANOVA test with Tukey’s multiple comparisons, * P <0.05, ** P <0.01, ***P < 0.001
Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th># of tumors analyzed</th>
<th>Average length on treatment regimen</th>
<th>MΦ cluster size (% MΦ among immune cells)</th>
<th>Tumor Phagocytic MΦ (% MΦ containing hERBB2 transcripts)</th>
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<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>28 days</td>
<td>2354/4527 (52 %)</td>
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<tr>
<td>αCD47</td>
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<td>36 days</td>
<td>1228/2154 (57 %)</td>
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<tr>
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<td>45 days</td>
<td>2938/3815 (77 %)</td>
<td>9.07 %</td>
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<tr>
<td>4D5-IgG2A + αCD47</td>
<td>4</td>
<td>56 days</td>
<td>4673/5079 (92 %)</td>
<td>48.44 %</td>
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Table 1. Single-Cell Transcriptome Analysis of macrophage cluster sizes and frequency of tumor phagocytic macrophages. Numbers represent mean of replicates in each treatment group.