The Therapeutic Effect of Anti-HER2/neu Antibody Depends on Both Innate and Adaptive Immunity

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SUMMARY

Anti-HER2/neu antibody therapy is reported to mediate tumor regression by interrupting oncogenic signals and/or inducing FcR-mediated cytotoxicity. Here, we demonstrate that the mechanisms of tumor regression by this therapy also require the adaptive immune response. Activation of innate immunity and T cells, initiated by antibody treatment, was necessary. Intriguingly, the addition of chemotherapeutic drugs, although capable of enhancing the reduction of tumor burden, could abrogate antibody-initiated immunity leading to decreased resistance to rechallenge or earlier relapse. Increased influx of both innate and adaptive immune cells into the tumor microenvironment by a selected immunotherapy further enhanced subsequent antibody-induced immunity, leading to increased tumor eradication and resistance to rechallenge. This study proposes a model and strategy for anti-HER2/neu antibody-mediated tumor clearance.

INTRODUCTION

The human epidermal growth factor receptor 2 (HER2, HER2/neu, or ErbB-2) is overexpressed in 20%–30% of breast carcinomas and is associated with aggressive disease, a high recurrence rate, and reduced patient survival (Hudis, 2007; Kiessling et al., 2002; Meric-Bernstam and Hung, 2006; Slamon et al., 1987). The use of trastuzumab (Herceptin), a humanized monoclonal antibody that binds the extracellular, juxtamembranous domain of HER2, has proved to be an effective treatment in animal and human studies (Hudis, 2007; Moasser, 2007). Many groups have demonstrated that anti-HER2/neu antibody can efficiently stop or slow the growth of HER2/neu+ tumors in vitro (Hudis, 2007; Kiessling et al., 2002; Meric-Bernstam and Hung, 2006). Growth inhibition is mainly due to the induction of G1 cell cycle arrest and is closely tied to increased p27Kip1 expression, and reduced cyclin E expression (Le et al., 2005; Mittendorf et al., 2010). In addition, antibody treatment was shown to inhibit the ability of tumor cells to repair damaged DNA (Pegram et al., 1999). The combination of antibody treatment with multiple chemotherapeutic agents showed additive and synergistic effects in in vitro studies and in vivo xenograft tumor models (Pegram et al., 1999; Pegram et al., 2004). As a result, interference with HER2 oncogenic signaling and increased

Significance

Although anti-HER2/neu antibody is an effective adjuvant therapy targeting HER2+ breast cancers, relapse often occurs even after prolonged treatment. Current understanding holds that this antibody therapy interrupts oncogenic signals and induces FcR-mediated cytotoxicity. This study reveals that the therapeutic effect of anti-HER2/neu antibody treatment also depends on adaptive immunity. Furthermore, this study demonstrates an interesting antibody-mediated mechanism whereby danger signals are required to mobilize and activate innate cells and prime the adaptive immune system for increase tumor clearance. However, antibody-initiated tumor regression can be impaired by certain chemotherapies regimens. This study has important clinical impact because various chemotherapy drugs have been used before or after antibody treatment.
susceptibility to chemotherapy-induced apoptosis (chemosen-
sitization) have been proposed as the central mechanisms
responsible for the clinical efficacy of trastuzumab (Hudis,
2007; Moasser, 2007; Pegram et al., 2004). Based on the con-
vincing preclinical studies, clinical trials were conducted and
demonstrated the benefits of combining chemotherapy admin-
istration with trastuzumab (Hudis, 2007; Piccart-Gebhart et al.,
2005; Romond et al., 2005). Despite of the initial clinical success
of antibody plus chemotherapy treatment for Her2\(^{+}\) tumors,
relapse has been reported after cessation of this treatment.

Considering reports that inhibition of oncogenic signals by
anti-Her2/neu antibody controls tumor growth in vitro, it was
surprising that the therapeutic effect of this antibody was dimin-
ished in the absence of Fc receptor (FcR) signaling in vivo
(Clynes et al., 2000). The role of FcRs in the efficacy of antibody
treatment is further supported by evidence that Fcr polymor-
phisms are associated with the clinical outcome in breast cancer
patients (Musolino et al., 2008). These data raise the possibility
that antibody-dependent cellular cytotoxicity (ADCC) may play
a major role in the antitumor effects of antibody therapy. Consis-
tently, an increase of tumor-infiltrating leukocytes, especially
FcR\(^{+}\) cells such as NK cells, has been observed in tumor tissue
after antibody treatment (Arnould et al., 2006; Varchetta et al.,
2007). Furthermore, it was reported that patients with partial or
complete remission after antibody treatment had higher in situ
infiltration of leukocytes and an increased capacity to mediate
in vitro ADCC activity (Gennari et al., 2004). Endogenous anti-
Her2 antibodies after vaccine can be detected in some patients
and can effectively suppress Her2 kinase activity and down-
stream signaling to inhibit the transformed phenotype of Her2-
expressing tumor cells (Montgomery et al., 2005). However,
most models, including xenografts used for preclinical evalua-
tion, fail to account for adaptive immunity in the antibody-
mediated therapeutic effect. Therefore, the essential role of T
and B cells in anti-Her2/neu antibody-mediated tumor regres-
sion remains unclear.

RESULTS

Adaptive Immunity Is Essential for the Therapeutic
Effect of Antibody Treatment

To evaluate whether targeted antibody treatment of Her2/neu\(^{+}\)
brust cancer could reduce tumor burden in syngeneic wild-type
(Wt) mice, we used the well-characterized anti-neu (rat homolog
of human Her2) monoclonal antibody 7.16.4 (Zhang et al.,
1999). This antibody competes with 4D5 (the original mouse
anti-Her2/neu antibody that was humanized to trastuzumab)
for binding to human Her2 and inhibition of tumor growth.
BALB/c mice bearing established TUBO tumors, a neu overex-
pressing cell line derived from a spontaneous carcinoma in
neu-transgenic mice (Rovero et al., 2000), were treated with
anti-neu antibody. Impressively, without the addition of chemo-
therapy, the majority (28/35) of Balb/c mice from several exper-
iments rejected tumors completely 4 weeks after treatment,
while control Ig-treated mice had to be sacrificed due to large
tumor burden (Figure 1A and Table 1).

To test the relative contribution of Her2/neu signal interfer-
ence with anti-neu antibody-mediated tumor regression, we
compared the efficacy of this antibody to erlotinib, a tyrosine
kinase inhibitor (TKI), in vitro and in vivo. A high dose of anti-
neu antibody slightly inhibited TUBO cell proliferation in vitro
(~20%), but effectively reversed the growth of or cleared all
established tumors in vivo (35/35). On the other hand, erlotinib
strongly inhibited TUBO cell proliferation in vitro (>80%) but
Together, using a system modeling human HER2/\textit{Fc} system, WT and this additional reduction of tumor masses in our in vivo model were inoculated, further explore the role of ADCC in anti-neu antibody therapy. Indeed, the therapeutic effect of anti-neu antibody was established tumors were treated with 7.16.4. Therefore, we considered the role of the adaptive immune response being essential for antibody-mediated tumor reduction.

CD8\textsuperscript{+} cytotoxic lymphocytes are a major adaptive immune cell population involved in controlling tumor growth. We observed increased lymphocytes, especially CD8\textsuperscript{+} cells, in TUBO tumor tissues 1–2 weeks after antibody treatment (see Figure S1 available online). To determine whether CD8\textsuperscript{+} T cells are essential for anti-neu antibody-mediated tumor regression, TUBO-bearing mice displayed delayed tumor growth lasting, as (A). One of three experiments is shown. Increase of lymphocytes inside tumor is seen in Figure S1.

<p>| Table 1. The Therapeutic Effect of Combined Chemotherapy and Antibody Treatment on Tumor Regression and Rechallenge |
|---------------------------------|-----------------|-----------------|----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Chemotherapy</strong></th>
<th><strong>Antibody Dosage</strong></th>
<th><strong>Drug</strong></th>
<th><strong>Dosage</strong></th>
<th><strong>Schedule</strong></th>
<th><strong>Rate of Tumor Regression</strong></th>
<th><strong>Rejected/Total (%)</strong></th>
<th><strong>Growth After Rechallenge Growth/Total (%)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg x 2</td>
<td>None</td>
<td>PTX</td>
<td>60 mg/kg x 2</td>
<td>3 days after</td>
<td>+</td>
<td>10/10 (100)</td>
<td>7/9 (78)</td>
</tr>
<tr>
<td>100 μg x 2</td>
<td>None</td>
<td>PTX</td>
<td>40 mg/kg x 2</td>
<td>3 days after</td>
<td>+</td>
<td>10/10 (100)</td>
<td>7/9 (78)</td>
</tr>
<tr>
<td>100 μg x 2</td>
<td>PTX</td>
<td>10 mg/kg x 4</td>
<td>3 days after</td>
<td>4/4 (100)</td>
<td>4/4 (100)</td>
<td>0/4 (0)</td>
<td></td>
</tr>
<tr>
<td>100 μg x 2</td>
<td>PTX</td>
<td>40 mg/kg x 1</td>
<td>1 day before</td>
<td>+</td>
<td>12/12 (100)</td>
<td>0/4 (0)</td>
<td></td>
</tr>
<tr>
<td>100 μg x 2</td>
<td>PTX</td>
<td>20 mg/kg x 1</td>
<td>1 day before</td>
<td>+</td>
<td>4/4 (100)</td>
<td>2/4 (50)</td>
<td></td>
</tr>
<tr>
<td>100 μg x 2</td>
<td>CTX</td>
<td>100 mg/kg x 2</td>
<td>3 days after</td>
<td>+</td>
<td>12/20 (60)</td>
<td>2/3 (67)</td>
<td></td>
</tr>
<tr>
<td>100 μg x 2</td>
<td>DOX</td>
<td>15 mg/kg x 2</td>
<td>Same day</td>
<td>–</td>
<td>0/5 (0)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>100 μg x 2</td>
<td>DOX</td>
<td>5 mg/kg x 2</td>
<td>Same day</td>
<td>–</td>
<td>0/5 (0)</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

CTX, cyclophosphamide; DOX, doxorubicin; ND, not determined; PTX, paclitaxel.

\textsuperscript{a} BALB/c mice were implanted with 4–5 x 10\textsuperscript{5} TUBO cells on day 0. Groups of 5–10 mice were injected i.p. with anti-neu antibody at 5–7 day intervals starting on day 12 or day 18 after tumor implantation, with or without chemotherapeutics. Tumors were measured twice a week.

\textsuperscript{b} Chemotherapeutic used. All drugs were injected i.p.

\textsuperscript{c} Chemotherapeutic administration relative to anti-neu antibody treatment.

\textsuperscript{d} Comparison of the incidence of regression to anti-neu antibody treatment (100 μg x 2) alone; +, greater than antibody alone; = , similar to antibody alone; –, less than antibody alone.

\textsuperscript{e} One month after complete tumor regression, tumor-free mice were rechallenged with 2–5 x 10\textsuperscript{6} TUBO cells and tumor growth was monitored for 30 days.

\textsuperscript{f} Experiments were performed in UC.

\textsuperscript{g} Experiments were performed in CAS.

Weakly impacted tumor growth rates in vivo without reversal of tumor growth (Figures 1B and 1C). These data support the recent model that anti-HER2/neu antibody treatment requires ADCC via FcRs for effective in vivo treatment of human HER2/neu\textsuperscript{+} tumors (Clynes \textit{et al.}, 2000). To test whether ADCC is accountable for this additional reduction of tumor masses in our in vivo model system, WT and FC\gamma KO mice were inoculated with TUBO and established tumors were treated with 7.16.4. Indeed, the therapeutic effect of anti-neu antibody was FC\gammaR-dependent, as FC\gammaR deficient mice failed to show antibody-induced inhibition (Figure 2A). Together, using a system modeling human HER2/neu\textsuperscript{+} tumor growth, our data consistently support the model that FcR\textsuperscript{+} cells are essential for inhibiting or even eradicating neu\textsuperscript{+} tumors in response to anti-neu antibody treatment. To further explore the role of ADCC in anti-neu antibody therapy, we inoculated \textit{Rag-1}\textsuperscript{−/−} mice with TUBO. Though \textit{Rag-1}\textsuperscript{−/−} mice lack T and B cells, these mice do have a complete population of FcR\textsuperscript{+} innate cells to mediate ADCC. Thus, we expected these mice to regress established TUBO tumors efficiently after anti-neu antibody treatment. Although WT mice treated with anti-neu antibody consistently demonstrate tumor regression, this antibody treatment had a very limited impact on the growth of established tumors in \textit{Rag-1}\textsuperscript{−/−} mice (Figure 2B). Although some \textit{Rag-1}\textsuperscript{−/−} mice displayed delayed tumor growth lasting, no tumor regression was observed in \textit{Rag-1}\textsuperscript{−/−} mice (total n = 13) after antibody treatment. Thus, these data reveal that the innate immune response alone is not sufficient to mediate the therapeutic effect of anti-neu antibody treatment. Therefore, we considered the role of the adaptive immune response being essential for antibody-mediated tumor reduction.
BALB/c mice were treated with an anti-CD8α-depleting antibody (YTS169.4.2) in conjunction with anti-neu antibody treatment. Initially, tumors in antibody-treated mice continued to regress, but relapsed rapidly in the absence of CD8+ T cells (Figure 3A). Anti-CD8α antibody had no detectable impact on tumor growth in control Ig treated mice. In addition, depletion of CD8+ cells using another clone (53.6.7) that has less impact on CD8+ T cells, specific antibody to CD8+ T cells, specific antibody to CD8β chain or mice deficient of CD8α DC might be useful.

This essential role for T cells in antibody-mediated tumor regression is not limited to the TUBO cell line. Anti-neu antibody treatment of mice bearing neu-dependent N202 tumors, derived from neu transgenic mice on the FVB background, also resulted in tumor regression. More importantly, antibody-mediated tumor regression of N202 tumor was also CD8+ T cell-dependent (data not shown). As WT Balb/c mice are not tolerated to the neu antigen, we decided to test whether CD8+ T cells contribute to the effect of this antibody in a tolerant model. We used F1 neu transgenic (Tg) mice (BALB/c × FVB/N MMTV-neu), which are tolerant to the neu antigen and resistant to various treatments (Machiels et al., 2001). Whereas TUBO-bearing WT mice consistently demonstrate tumor regression after anti-neu antibody treatment, only 20% of tumors implanted in neu Tg mice demonstrate complete regression with the other 80% relapsing a few weeks after antibody cessation (Figure 3B). Nevertheless, short-term treatment with the anti-neu antibody still resulted in significant reduction of tumor growth in a CD8-dependent fashion. Thus, tumor relapse in neu-Tg mice mimics frequently relapse observed in the clinic. Therefore, in addition to supporting the role of CD8+ T cells in mediating neu-antibody therapy, this data also raises an interesting possibility that anti-neu antibody may transiently break tolerance in neu-Tg mice and generate immunity against HER2/neu+ tumors.

To determine whether the immune response initiated by anti-neu antibody results in memory, the hallmark of adaptive immunity, we evaluated cured mice for long-term protection by tumor rechallenge. Mice that underwent complete tumor regression following antibody treatment (n = 14) were rechallenged s.c. with 5 × 10^6 TUBO cells on different site from primary tumor at least 1 month after complete rejection of primary tumors. All of mice rejected the secondary tumor. One of two experiments is shown.

To define whether tumor-specific T cell responses are increased by antibody treatment, splenocytes were collected after cessation of antibody treatment, and IFN-γ production from anti-neu or control antibody-treated TUBO-bearing WT mice was evaluated by ELISPOT. Antibody-treated group has much higher neu-reactive T cells (Figure 3D). To further define whether tumor-specific T cell responses are increased by antibody treatment, even in antibody-treated neu-Tg mice, the splenocytes from Tg mice was evaluated by ELISPOT (Figure 3E).
1475 ± 120.7/10^6 TUBO-specific IFN-γ producing T cells were detected in Tg mice, which was about 7–14-fold more than control Ig-treated group. These data suggest that anti-neu antibody treatment induces neu- and TUBO-specific CD8+ T cell responses.

To correlate this data with clinical observations, we compared pre- and posttreatment breast tissue biopsies from patients with HER2+ and HER2- tumors treated with trastuzumab and chemotherapy or chemotherapy alone respectively. There was no significant difference in tumor-infiltrating lymphocytes (TIL) between HER2+ versus HER2- tumor tissues before treatment, with few cases having high TIL numbers before treatment. In our medical center, obtaining paired pre- and post-antibody samples is rare because trastuzumab treatment is reserved mainly for patients with potential metastasis after primary tumors have been surgically removed. However, we were able to obtain multiple posttreatment samples and observed that primary human HER2+ breast cancers had increased lymphocyte infiltrates post trastuzumab treatment whereas no detectable increase in lymphocyte infiltrates was observed in the chemotherapy alone group. Though both groups received chemotherapy, the addition of trastuzumab significantly increased lymphocyte, especially CD8+ infiltrates (Figure S2C). Defining the role of adaptive immunity initiated trastuzumab alone in human studies will be challenging because standard care requires that trastuzumab be combined with chemotherapy after surgery, and additional biopsy of metastasis requires strict IRB approval. Thus, formal clinical trials are needed to determine whether and when trastuzumab alone can increase TIL in better controlled cases.

**Anti-Neu Antibody Induces HMGB-1 Release for Strong Innate Responses**

To test whether the MyD88 pathway is essential to tumor regression after antibody treatment, TUBO-bearing WT and Myd88−/− mice were treated with anti-neu antibody. The therapeutic effect of anti-neu antibody was abolished in Myd88−/− mice (Figure 4A). Recent studies have shown that HMGB-1 can function as an endogenous danger signal that stimulates DC cross-priming in a MyD88-dependent fashion (Apetoh et al., 2007; Burgdorf et al., 2008). To determine whether antibody-mediated tumor regression is HMGB-1-dependent, free HMGB-1 was neutralized by administration of an anti-HMGB-1 antibody (3B1) in conjunction with anti-neu antibody treatment. Anti-HMGB-1 alone had no significant impact on tumor growth (data not shown) whereas coinjection of anti-HMGB-1 antibody partially diminished the therapeutic effects of anti-neu antibody (Figure 4B). These data indicate that HMGB-1, an endogenous danger signal, is essential for antibody-mediated tumor regression. It is conceivable that anti-neu antibody induces HMGB-1 release in the tumor microenvironment that enhances innate responses via the MyD88 pathway.

**Combination Treatments of Anti-Neu Antibody with Chemotherapeutics**

The essential role of T cells in anti-neu antibody-mediated tumor regression warrants reevaluation of current anti-HER2/neu treatment protocols because many current combination treatments of antibody plus chemotherapy may have negative effects on the host immune response to tumor antigens. For instance, high dose chemotherapy is used clinically to significantly reduce tumor burden; yet this treatment could possibly inhibit immune-mediated tumor regression by limiting immune responses. To test whether chemotherapy drugs used for breast cancer synergize with or antagonize anti-neu antibody, this treatment was combined with clinically equivalent doses of doxorubicin (DOX), cyclophosphamide (CTX), or paclitaxel (PTX). These chemotherapeutic agents are combined with anti-HER2/neu antibody in the clinic and are effective for TUBO regression in the presence or absence of antibody (Machiels et al., 2001). CTX (100 mg/kg) or PTX (40–60 mg/kg) was administered 3–5 days after anti-neu antibody, and accelerated tumor mass regression was observed compared to anti-neu antibody treatment alone (Figures 5A and 5B and Table 1). On the contrary, DOX treatment (5 or 15 mg/kg) concomitant with anti-neu antibody resulted in slower tumor regression than antibody alone, and mice receiving this combined treatment demonstrated tumor relapse whereas the antibody-alone treated group eradicated tumors (Table 1). The lack of a strong effect by drugs is not due to an insufficient dose, because the highest doses of DOX (15 mg/kg) and PTX (60 mg/kg) increased morbidity and mortality in a fraction of tumor bearing hosts (data not shown).

It was unclear whether the accelerated tumor regression observed by combining anti-neu antibody treatment with CTX or PTX was attributed to enhanced killing of tumor cells directly by the chemotherapy or to enhancement of the immune system. To test the impact of chemotherapeutics on the immune response and subsequent memory generated by anti-neu antibody treatment, mice were rechallenged with 5 x 10^6 TUBO cells (10 times more than primary tumor inoculum) 1–2 months after tumor masses were undetectable. All mice whose primary tumor underwent complete regression following anti-neu antibody treatment alone rejected the tumor rechallenge (Table 1). Surprisingly, a majority of the mice treated with anti-neu antibody and chemotherapy were susceptible to tumor rechallenge (66%
Bridging Innate and Adaptive Immunity by Anti-Neu

Figure 5. Administration of Chemotherapeutics After Antibody Treatment Enhances Primary Tumor Reduction but Reduces Immunity Induced by Anti-Neu Antibody

WT BALB/c mice (n = 5–10/group) were injected s.c. with 5 × 10⁶ TUBO cells and treated with 100 µg of anti-neu antibody (α-neu) on days 11 and 16. Select chemotherapeutic agents were injected i.p. at different time points. One of three experiments is shown. Reduced T cell proliferation was seen in Figure S3. (A) 100 mg/kg of CTX was injected i.p. on days 16, 23, and 33. (B) 40 mg/kg of PTX was injected i.p. on days 14 and 19. Treated, tumor-free mice were rechallenged with 2 × 10⁶ TUBO cells when primary tumor was not detected for at least 30 days. (C) Percent tumor-bearing mice. (D) Percent mean tumor volume.

We have previously shown that 20 mg/kg of PTX could suppress the priming of antigen-specific CD8+ T cell response initiated by local radiation of primary tumor (Lee et al., 2009). Although the addition of chemotherapeutic agents induce a more robust control of the primary tumor, they also confer a loss of protection from tumor rechallenge, a potential problem related to late relapse observed in the clinic once primary tumor is diminished after treatment. Reduced white blood cell count is a common side effect in patients undergoing chemotherapy. Although we did not observe significant differences in the numbers or percentages of T and B cells in blood, LN, and the spleen after PTX treatment, the number of Ki67+ cells was reduced in PTX treated group, suggesting early immune suppression (Figure S3).

We have previously shown that 20 mg/kg of PTX could suppress the priming of antigen-specific CD8+ T cell response initiated by local radiation of primary tumor (Lee et al., 2009). Similar suppression of Ki67 on lymphocytes was detected in DOX treated group (data not shown). CTX and DOX have been implicated in immunosuppression leading to fulminant hepatitis B hepatitis after chemotherapy for non-Hodgkin’s lymphoma in an HBV carrier (Aomatsu et al., 2010). However, some drugs can also boost immune responses, likely depending on doses and patients’ immune status (Emens et al., 2001). More studies are needed to address those complicated issues.

We speculated that there might only be a window of time when chemotherapy drugs may effectively reduce tumor burden without inhibiting antibody-induced immunity, as the half-life of these drugs is rather short. To test whether PTX given before anti-neu antibody also inhibited immune memory, an identical dose of PTX (40 mg/kg) was injected 24 hr before antibody instead of 3 days after antibody treatment. Impressively, the combination of PTX at this time and dose not only synergized

Combination Treatments of Anti-Neu Antibody with Ad-LIGHT

Although anti-neu antibody therapy induces initial immunity against tumor, sustained immunity could be transient or diminished if the tumor is able to bolster immune suppressive mechanisms. One major hurdle for antibody treatment of an established tumor is the tumor barrier that prevents effective infiltration of antibody, FcR+ cells (NK and macrophages), DC, and T cells. Expression of LIGHT intratumoral can attract various immune cells, including substantial numbers of FcR+ cells, DC, and T cells (Fan et al., 2006; Wang et al., 2005; Yu et al., 2004; Yu et al., 2007). We reasoned, therefore, that given the necessity of the adaptive immune system in anti-neu antibody therapy, targeting tumors with an adenovirus expressing LIGHT (Ad-LIGHT) by intratumoral injection could enhance tumor barrier breakdown, attract more immune cells, and amplify or sustain the therapeutic effect of anti-neu antibody treatment. To test this hypothesis and determine the contribution of Ad-LIGHT, mice bearing established TUBO tumors (18 days after inoculation) were treated with a suboptimal dose of anti-neu antibody in close combination with intratumoral injection of Ad-LIGHT. Neither suboptimal anti-neu antibody (two injections of 50 µg/mice), nor Ad-LIGHT alone was sufficient to control tumors (Figure S6A). Tumor growth in the Ad-LIGHT-treated group was only delayed by 1–2 weeks, whereas all mice in the anti-neu antibody-treated group showed relapse 4–6 weeks after the end of drug administration or dose could have a major impact on the immunological memory response to tumor antigens. It is likely that different doses and schedules related to drugs and antibody as well as patient immune status might be critical for overall antitumor effect, more studies, including animal models and clinical trials, are needed to optimize the antitumor effects by various protocols in the future.
protect from lethal rechallenge of TUBO but not 4T1, a neu 
This combination treatment generated immunity sufficient to 
weeks after the completion of treatment (Figure S4A). Notably, 
planted TUBO tumors, and no relapse was detected up to 8 
antibody treatment. In contrast, combination treatment of anti-
combination treatment of anti-neu antibody and Ad-LIGHT resulted in rejection of all trans-
antumor was measured twice a week. One of two representative experiments is 
Increase of antitumor immunity by antibody plus ad-LIGHT was seen in 
Figure S4.

antibody treatment. In contrast, combination treatment of anti-
neu antibody and Ad-LIGHT resulted in rejection of all transplanted TUBO tumors, and no relapse was detected up to 8 weeks after the completion of treatment (Figure S4A). Notably, this combination treatment generated immunity sufficient to protect from lethal rechallenge of TUBO but not 4T1, a neu mammary carcinoma (Figure S4B), demonstrating that this combination treatment induced neu-specific immunity. The combination of anti-neu antibody and Ad-LIGHT was also tested in neu Tg mice that frequently relapse after anti-neu antibody single treatment. Impressively, the combination treatment of anti-neu antibody and Ad-LIGHT rapidly controlled tumor growth in 80% of tolerant mice, whereas neither of the single treatment was able to control tumor growth effectively at the same dose (Figures S4C and S4D). We believe these data support the necessity of the adaptive immune system in anti-neu mediated tumor regression and suggest that proper immunotherapy can amplify or maintain antibody-initiated antitumor immunity to clear residual cancer and prevent relapse, even in a tolerant environment.

DISCUSSION

Over the past two decades, several studies aimed at understanding the mechanism(s) of anti-HER2/neu antibody therapy have shown that this therapy can efficiently slow the growth of HER2/neu+ tumors in vitro and in vivo, suggesting the importance of oncogenic signaling blockade in antibody-mediated tumor reduction. However, FcR+ cells were also shown to be essential for mediating the therapeutic effects of the HER2/neu antibody (Clynes et al., 2000). Although not mutually exclusive,
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immune complexes (Dhodapkar et al., 2002; Kalergis and Ravetch, 2002; Rafiq et al., 2002). Given the importance of danger signals in initiating immunity, we propose that blocking oncogenic signals by the anti-HER2/neu antibody may be an important initiator of, and positive-feedback loop for, adaptive immunity. For instance, antibody treatment of a neu" transfected tumor line, that is not dependent on a HER2/neu signal for its growth, does not reverse tumor growth in vitro and fails to demonstrate any effect on in vivo tumor growth (Whittington et al., 2008). We also observed that tyrosine kinase inhibitor treatment is very potent in blocking oncogenic signals in vitro, but anti-neu antibody is more potent in vivo, presumably because of the additional FcR-mediated effect. More studies are needed to explore the mechanisms and efficacy of the two different treatment strategies to develop better combination protocols.

Despite the initial clinical success of trastuzumab plus chemotherapy treatment for Her2" tumors, in metastatic and adjuvant settings, relapse still occurs. Frequently, this relapse is thought to be acquired resistance to the antibody, but our data suggest that this may also be the result of reduced T cell responses by chemotherapy. It has been shown previously that agents traditionally used for tumor reduction can have both positive and negative effects on host immunity (Emens et al., 2001). Most conventional chemotherapeutic drugs inhibit rapidly dividing cells, including some tumor cells and high proliferative T cells. However, small numbers of tumor cells might undergo dormancy or a very slow growth rate resulting in “drug resistance.” These cells could be the source of relapse. Thus far, the traditional combination of anti-HER2/neu antibody with chemotherapeutic agents has shown additive and synergistic effects in in vitro studies and in vivo xenograft tumor models (Hudis, 2007; Pegram et al., 1999; Pegram et al., 2004). Chemotherapeutic agents can not only reduce the size of the primary tumor, but also alter immune responses. Recent studies showed that the success of some protocols using low dose drug administration for anticancer therapy might stimulate innate and adaptive antitumor immune responses (Apetoh et al., 2007) or enhance vaccine-mediated immunity (Emens et al., 2001). During low dose chemotherapy, DCs require signaling through TLR4 and its adaptor MyD88 for efficient processing and cross-presentation of antigen from dying tumor cells. Patients with breast cancer who carry a Tlr4 loss-of-function allele relapse more quickly after radiotherapy and chemotherapy than those carrying the normal Tlr4 allele (Apetoh et al., 2007). Anti-neu antibody treatment could play a unique role for combination with low dose chemotherapy because it can significantly reduce tumor burden while boosting immune responses. A recent study showed that combined therapy with anti-DR5 and anti-ErbB-2 monoclonal antibodies significantly enhanced suppression of the growth of advanced spontaneous tumors in ErbB-2-neuT transgenic mice, even when treatment was delayed until tumors were palpable (Stagg et al., 2008). Recent clinical study showed that anti-HER-2/neu humoral responses significantly increased during combinational chemo and trastuzumab therapy, supporting our clinical observation (Taylor et al., 2007). Thus, current common uses of xenograft to evaluate antitumor effect by various treatments and protocols have their limitation and could have a biased selection of direct antitumor effect but ignore immune responses. As more preclinical and clinical studies are conducted, taking immune responses into consideration for future combination treatments will be necessary.

Based on numerous preclinical studies, clinical trials were conducted and demonstrated benefits of chemotherapy administration with trastuzumab over chemotherapy alone. Thus, antibody-mediated tumor vaccine is currently administered in conjunction with chemotherapy. However, tests done by cell culture or xenograft of human tumors into T cell deficient mice, may have been biased in favor of high dose chemotherapy. It is unclear whether chemotherapy plus anti-HER2/neu antibody is always better than antibody alone (Hudis, 2007; Piccart-Gebhart et al., 2005; Romond et al., 2005). Thus it remains to be determined if, when, and how anti-HER2/neu antibody should be combined with chemotherapy. It is likely that careful monitoring of the effect of chemotherapy on the tumor and the individual’s immune response. Our study suggests that sequential administration of anti-HER2/neu antibody after chemotherapy may allow chemotherapists to enhance the antibody mediated antitumor effect. Yet, formal clinical trials are needed to revisit this issue and to address whether and which chemotherapy drugs can enhance antibody mediated anti-cancer effect in neoadjuvant, adjuvant, and metastasis settings.

Optimal impact of anti-neu antibody treatment on tumor growth likely depends in part on the amount of antibody, and the number of FcR+ cells antigen presenting cells inside the tumor microenvironment. Therefore, combining this antibody treatment with an immunotherapy that can break tumor barriers and attract immune cells may have great impacts on the efficacy of anti-HER2/neu antibody treatment. Ad-LIGHT has multiple potential effects for enhancing antibody-mediated immunity. We have previously shown that intratumoral delivery of LIGHT, but not extra-tumoral delivery, can increase the expression of chemokines and adhesion molecules within the tumor, which then attract various immune cells, including T cells, NK, and DC (Fan et al., 2006; Ishihara et al., 2004; Wang et al., 2005; Yu et al., 2007). Recruiting FcR+ cells to the tumor might facilitate the FcR-mediated effect of HER2/neu antibody. In addition, LIGHT can also activate NK and T cells via the HVEM receptor to enhance antitumor cytotoxicity (Fan et al., 2006). Finally, driving high expression of LIGHT in the tumor by Ad-LIGHT may elaborate lymphatic vessels and alter the permeability of tumor vasculature to favor infiltration of both antibody and immune cells. LIGHT signaling on vessels has been shown to promote the neogenesis of vasculature and regulate LN hyper-trophy (Liao and Ruddle, 2006). Thus, the synergy we observe from intratumoral injection of Ad-LIGHT during anti-neu antibody treatment may result in increased infiltration of FcR+ cells and enhanced interactions between antibody and FcR+ cells leading to antibody-mediated tumor clearance and the generation and local accumulation of T cells for the increase of host resistance to rechallenge.

Our data demonstrate that T cells are necessary for anti-HER2/neu antibody-mediated tumor reduction. Because the antibody mediated blockade of oncogenic signals and the induction of ADCC have been previously demonstrated, we propose that both of these outcomes can induce a significant release of danger signals to alarm/activate DC and promote cytokine production. FcR-mediated signaling can induce additional
cytokine/danger signal production, and FcR-mediated phagocytosis and MyD88-enhanced adaptive cross-presentation may more effectively activate the adaptive immune system for enhanced tumor control. However, even with efficient blockade of oncocgenic signals and induction of proper danger signals, antibody-initiated immunity may still be transient and weak, making further combination immunotherapy necessary to reach clinical significance. We demonstrate that timing of chemotherapeutic doses has a distinct impact on antibody initiated immunity, and that proper immunotherapy can synergize with antibody treatment. Thus, this study provides insight into an interesting antitumor mechanism of anti-HER2/neu antibody that promotes cooperation between innate and adaptive immunity and warrants the use of combination therapies promoting antibody-initiated antitumor immune responses.

EXPERIMENTAL PROCEDURES

**Mice**

Balb/c, Balb/c Rag-1, and Rag-2 KO and FVB/N-Tg (MMTV-neu) mice were purchased from Jackson Laboratory and Balb/c FcγRII-I-/ mice were purchased from Taconic at 6 to 7 weeks of age. BALB/c MyD88-/- mice were kindly provided by Dr. Anita Chong, University of Chicago. Neu Tg F1 (FVB/N-Tg/ MMTV-neu x BALB/c) were bred and housed at the University of Chicago. All mice were maintained under specific pathogen free conditions and used according to animal experimental guidelines set by the Institutional Animal Care and Use Committee. The study has been approved by the Institutional Animal Care and Use Committee of the University of Chicago and Institute of Biophysics and all experiments conform to the relevant regulatory standards.

**Cell Lines and Reagents**

TUBO was cloned from a spontaneous mammary tumor in a BALB Neu Tg mouse (Rovero et al., 2000), and N202 was cloned from a spontaneous tumor line. N202 was approved by the Institutional Animal Care and Use Committee. The study has been approved by the Institutional Animal Care and Use Committee of the University of Chicago. Neu Tg F1 (FVB/N-Tg/ MMTV-neu x BALB/c) were bred and housed at the University of Chicago. All mice were maintained under specific pathogen free conditions and used between 6–16 weeks of age in accordance to the animal experimental guidelines set by the Institutional Animal Care and Use Committee. The study has been approved by the Institutional Animal Care and Use Committee of the University of Chicago and Institute of Biophysics and all experiments conform to the relevant regulatory standards.

**Proliferation Assay**

Cell proliferation was measured indirectly by mitochondria metabolic activity using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazodium bromide (MTT) assay. Briefly, quadruplicate wells of cells were plated in flat-bottomed 96-well plates at 10,000 cells/well. Approximately 16 hr after plating, when cells reached 40%–50% of confluence, dilutions of anti-neu mAb 7.16.4 or erlotinib were added. Replicate plates were terminated at 24, 48, and 72 hr posttreatment. At each time point, 10–20 µl of 5 mg/ml MTT in PBS was added and incubated for 4 hr at 37°C before the stop reagent (isopropanol with 0.04 N HCl) was added and the absorbance measured at 600–650 nm.

**Measurement of IFN-γ–Secreting T Cells by ELISPOT Assay**

Neu reactive T cells were measured by ELISPOT assay (Jacob et al., 2006). In this assay, the APCS were transfected to stably express the MHC-I molecule H-2Kd and B7.1 (3T3KB) or H-2Kd, B7.1, and neu (3T3NKb), thus allowing for measurement of neu-specific CD8+ T cell responses. Spleen or lymph node cells (responder cells) were resuspended in RPMI 1640 supplemented with 10% FCS, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. A total of 1 × 10^5 spleen or lymph node cells were added to each well of a 96-well IP plate (Millipore), which was precoated with 2.5 µg/ml rat anti-mouse IFN-γ (clone R4-6A2; BD-PharMingen). 3T3/NKb cells were added as APC over the spleen cells. 3T3/KB cells were used as control. The ratio of responder cells to APC was 10:1. After 48 hr of incubation, cells were removed and 2 µg/ml biotinylated rat anti-mouse-IFN-γ (clone XMG 1.2; BD-PharMingen) was added. Plates were incubated for another 12 hr at 6°C, then washed to remove unbound antibody. Bound antibody was detected by incubating the plates with 0.9 µg/ml avidin-horseradish peroxidase (BD-PharMingen) for 2 hr at room temperature. The substrate 3-amin-9-ethylcarbazole (AEC; PharMingen) diluted in 0.1 mol/l acetic acid and 0.003% hydrogen peroxide was added, and the plate was incubated for 3–5 min. AEC solution was discarded, and the plates were washed six times with water. The visualized cytokine spots were enumerated with the ImmunoSpot analyzer (CTL), and the results were expressed as the number of cytokine producing cells per 10^3 cells.

**Analysis of TIL**

Tumor masses established by TUBO were resected 3 days after antibody treatment. For FACS analysis, single-cell suspensions were obtained by collagenase digestion and incubated with fluorochrome-conjugated mAbs against surface markers. Cells were acquired on FACSCanto flow cytometer (BD Cytometry Systems). For immunohistochemical staining, resected tumors were embedded in Tissue-Tek (Sakura Finetek, Netherlands) and snap frozen in liquid nitrogen. The following primary antibodies were used for immunohistochemistry of human tissues: CD4 (4B12; Leica Biosystems Newcastle Ltd, UK), CD8 (C8/144B) and CD3 (2L6; DakoCytomation, Carpinteria, CA). Immunostaining was performed on the automated Bond TM system (Leica-Microsystems, Melbourne, Australia) according to the modified manufacturer’s protocol using Bond TM Polymer Refine Detection system (Leica Biosystems Newcastle Ltd.). Peroxidase reaction was developed with 3,3-diaminobenzidine (DAB) provided in the kit.

**Statistical Analysis**

Differences between groups were analyzed using the two-tailed Student’s t test or 2-way ANOVA. Error bars represent standard deviations (±SD). For survival curves, differences between curves were analyzed using the log-rank (Mantel-Cox) test.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.ccr.2010.06.014.
the mechanism of action of preoperative trastuzumab in patients with HER2.

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