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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, juxtamembranal 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 G_1 cell cycle arrest and is closely tied to increased $p27^{Kip1}$ 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 chemotherapy regimens. This study has important clinical impact because various chemotherapy drugs have been used before or after antibody treatment.

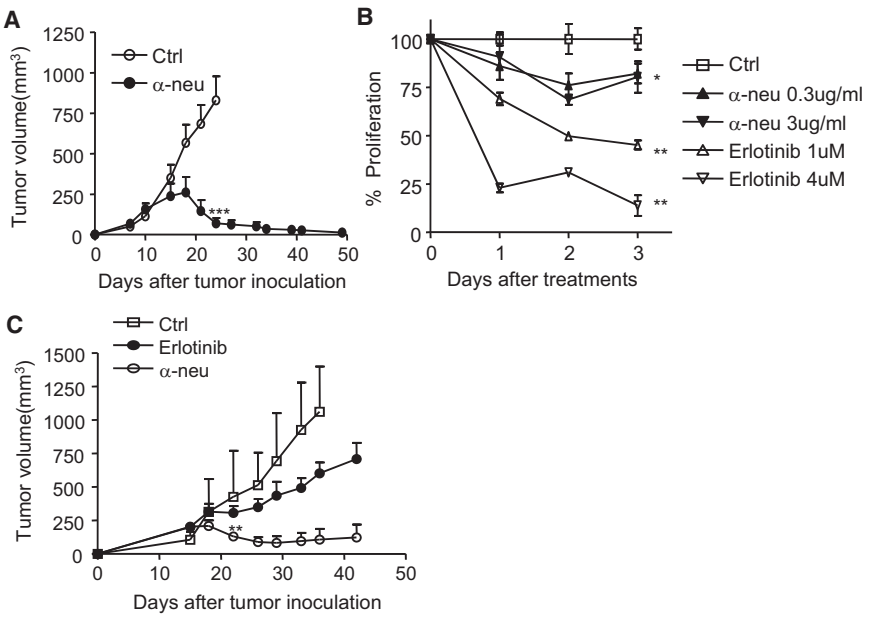

Figure 1. Anti-Neu Antibody Has Limited Effect In Vitro but Has Strong Effect Against Tumor In Vivo

(A) WT BALB/c mice (n = 5/group) were injected s.c. with 5 × 10^5 TUBO cells and treated with 100 μg of anti-neu (α-neu) or isotype control (Ctrl) antibody on days 14 and 21. The tumor growth was measured and compared twice a week. ***p < 0.005 compared with isotype control group after day 23. One of five representative experiments is shown.

(B) TUBO cells (1 × 10^5 cells/well) were plated in a monolayer and incubated with Erlotinib (1–4 μmol/l) or anti-neu antibody (0.3–3 μg/ml). Control groups received isotype-control antibody (Ctrl). Relative proliferation, reflected by metabolic activity, was evaluated at indicated times by MTT assay and graphed as percent of isotype control. Mean ± SD; *p < 0.05; **p < 0.005 compared with isotype control. One of two representative experiments is shown.

(C) TUBO-bearing BALB/c mice (n = 5/group) were treated four times with 100 μg of anti-neu antibody (α-neu) every other day and with 500 μg of Erlotinib every day for 7 days from day 18. *p < 0.05 compared with the control group from day 33; **p < 0.01 compared with erlotinib-treated group after day 26. One of two representative experiments is shown.

susceptibility to chemotherapy-induced apoptosis (chemosensitization) 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 convincing preclinical studies, clinical trials were conducted and demonstrated the benefits of combining chemotherapy administration 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 diminished 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* polymorphisms 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. Consistently, 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 downstream signaling to inhibit the transformed phenotype of HER2-expressing tumor cells (Montgomery et al., 2005). However, most models, including xenografts used for preclinical evaluation, 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 regression remains unclear.

RESULTS

Adaptive Immunity Is Essential for the Therapeutic Effect of Antibody Treatment

To evaluate whether targeted antibody treatment of $HER2/neu^+$ breast 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 overexpressing 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 chemotherapy, the majority (28/35) of Balb/c mice from several experiments rejected tumors completely 4 weeks after treatment, whereas 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 interference 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

Table 1. The Therapeutic Effect of Combined Chemotherapy and Antibody Treatment on Tumor Regression and Rechallenge

	Chemotherapy					
Antibody Dosage[a]	Drug[b]	Dosage	Schedule[c]	Rate of Tumor Regression[d]	Rejected/Total (%)	Growth After Rechallenge Growth/Total (%)[e]
100 μg × 2		None			28/35 (80	0/19 (0)[f,g]
100 μg × 3		None			20/24 (83%)	0/6 (0)[f,g]
100 μg × 4		None			1/5(80)	ND[f]
100 μg × 2	PTX	60 mg/kg × 2	3 days after	+	10/10 (100)	7/9 (78)[f]
100 μg × 2	PTX	40 mg/kg × 2	3 days after	+	10/10 (100)	7/9 (78)[f]
100 μg × 2	PTX	10 mg/kg × 4	3 days after	=	4/4 (100)	0/4 (0)[f]
100 μg × 2	PTX	40 mg/kg × 1	1 day before	+	12/12 (100)	0/4 (0)[f,g]
100 μg × 2	PTX	20 mg/kg × 1	1 day before	+	4/4 (100)	2/4 (50)[f,g]
100 μg × 2	CTX	100 mg/kg × 2	3 days after	+	12/20 (60)	2/3 (67)[f]
100 μg × 2	DOX	15 mg/kg × 2	Same day	–	0/5 (0)	ND[g]
100 μg × 2	DOX	5 mg/kg × 2	Same day	–	0/5 (0)	ND[g]

CTX, cyclophosphamide; DOX, doxorubicin; ND, not determined; PTX, paclitaxel.
[a] BALB/c mice were implanted with 4–5 × 10^5 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.
[b] Chemotherapeutic used. All drugs were injected i.p.
[c] Chemotherapeutic administration relative to anti-neu antibody treatment.
[d] Comparison of the incidence of regression to anti-neu antibody treatment (100 μg × 2) alone; +, greater than antibody alone; = , similar to antibody alone; –, less than antibody alone.
[e] One month after complete tumor regression, tumor-free mice were rechallenged with 2–5 × 10^6 TUBO cells and tumor growth was monitored for 30 days.
[f] Experiments were performed in CAS.
[g] Experiments were performed in UC.

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$^+$ tumors (Clynes et al., 2000). To test whether ADCC is accountable for this additional reduction of tumor masses in our in vivo model system, WT and *Fcγr* KO mice were inoculated with TUBO and established tumors were treated with 7.16.4. Indeed, the therapeutic effect of anti-neu antibody was FcγR-dependent, as FcγR deficient mice failed to show antibody-induced inhibition (Figure 2A). Together, using a system modeling human HER2/neu$^+$ tumor growth, our data consistently support the model that FcR$^+$ cells are essential for inhibiting or even eradicating neu$^+$ tumors in response to anti-neu antibody treatment. To further explore the role of ADCC in anti-neu antibody therapy, we inoculated *Rag-1*$^{-/-}$ mice with TUBO. Though *Rag-1*$^{-/-}$ mice lack T and B cells, these mice do have a complete population of FcR$^+$ 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 *Rag-1*$^{-/-}$ mice (Figure 2B). Although some *Rag-1*$^{-/-}$ mice displayed delayed tumor growth lasting, no tumor regression was observed in *Rag-1*$^{-/-}$ 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.

Anti-Neu Antibody-Induced CTL and Immunological Memory Are Required for Protection

CD8$^+$ cytotoxic lymphocytes are a major adaptive immune cell population involved in controlling tumor growth. We observed increased lymphocytes, especially CD8$^+$ cells, in TUBO tumor tissues 1–2 weeks after antibody treatment (see Figure S1 available online). To determine whether CD8$^+$ T cells are essential for anti-neu antibody-mediated tumor regression, TUBO-bearing

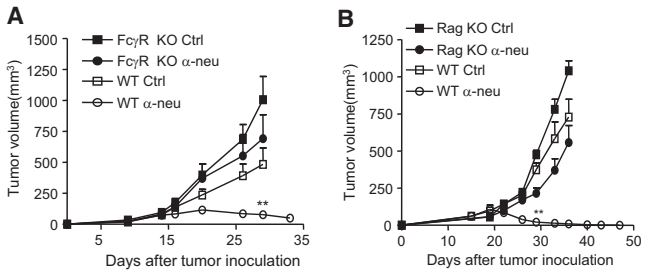

Figure 2. Antitumor Effect of Antibody Depends on Both FcR and Adaptive Immune System

(A) TUBO-bearing *Fcγ* receptor KO and WT BALB/c mice (n = 6–8/group) were treated with 100 μg of anti-neu or isotype control antibody on days 14 and 21. **p < 0.01 compared with WT isotype control group after day 29. One of three experiments is shown.

(B) TUBO-bearing Wt and *Rag-1*$^{-/-}$ mice (n = 5–7/group) were treated with 100 μg of anti-neu antibody (α-neu) or isotype control (Ctrl) on days 18 and 25. *p < 0.05; **p < 0.005 compared to isotype control groups of each mouse strain as (A). One of three experiments is shown. Increase of lymphocytes inside tumor is seen in Figure S1.

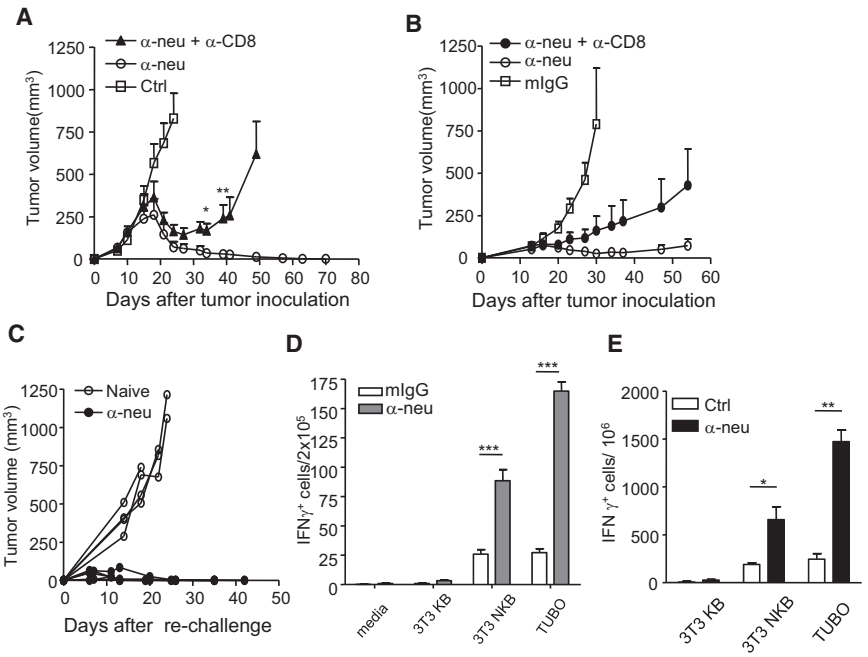

Figure 3. Therapeutic Effect of Anti-Neu Antibody Treatment Requires CD8$^+$ Cells and Induces Memory T Cell Responses

(A) WT BALB/c mice (n = 5–10/group) were injected s.c. with 5 × 10^5 TUBO and treated with 100 μg of anti-neu antibody (α-neu) on days 10, 17, and 24. CD8-depleting antibody (YTS169.4.2, 200 μg/mouse) was administered every 3 days, starting on day 9. *p < 0.05; **p < 0.005 compared to anti-neu antibody-treated WT mice. One of three experiments is shown.

(B) Neu Tg F1 mice (n = 6/group) were injected with 3 × 10^5 TUBO cells and treated with 100 μg of anti-neu antibody (α-neu) on days 11 and 18. CD8-dpeleting antibody (YTS169.4.2, 200 μg/mouse) was administered on the same days. *p < 0.05 compared to anti-neu antibody-treated group. One of two experiments is shown. The data from other CD8-depleting antibodies is shown in Figure S2.

(C) Tumor-free, antibody-treated BALB/c mice (n = 14 pooled from two experiments) 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.

(D) TUBO bearing mice were treated twice with 150ug of either anti-neu (n = 3) or mIgG (n = 3) on days 11 and 18. Mice were sacrificed 12 days after the final treatment and splenocytes were isolated for ELISPOT analysis as described in Experimental Procedures. ***p < 0.0001.

(E) Splenocytes from neu Tg F1 mice (n = 3–5) treated with anti-neu or isotype control antibody were stimulated with 3T3/KB, 3T3/NKB, or TUBO cells. The ratio of splenocytes to APC was 10:1. IFN-γ–producing cells were enumerated by ELISPOT assay. Results were expressed as number of spots per 10^6 splenocytes. *p < 0.05; **p < 0.005 compared with isotype control group. One of three experiments is shown for (D) and (E). Increase of tumor infiltrated lymphocytes was also seen in human samples after antibody treatment (see Figure S2C)

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-CD8 α antibody had no detectable impact on tumor growth in control Ig treated mice. In addition, depletion of CD8 α$^+$ cells using another clone (53.6.7) that has less impact on CD8 α$^+$ DC displayed the same phenotype but antibody-mediated effect is still dependent on CD8$^+$ cells (Figures S2A and S2B). To completely separate the direct role of CD8 α$^+$ DC and CD8$^+$ T cells, specific antibody to CD8β chain or mice deficient of CD8 α 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 tolerized 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 F_1 neu transgenic (Tg) mice (BALB/c x 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 with 5 × 10^6 TUBO cells (10 times of the primary tumor inoculation) after primary tumors had not been detected for at least 1 month. Impressively, all mice rejected the rechallenged tumors (Figure 3C and Table 1). This data strongly supports the idea that anti-neu antibody-mediated tumor regression generates detectable long-term immune memory capable of protecting the host from rechallenge, and presumably against relapse.

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). In the antibody-treated group, 658 ± 133/10^6 neu-specific and

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

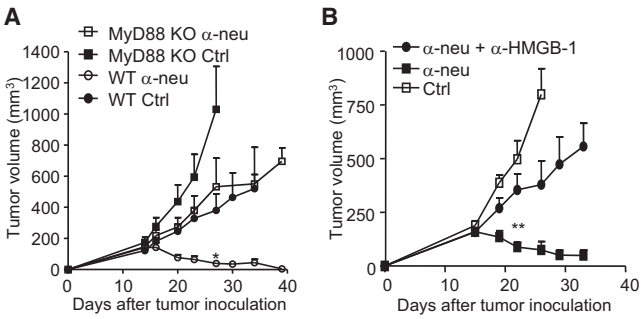

Figure 4. Therapeutic Effect of Anti-Neu Antibody Depends on Endogenous Danger Signals

(A) WT and *Myd88*$^{-/-}$ BALB/c mice (n = 5–7/group) were injected s.c. with 4 × 10^5 TUBO cells and treated with 100 μg of anti-neu (α-neu) or isotype control (Ctrl) antibody on days 21 and 28. *$p < 0.01$ compared to anti-neu antibody treated *Myd88*$^{-/-}$ mice. One of two experiments is shown.

(B) TUBO-bearing WT BALB/c mice (n = 4/group) were treated with 100 μg of anti-neu antibody (α-neu) and 100 μg of neutralizing anti-HMGB-1 antibody on days 14 and 21. **$p < 0.005$, compared to anti-HMGB-1 antibody-treated group. One of four experiments is shown.

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 enhance activation 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 × 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%

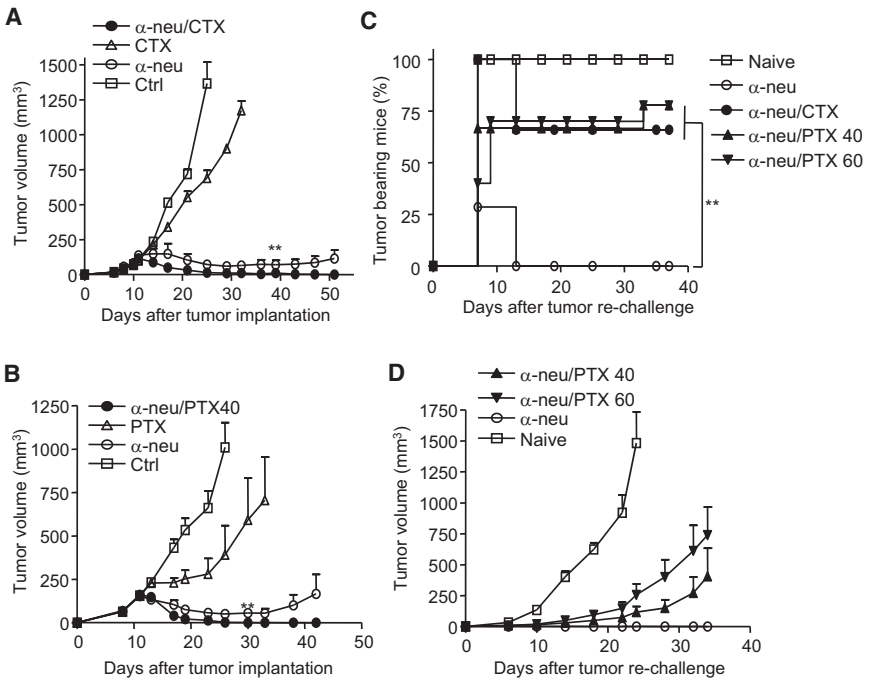

Figure 5. Administration of Chemotherapeutics after Antibody Treatment Enhances Primary Tumor Reduction but Reduces Immunity Induced by Anti-Neu Antibody

WT BALB/c mice (n = 5–10/group) were injected s.c. with 5 × 10^5 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 × 10^6 TUBO cells when primary tumor was not detected for at least 30 days.

(C) Percent tumor-bearing mice.

(D) Percent mean tumor volume.

of CTX combination mice; 77% of 40 mg/kg of PTX combination mice) (Figures 5C and 5D and Table 1). When a higher dose (60 mg/kg) of PTX was used, the treated mice more effectively controlled the growth of the primary tumor burden but were less resistant to rechallenge (Figure 5D and Table 1). 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, same 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 with anti-neu antibody to control the primary tumor, but also preserved the ability of the host to clear a lethal tumor rechallenge (Figures 6A and 6B and Table 1). Therefore, a simple alteration 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.

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

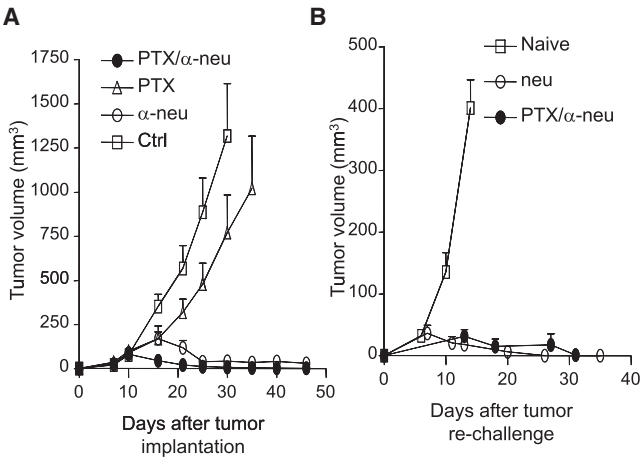

Figure 6. Administration of Chemotherapeutics before Antibody Treatment Could Enhance Primary Tumor Reduction without Affecting Immunological Memory

(A) WT BALB/c mice (n = 5–10/group) were injected s.c. with 5 × 10^5 TUBO cells and treated with 40 mg/kg of PTX on day 10. Mice were then treated with 100 μg of anti-neu antibody (α-neu) i.p. on days 11 and 18.

(B) Tumor-free mice from both groups were rechallenged with 5 × 10^6 TUBO cells when primary tumor was not detected for at least 40 days. The growth of tumor was measured twice a week. One of two representative experiments is shown. 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, these data promoted ADCC as the major mechanism for the in vivo effects of antibody treatment. Here, we demonstrate that T cells are necessary for the tumor reduction by anti-neu antibody alone. Our current findings support the critical role of T cells through the following findings: (1) the therapeutic effect of HER2/neu antibody treatment on tumor growth is greatly reduced in T cell-deficient mice; (2) WT mice depleted of CD8$^+$ T cells show rapid relapse of tumor; (3) increased anti-neu reactive T cells can be measured by ELISPOT after antibody treatment; (4) antibody-treated, tumor-free mice are subsequently resistant to high dose tumor rechallenge, suggesting the presence of immune memory; (5) increased T cell infiltration, especially CD8$^+$ cells, can be detected in tumor tissue of mice treated with anti-neu and patients treated with adjuvant HER2/neu antibody, compared to untreated controls; and (6) CD8-dependency occurs in both WT and tolerized neu-transgenic mice. We believe this study reveals an essential role for the adaptive immune system in the therapeutic effect of antibody treatment on HER2/neu tumors.

The treatment of WT mice with anti-neu antibody did result in more potent antitumor effects than treatment of neu-Tg mice. Modeling human HER2$^+$ cancer poses many complications. On one hand, humans are partially tolerant to the HER2 tumor antigen, so using neu transgenic mice provides an alternative model. On the other hand, transgenic mice greatly overexpress the neu antigen in every mammary epithelial cell soon after birth and potentially induce stronger tolerance than that observed in humans (Park et al., 2008). Endogenous anti-HER2 antibodies have been detected and shown to suppress HER2 kinase activity and to inhibit the transformed phenotype of HER2-expressing tumor cells (Montgomery et al., 2005). Moreover, a recent study demonstrated that treatment of neu transgenic mice with a combination of anti-DR5 and anti-ErbB-2 monoclonal antibodies induced complete responses in a majority of the transgenic mice (Park et al., 2008). Notably, depletion of CD8$^+$ T cells provoked primary and secondary tumor relapse, revealing the induction of antitumor immunity by the combination treatment (Stagg et al., 2008). Because anti-DR5 antibody mainly kills tumor cells, this study raises the possibility that anti-neu antibody can also induce immunity even in Tg mice. Therefore, using both WT and transgenic models to assess anti-neu antibody treatment of Her2/neu$^+$ tumors is complementary .

Because FcR-deficient mice fail to reduce or eradicate tumor after antibody treatment, ADCC was proposed as the ultimate mechanism for tumor clearance. Our study, however, raises the possibility that ADCC might not be the only mechanism dependent on FcR$^+$ cells. In addition to their cytotoxic effects, FcR$^+$ cells can also produce cytokines and/or danger signals in response to signals received via FcR. Indeed, various FcR$^+$ cells can release HMGB-1 that increases cross-priming and activation of DC in both mice and humans (Apetoh et al., 2007; Urbonaviciute et al., 2008). We have shown that the blockade of even this one danger signal greatly reduces the efficacy of antibody treatment. Thus, danger signals might coordinate with FcR signaling to activate DC and NK cells. Furthermore, APCs may use FcR to internalize antigens for enhanced presentation. Several studies have shown that antitumor specific antibody treatment enhances cross-priming of CD8$^+$ T cells through FcR-mediated phagocytosis, especially through the formation of

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, anti-HER2/neu antibody therapy is currently administered in conjunction with chemotherapy. However, tests done by cell culture or xenograph 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 chemotherapy 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. LTβR signaling on vessels has been shown to promote the neogenesis of vasculature and regulate LN hypertrophy (Liao and Ruddle, 2006). Thus, the synergy we observe from intratumoral injection of Ad-LIGHT during anti-neu antibody treatment may result from 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 cross-presentation may more effectively activate the adaptive immune system for enhanced tumor control. However, even with efficient blockade of oncogenic 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 *Fcr*$\gamma^{-/-}$ 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 × 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.

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 mammary tumor in a FBV Neu Tg mice. Both cell lines are gift from Joseph Lustgarten (Mayo Clinic, Arizona). TUBO was cultured in 5% CO_2, and maintained in vitro in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 10% NCTC 109 medium, 2 mmol/l L-glutamine, 0.1 mmol/l MEM nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin.

APC 3T3/KB and 3T3/NKB were provided by Dr. Wei-Zen Wei, Wayne State University (Wei et al., 2005). Anti-CD8 depleting antibody 53.6.7 was purchased from BioXcell (West Lebanon, NH). Anti-neu mAb 7.16.4, anti-CD8 depleting antibody YTS 169.4.2 (ATCC), and anti-HMGB-1 neutralizing mAb 3B1, were produced in house. Anti-neu antibody (7.16.4) recognizes the juxtamembranal region of rat neu and competes with 4D5, the precursor of trastuzumab, for binding and inhibition of tumor growth (Zhang et al., 1999). Anti-HMGB-1 mAb is capable of neutralizing HMGB-1 in vivo (Chen et al., 2009). Chemotherapeutic agents including erlotinib (Tarceva; Genentech, CA), cyclophosphamide (CTX; Baxter, IL), paclitaxel (PTX; Mayne Pharma, NJ), and doxorubicin (DOX; Teva Parenteral Medicine, CA) were purchased and prepared according to manufacturer recommendations. All antibodies for FACS were purchased from BD Biosciences. The generation of Ad-LIGHT was described previously (Kim et al., 2007). Endotoxin inside antibody was measured by the limulus amebocyte lysate assay (Cambrex inc. MD). For all mAb preparations, the amount of endotoxin was determined to be <0.2 E.U./mg mAb (limit of detection).

In Vivo Treatments

TUBO cells (3–5 × 10^5) were injected subcutaneously (s.c.) in the back of 6- to 8-week-old mice. Tumor volumes were measured along three orthogonal axes (a, b, and c) and calculated as tumor volume = abc/2. Mice were treated with two or three intraperitoneal (i.p.) injections of 80–100 μg of anti-neu antibody (7.16.4). For CD8 depletion experiments, 200 μg of anti-CD8 antibody (YTS 169.4.2 or 53.6.7) was injected i.p. at the same time as anti-neu antibody treatment. For the HMGB-1 neutralizing experiment, 100 μg of mouse anti-HMGB-1 antibody (3B1) was injected i.p. on the day of anti-neu antibody treatment. For chemotherapeutic agent combination, 500 μg/mouse of erlotinib, 100 mg/kg of CTX, 40–60 mg/kg of PTX, or 5–15 mg/kg of DOX were administered i.p. at the indicated times.

Proliferation Assay

Cell proliferation was measured indirectly by mitochondria metabolic activity using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium 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 APC were transfected to stably express the MHC-I molecule H2-K^d and B7.1 (3T3KB) or H2-K^d, 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 1–4 × 10^5 spleen or lymph node cells were added to each well of a 96-well HTS 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 4°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-amino-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 10^6 cells.

Analysis of TIL

Tumor masses established by TUBO were resected 3 days after antibody. 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 CD20cy (L26); DakoCytomation, Carpinteria, CA. Immunostaining was performed on the automated Bond TM system (Leica-Microsystems, Melbourne, Australia) according to the modified manufacturer 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.

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